ECOLOGICAL ASPECTS OF THE BIODEGRADATION OF TOWN WASTE

THOMAS GRAHAM BARNES, B.Sc. (Hons.), M.I.Biol.

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SUMMARY

Investigations concerning the microbiology of the composting of town waste were carried out and these indicated that refuse, even on entry into the disposal system, is a rich source of micro-organisms. The myco-flora consisting of ten species of thermophilic and thirty-three species of mesophilic fungi.

The microbiological changes which occurred during the composting of refuse were studied. The population of thermophilic actinomycetes became dominant with Thermomonospora curvata being particularly prevalent. Chaetomium thermophile was the most frequently isolated thermophilic cellulolytic fungus.

The completion of these investigations coincided with the publication of a governmental report on refuse disposal which criticised and discredited the composting of refuse. Thus, the results of the ecological research completed at this stage were applied to the biodegradation of the main component of domestic refuse which is cellulose.

A screening programme revealed that thermophilic fungi, generally have a much faster growth rate than mesophilic fungi. In shake-flask cultures with suspensions of ball-milled newsprint Chaetomium thermophile var dissitum and a variety of Sporotrichum thermophile combined the greatest degrees of substrate degradation with fungal protein synthesis.

Two processes of fermentation of newsprint by thermophilic fungi were developed at the laboratory scale for the production of an animal feedstuff. Using a system of submerged fermentation it was found that two species were capable of utilising nearly all the available substrate indicating that they could be lignolytic as well as cellulolytic.

A novel technique of solid substrate fermentation was developed using the ecological principle of competitive substrate colonisation as a means of obtaining the growth of the desired organism; instead of substrate sterilisation. Feeding trials demonstrated that the product of such a process is not acutely toxic and is of nutritional benefit.

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To my parents.

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Chapter 1

INTRODUCTION

The original project was concerned with the biodegradation of town waste (refuse) and was conceived by Dr. Eggins of the University of Aston in Birmingham and Dr. Stribling of Lucas Furnace Developments Limited. The project was included in the Inter-disciplinary Higher Degree Scheme operated by the University. The suggested project had three main aims which were:-

(i) Economic evaluation of the suggested process, including constraints of legislation, potential markets for town waste treatment equipment and predictions of the marketability of products.

(ii) Careful determination of the parameters affecting the thermophilic degradation of town waste components and in particular ecological studies of the organisms most likely to be involved in such activities and their relation to individual components of town waste.

(iii) Design of equipment to facilitate such processing.

Biodegradation can be defined as the useful breakdown or conversion of materials, usually waste products such as refuse, to an economically more useful state. This may be because the product is more versatile, has a higher nutritive value or is more simply aesthetically pleasing or less offensive. In this connection it may be termed a pragmatically positive action of micro-organisms. The process may remove pollutants odours or simply reduce the final bulk of the material (Seal and Eggins, 1972). The

process of biodegradation most frequently associated with refuse is known as composting, which is usually defined as a controlled process of decay of organic wastes effected by micro-organisms (a fuller definition of composting is given in Chapter 3).

Refuse disposal processes

Refuse is usually defined as the solid waste emerging from all household and commercial premises (Anon, 1968) and is generally produced as a result of man's urban activities. In 1966/67 in the United Kingdom approximately 16 million tons of house and trade refuse were disposed of by local authorities (H.M.S.O., 1971). In the U.S.A. in 1968 the Bureau of Solid Waste Management estimated that the total of household, commercial and industrial solid wastes generated amounted to over 360 million tons per year (Breidenbach and Eldredge, 1969). These large quantities of solid wastes are continually increasing, the annual

Table 1.1 Treatment methods for British and U.S. municipal waste; the figures show percentage by weight

Method of Treatment	U.K.	U.S.
Uncollected	-	24
Incineration	8.5	6
Controlled Tipping	64	10
Open Dumps	26	58
Tipping at Sea	0	2
Pulverisation	1	
Composting	0.5	-
	100	100

increase being between 1-2% per year in the U.S.A., (Gilbertson 1969) and are overloading the present methods of refuse disposal. The municipal refuse disposal processes in use today in the U.K., are controlled tipping, incineration and composting (H.M.S.O., 1971; Porteous, 1971a). Of the house and trade refuse handled by local authorities in 1966/67 90 per cent was tipped directly onto the land, a further 1 per cent being tipped after pulverisation, under 9 per cent was incinerated and an insignificant quantity 0.3 per cent was composted. Since 1966/67 a greater quantity of the refuse is being incinerated or treated by pulverisation and before long it is estimated that 25 per cent of the refuse will be treated by these methods (H.M.S.O., 1971). More recent estimates for the methods used to dispose of refuse in the U.K. and the U.S.A. are given in Table 1.1 taken from Tinker (1972).

In the United Kingdom controlled tipping may be defined as the disposal of refuse by tipping on suitable sites in accordance with the recommendations made by the Ministry of Health in the early 1930's (Bevan, 1967). The disposal of refuse by this method is not new, it has been practised since the very earliest days when man realised that his discards were untidy and unpleasant, the new aspect of controlled tipping is the list of regulations produced in the U.K. 40-50 years ago to ensure that the tipping of refuse was done without giving offence and without risk to the public health. The refuse is tipped in layers of not more than a 6ft. depth and covered within 24 hours with at least 9 inches of soil or other inert material. The types of sites used have included low lying land, marshland

settings and foreshores, river valleys, marginal land, moorland, poor agricultural land, disused railway cuttings and canals, ponds, ravines and mineral excavations such as stone quarries and clay pits. Generally sites have been selected where land could be improved by filling or raising of levels. After tipping the refuse slowly decomposes and over a number of years becomes compact in the tip. After this process has been completed the land may be used for building, providing playing fields, etc. The main advantage of controlled tipping is that it is the only method capable of disposing of all the types of solid waste irrespective of size, moisture content and other characteristics. No secondary disposal is required and no special preparation of the refuse is involved (Gilbertson, 1969). The low costs, and the advantages just described have made this the method of choice for most communities both in the U.K. and the U.S.A. The drawbacks often associated with controlled tipping have arisen from a failure to fully observe the recommended tipping precautions, especially tipping in excessive depths leading to voids in the tip and providing inadequate covering by inert materials which has led to difficulties from smells, fires, vermin, breeding of flies and disposal of the light refuse over adjoining areas. Changes in the volume and nature of the refuse to be tipped have combined to make controlled tipping increasingly difficult to control in the manner which was intended when it was introduced in the 1930's. Two trends in particular are of far reaching importance, first the volume of refuse generated by each household is rising rapidly so that existing tip sites are being filled at an ever increasing

rate and secondly modern refuse contains a far higher proportion of paper and plastics than ever before resulting in a lighter and bulkier refuse which is an inefficient filling material (Brookes, Green and Nice, 1968). The practice of controlled tipping has been extensively used by most cities in this country and there is now an acute shortage of convenient tipping sites, close to these cities, which is being aggravated by the changes in the nature of the refuse described above. The tendency as existing tips are exhausted will be for alternative sites to become both harder to find and more remote from the areas where refuse is generated. This means that the distance which collection vehicles must travel in order to discharge their loads will increase and because of this the time during which collection can take place will decrease causing the costs of collection to rise. Two developments have evolved to alleviate the current and future shortage of tipping space and these are the processesof transfer loading and pulverisation. The system of transfer loading has been developed to halt the rise in transport costs as final disposal sites become further removed from the area of collection, it involves the introduction of a plant where refuse collection vehicles discharge their loads and from which refuse is transferred to the final disposal point by specially designed bulk haulage vehicles. The fixed plant means that the distance which collection vehicles must travel is no longer dependent on the final disposal point, thus stabilising collection costs. (Brookes and Green, 1968). The second method of conserving tip space involves the use of pulverisation to reduce the volume of the refuse before tipping. Pulveris-

ation initially reduces the volume of refuse by 50-60 per cent (Anon, 1968) but this saving does not manifest itself into extended tip life as after a very short period of time the density of pulverised or crude refuse becomes approximately the same after settlement of the tip. The overall saving in tip space produced by pulverisation is estimated at only 30 per cent (H.M.S.O., 1971) and this is brought about largely by the greatly reduced need for covering material. Other advantages of pulverising the refuse before tipping are, that it is more easily controlled in landfill, it is visually less objectionable and can therefore be tipped on sites which would be considered unsuitable for crude refuse and because of its higher density it can be used in shallow depressions and therefore used for landscaping. It has been stated (H.M.S.O., 1971) that controlled tipping in the U.K. will continue to provide a satisfactory method of refuse disposal for the 'foreseeable future' but as tipping sites become even scarcer and transport costs more expensive the development of alternative methods of refuse disposal will assume greater importance.

Incineration is the second most widely used method of refuse disposal in the U.K. Modern refuse burns readily and leaves a residue that is virtually sterile and unrecognisable as refuse. Incineration will normally reduce the volume of the original refuse by 80-90 per cent and the weight by about half since a large part of the refuse is disposed of as a flue gas. The residue produced by the incineration of refuse is physically inert and biologically stable but it is not entirely free from poisonous metallic

residues derived from tins (Brookes and Green, 1968). The residue can be tipped with a minimum of nuisance and tip sites may be used for considerably longer than if the refuse is tipped crude. Other uses for the residue from incineration are found in the construction and road building industries (Tinker, 1972). In the United Kingdom for the 40 years preceding 1965 incineration had been based on the 'separation-incineration plants' which provided for the mechanical separation of dust and cinder from the refuse prior to its incineration. The separation part of these plants provided an easy opportunity for the manual salvage of paper, rags, non-ferrous metals, bottles, etc. with the ferrous metals being extracted magnetically. Now, however, because of the changes in the nature of refuse and the expense and difficulties in finding labour for such sorting these plants are no longer economically viable (H.M.S.O., 1971) and the incineration of refuse is now performed in 'direct incineration' plants. These plants do not offer any scope for the recovery of the wastes described above, the only recovery possible so far being of tins and similar scrap from the ash. Periodically schemes are developed for the recovery and utilisation of the heat produced by the combustion of refuse for use in district heating schemes or the generation of electricity. Examples of such schemes can be found at Edmonton in Greater London where the country's largest refuse incineration plant was designed for the generation of electricity and at Nottingham which has just provided a district heating scheme using refuse as the source of energy (Richardson, 1973). In most highly developed industrial societies the calorific value of refuse fluctuates between 6 and 9 M.J./kg and this can be

compared with the calorific value of coal of 32-36 M.J./kg and of oil with a calorific value of 42-45 M.J./kg. The calorific value of refuse does not therefore compare very favourably with the standard fuels and furthermore because of the high concentration of moisture in refuse, usually about 40 per cent of the total weight, combustion is often extremely difficult (Diamant, 1971). Calculations have shown that the total energy demand for this country in 1970 was equivalent to 310 million tons of coal and that the heat recoverable from the incineration of the whole of the country's production of refuse (estimated at 17 million tons) would be less than 2% of the above estimated demand (H.M.S.O., 1971). Thus the incineration of refuse is unlikely to become important as a means of providing and utilising energy. Additional factors which complicate incineration are the heterogenous nature of refuse making the design of efficient incinerators very difficult (Powell, 1960; Diamant, 1971) and the plastics content, especially polyvinyl chloride which on combustion at low temperatures produces extremely corrosive vapours of hydrogen chloride which corrodes the metallic parts of the incinerator. (Rolfe, 1969; Diamant, 1971; Porteous, 1971a; Stickelberger, 1971). Although it has been claimed that incineration is the most perfect method of removing refuse from man's environment (Howard, 1972) recent research indicates that even where flue gas cleaning equipment is used atmospheric pollution still occurs (Porteous, 1971a; Stickelberger,1971). The most harmful gaseous contaminants released from refuse incinerators are the polychlorinated bi-phenyls (P.C.B.) which like D.D.T. can be concentrated biologically to

significant amounts in the tissue of animals (Tinker, 1973; Porteous, 1971b). Also present in the flue gases are, fluorides produced from the combustion of trichlorfluomethones the volatising agent in hair spray, etc. and sulphur dioxide the most common invisible contaminant. It is true that in the most modern incineration plants the degree of air pollution is reduced to a minimum but unfortunately the majority of the plants operated are of an old design and they consequently have very little provision for the prevention of air pollution. The total achievement of refuse incineration is therefore an 80-90 per cent reduction in the volume of refuse producing a residue of very little value, the pollution of the atmosphere and the removal of carbon from the natural cycle.

The remaining method of refuse disposal used to any significant extent is composting. Modern composting as employed in the disposal of refuse involves aerobic thermophilic decomposition during at least one phase of the process. Aerobic conditions are used because they are not associated with the production of foul odours and they also promote more rapid decomposition than anaerobic conditions. Thermophilic conditions are required because at the high temperatures usually attained in the composting of refuse weed seeds and pathogens are killed and decomposition proceeds much quicker than under mesophilic conditions (Goleuke, 1972b). Modern mechanised composting systems always involve some degree of pulverisation and pre-sorting to remove bulky objects and inorganics such as metal glass and plastics. After this pre-treatment the refuse is either

composted directly in windrows or fermented in an enclosed vessel, e.g. the Dano Biostabiliser and the John Thompson Fermascreen for anything up to 5 days and then composted in windrows (Kershaw, 1968; Gilbertson, 1969; H.M.S.O., 1971; Goleuke, 1972b). It has been claimed that this short period of fermentation in the enclosed chambers mentioned above reduces the time required to produce a mature compost. The products from these fermentation chambers require from five to ten weeks to mature and recently doubts have been expressed about their value (Goleuke, 1972b). Mechanical composting evolved when composting was developed as a means of refuse disposal and a situation was reached in the U.S.A. where over mechanisation became as much a threat to the future of composting as anything else. The emphasis of research was placed on the equipment rather than on the composting process itself (Goleuke, 1972c) which usually increased the cost but contributed little to the process (Randles, 1963). Because of the relatively high capital and running costs of these fully mechanised plants incorporating an enclosed fermentation chamber of doubtful benefits, a tendency has recently developed to consider more simple plants in which the refuse is conditioned by pulverisation followed by composting in windrows (H.M.S.O,, 1971). It has also been reported that despite intensive investigations into the technology of composting both in Europe and the U.S.A. that no great breakthroughs are in the offing (Kolb, 1968) and that when space is not limited and time is not important then the windrow method of composting is to be preferred. The purpose of composting refuse and other organic wastes is the production of a

relatively stable humus like material (Burman, 1961; Wiley, 1963; Randles, 1963; Kershaw, 1968; Gilbertson, 1969; Porteous, 1971a; H.M.S.O., 1971; Stickelberger, 1971) which is usually described as a soil conditioner (Burman, 1961; Kershaw, 1968; Porteous, 1971a; H.M.S.O., 1971; McFarland, 1972), a soil builder (Gilbertson, 1969), a good organic mulch (Tinker, 1972) or as an organic product to ameliorate the soil (Keller, 1962). The principal by-products in the production of compost are carbon dioxide water and heat, none of which in the small quantities they are produced in composting is harmful to the environment. Composting as a means of refuse disposal suffers from a similar drawback to incineration in that a sizeable fraction of the refuse still remains to be disposed of. In many cases probably up to 50 per cent of the crude refuse intake is rejected by the process and has to be tipped (H.M.S.O., 1971).

Newer methods which have been proposed for the disposal of refuse are pyrolysis, baling and compression and the production of alcohol and food yeasts. Pyrolysis refers to the thermal breakdown of organic material in the absence of oxygen at a temperature of between 400°C and 1000°C. Depending upon the temperature and the exposure time end products are combustible gases such as carbon monoxide, hydrogen and methane, heavy oils and tar and carbon char. (Goleuke, 1972b; H.M.S.O., 1971). It has been claimed that one barrel of crude oil can be produced from each ton of refuse processed. The baling and compression of refuse has been developed by a Japanese firm and the bales are produced by compressing refuse beyond its yield point, the bales will therefore not expand after production and thus

may be tipped at sea or used in the construction industry (H.M.S.O., 1971). A scheme has been devised for the production of alcohol from the hydrolysed cellulosic fraction of refuse and it is claimed that this process could be operated at a profit (Porteous, 1971a). In the U.S.A. an alternative scheme has been developed for the conversion of cellulosic wastes in refuse to fodder yeasts (Meller, 1969) but it has also been reported that at the present time the economics of such a process are highly unfavourable (Goleuke, 1972b).

Estimates of the costs of each of the methods of refuse disposal are given in Table 1.2, these costs do not include the cost of collection which in 1966/67 was estimated at £45.5 million compared with the total disposal costs of £13.5 million for an approximate total of 17 million tons of house and trade refuse (H.M.S.O., 1971). The cheapest method of refuse disposal at the moment is obviously controlled tipping and for the future well over 50 per cent of local authorities have stated that they intend to continue to dispose of refuse by this means. A further 15 per cent who intended to tip their refuse have now got to pulverise it first because of shortages of tip space and covering material. Another 20 per cent of the authorities have considered incineration because of the difficulty in finding tip space and the remaining authorities were considering disposal schemes incorporating more than one method. It is clear from these figures that the shortage of available tipping sites is beginning to be felt as local authorities are now looking at alternative methods of refuse

Table 1.2 Treatment costs per ton of refuse

	Cost of disposal/ton of crude refuse		
Method of Disposal	H.M.S.O.(1971)	Porteous(1971a)	Tinker(1972)
Controlled Tipping			£0.75
Pulverisation	*£1.5-£2		£1.60
Composting	£3.00	£3.7	
Incineration	£1.85 - £4.55	£3.04	£3.00
Pyrolysis		£3.43	
Hydrolysis		Profit of ** £0.383 - £2.72	

*Cost per ton of refuse treated and tipped
Includes income from sale of compost at £1/ton and income obtained from salvage of the uncompostable material
These processes are at the moment in the laboratory or pilot stages.

**The lowest estimate is based on refuse with a paper content of 40% of the weight and the higher estimate on refuse with a paper content of 60% of the weight.

disposal. Porteous (1970) reported that the conventional method of disposal by tipping was drawing to a close because of this unavailability of suitable sites, typical estimated lives of sites for landfilling were for Wolverhampton 5 years, Leeds 10 years and Birmingham 10 years. On the basis of the above facts it is now safe to assume that the tipping of refuse can only be considered a short term method of refuse disposal for many large urban areas. This statement is supported by the findings of Brookes and Green (1968) who were members of a Local Government Operational Research Unit set up to investigate

the problems of refuse disposal in South Hampshire to consider long term solutions to the problem. They found that initially controlled tipping would be the cheapest method of disposal but that after the mid 1970's the costs of transfer loading and bulk haulage to distant tip sites made incineration cheaper than tipping. At the present time incineration is the only alternative method (H.M.S.O., 1971) to crude land filling in the U.K., (see Table 1.1) being used at the moment to handle 8.5 per cent of the total refuse; pulverisation also requires tipping sites.

From Table 1.2 it can be seen that the costs of incineration and composting, the present alternatives to tipping, are approximately the same and present similar problems in that they leave either a residue or rejected material which requires disposal. The most serious criticisms of incineration apart from the aspect of pollution are that it results in the destruction of reclaimable resources (Goleuke, 1972a) and that it can also destroy the value of non-combustible materials so that they are not worth recycling (Randles, 1963). The need for lasting and satisfactory answers to the solid waste problem has been stressed by Gilbertson (1969), who states that future solutions must avoid creating or adding to pollution of air, water and land and other environmental health hazards. As far as waste management is concerned we live in a closed environment which comprises soil, water and air and the disposal of wastes means discharging them into one or more of these sectors of the biosphere and possibly polluting one or more of them. Thus in the future proper waste management must

decide which of these sectors can accept refuse without detriment because disposal of wastes must not be synonymous with causing pollution (Stickelberger, 1971). It has been estimated that 40-80 per cent of domestic refuse consists of fermentable matter which theoretically could be converted into compost, which should be a material which can readily be accepted by the soil as an allied substance (Stickelberger, 1971). This evidence would indicate that when controlled tipping is no longer an option of disposal composting rather than incineration should be the method which is developed for the disposal of refuse.

These are the main reasons why the current research programme was initiated because in 1969 composting appeared to be the most logical solution to the solid wastes problem. Accordingly research was started on what has previously been stated to be the area of composting most urgently requiring clarification, that is an understanding of the microbial ecology of the composting process. Composting is a process of fermentation initiated by micro-organisms yet in all the literature concerned with the composting of refuse there is a scarcity of information on the microbiological aspects of the process particularly the cellulolytic fungi. It has been assumed that the micro-organisms essential for composting are indigenous to the wastes themselves yet even the identities of these organisms are not known. It has been stated by the American Public Works Association (1961) that an understanding of the microbiology of the process is not necessary for the operation of a composting plant and it is thus no surprise that composting has fallen

so widely into dispute in that country. The opinion has also been expressed (Gray, K.R., 1966) that the development of a better composting process must start with an understanding of the behaviour of the microbial population during composting. Thus the first steps taken in this project were, (i) the identification of the micro-organisms present in refuse and (ii) of those micro-organisms active in the composting process. Particular attention was paid to the activities of the thermophilic fungi in accordance with point (2) of the original proposals. The reasons for undertaking these studies are discussed in greater detail in the relevant chapters of this thesis.

At the beginning of 1971 the report of the Government's Working Party on Refuse Disposal became available (H.M.S.O., 1971) in which a number of observations and recommendations were made about the methods of refuse disposal in the U.K. In its recommendations the Working Party states, "It is considered that composting as a method can make little contribution to the disposal of refuse though in certain limited local circumstances there may be a case for adopting simplified systems for the production of material suitable for treating marginal land or for use in combination with the disposal of inert wastes". From this statement it is clear that there is not, in the foreseeable future, likely to be a wide scale adoption of composting as a means of refuse disposal. There were several reasons for this judgement by the Working Party and these are summarised below. The most serious criticism of the composting of refuse is the possibility of toxicity to plant life arising from the

concentration of metals present in the compost, these are especially prevalent in composts from processes incorporating sewage sludge. The report also carries a recommendation from the Forestry Commission which has banned the use of such composts because of the high levels of toxic metals notably zinc and copper. Other criticisms of refuse composts concerned the inconsistent quality of the product because of the impossibility of controlling the composition of the raw material from which it is made, the limited contribution that compost could make to the supply of humus in the soil compared with normal farming practice and the relatively short life of the organic fraction of composts compared with the life of other organic materials in the soil means that a saving in the use of fertilisers is unlikely. These criticisms have been supported by other reports, Kershaw (1968) had reported on the possibility and growing concern that municipal compost might have certain toxic properties and in the U.S.A. Goleuke (1972c) reported that areas in need of further study before the widespread use of compost could be recommended were the fate of the heavy metals and the long term effect of municipal compost on the soil and hence ultimately on crops. In recommending the use of composting for dealing with the ever increasing amount of sewage sludge in the U.S.A. the United States Department of Agriculture stressed that amongst other things the fate of the heavy metals must not be ignored (Goldstein, 1973). Another problem which has consistently plagued the use of municipal composts are the fragments of broken glass, plastics, leather, rubber and textiles which cannot be removed by the mechanical separation processes thus making the product aesthetically undesirable and therefore

unwanted for agricultural or horticultural purposes (Keller, 1962). The criticisms of the quality of refuse composts and the finding that they can even be toxic are more serious than the traditional criticisms of composting which are concerned with the economics of the process. When composting was first adopted as a method of refuse disposal it was seen as a potential source of revenue for it was the only process of disposal which produced a potentially saleable product. From then on composting was thought of as a business and as such should be self supporting. The main reason why this profitable state of affairs failed to materialise was the poor demand for the product. In a study of 21 composting plants around the world Kupchik (1966) found that they all ran at a loss because the expected revenue from the sale of compost did not materialise. The reason for the failure of a market to develop for compost was that farmers and horticultural workers preferred their fertiliser in the convenience of a bag (Abrahams, 1969) because in this form it is cheap (subsidised in the U.K.), concentrated and can be distributed on the land cheaply (Howard, 1972). Refuse composts have an infinitely variable composition and are expensive to distribute on the land. Goleuke (1972c) thinks that the development of composting as a widespread means of refuse disposal has been hindered because its success has been measured solely on this basis of expecting a financial return from the sale of the product whereas other methods are now judged almost solely by their effect on the environment. Refuse disposal is after all a public service and consequently it has been proposed that composting should not be considered as a money maker but as a means to provide economical sanitary waste disposal (Wiley,

1963). The non-marketability of compost does not make the process infeasible as a disposal method, after all there is very little demand for the output of an incinerator either (McFarland, 1972). This contention that composting should be treated the same as other methods of disposal and be subsidised is perfectly valid until further market exploration is performed. It has often been stated that compost did not sell because it was considered as a fertiliser and then sold on the merits of its nitrogen phosphorous and potassium contents which are now known to be quite minor in comparison with the subsidised chemical fertilisers (Goleuke, 1972c; H.M.S.O., 1971). If it is accepted just for the sake of the argument that compost is a good soil conditioner providing valuable humus and organic matter which are beneficial to the soil the expense of the high rates of application, 30 tons/acre, (H.M.S.O., 1971) would still be prohibitive because of the disadvantages of handling and distribution for which there are still no simple cheap mechanical remedies. Even so if composting were still to be adopted as a means of refuse disposal it would suffer one more very important drawback. In the U.S.A. the total consumption of natural organic fertilisers such as compost, estimated by the U.S. Department of Agriculture was 460,000 tons/year and even assuming a doubling of this total by the year 2,000, then 60 per cent of the demand could be met by the production of compost from the refuse of 'Orange County', California, A similar situation almost certainly exists in the U.K. (Randles, 1963). This situation shows that even if the composting of municipal refuse was subsidised there would not be sufficient agricultural consumption for it

Table 1.3 *Changing composition of British municipal refuse (Percentages by weight).

Components	1888	1935/36	1968	1980
Dust, ash, cinders	81	57	22	12
Paper, cardboard	1	14	37	43
Glass	2	3	9	9
Metal	1	4	9	9
Plastics	-	-	1	5
Rags and Textiles	1	2	2	3
Vegetables, Garden Waste etc.	13	14	18	17
Miscellaneous	1	6	2	2

* Taken from Tinker (1972)

Estimates

even if it were supplied free of charge, which means that it would have to be disposed of in a landfill. If compost had to be disposed of in this manner the savings in tip space would be comparable to those achieved by pulverisation. Although the product does not present the same hazards of water pollution and public complaint as pulverised refuse composting would be impracticable in these circumstances since the end result in terms of tip conservation would be much the same as with pulverisation but at a much higher price because of the high cost of the provision and operation of mechanical composting plants (Brookes and Green, 1968; H.M.S.O., 1971). However it must not be forgotten that the most serious problem concerning refuse compost is the

possibility of its toxicity to plant life.

The problem of refuse disposal today is complicated by the fact that refuse is a rapidly changing material. The differences in composition of refuse since 1888 are shown in Table 1.3. The main trends in the composition of refuse are the steadily declining concentrations of ash and cinder (due to the falling consumption of solid fuels) and the rapidly increasing quantities of paper, glass bottles, tin cans and plastics, the hallmarks of the 'throw-away' society. Since 1935/36 there has been an increase of about 44 per cent in the volume of refuse generated per household and a halving of the density of this refuse during the same period of time (H.M.S.O., 1971). The most significant increase in the constituents of refuse has been in the paper content, it now approximately constitutes 40 per cent by weight of the refuse and it has been estimated that in the next ten years it will increase in weight by about 30-50 per cent (H.M.S.O., 1971). The situation is much the same in the U.S.A. where the increases in the use of packaging materials has produced a situation where typical samples of refuse may contain up to 70 per cent by weight of paper and other cellulosic materials (Regan and Jeris, 1970). Other estimates have placed the paper content of the refuse in the larger cities of Britain such as Birmingham as high as 50 per cent.

The high paper content of refuse has affected all three methods of disposal. A high volume low density material is not desirable for tipping purposes. Although the higher paper content has increased the calorific value

of refuse the process of incineration has been complicated by the low density of refuse which has created problems in loading incinerators (Diamant, 1971). The high paper content has also affected the already doubtful future of composting because although cellulose in the form of paper is now the dominant organic fraction in refuse, it is in this form, relatively resistant to decay in existing composting processes unlike the cellulose of leaves and straw (H.M.S.O., 1971; Regan and Jeris, 1970). The presence of undegraded cellulosic materials has already been reported in the composts of municipal wastes at the end of the composting process (Stutzenberger et al, 1970) and cellulose degradation has now become the most important aspect of the whole process.

The above review of the three main methods of refuse disposal, controlled tipping, incineration and composting reveals that at the present time there is not a satisfactory way of dealing with a mixed or multiple stream waste such as refuse. As the present methods have all evolved on the least cost basis (Burch, 1971) this is not surprising. It is because of the problems associated with the traditional methods of disposal that thought has recently been given to developing a system in which the various wastes present in refuse would be kept separate at source and then collected and dealt with as separate entities in order to promote recycling (Tinker, 1972).

Recycling

In 1966/67 in the U.K. a little more than 2 per cent of the total quantity of household refuse was recovered for

re-use (H.M.S.O., 1971) and it has been predicted that although new methods of recovery and salvage will be developed for industrial wastes, recovery from domestic refuse will decline in importance (Howard, 1972). The reason for this decline is the need for sorting which in the absence of an efficient mechanised process is of necessity a manual job (Goleuke, 1972a). Unfortunately the cost of such a labour intensive system is not offset by the revenue obtained from the salvaged articles. Thus unless the refuse and collection disposal systems are altered large quantities of potentially re-usable materials will be wasted. Domestic refuse is a mixed or multiple stream waste which is more difficult to reclaim than a single stream waste because it is apparently very easy to mix different types of waste products but very difficult and expensive to separate them (Fulbrook et al, 1973). The concept of thermodynamics has been used as an analogy for the treatment of solid wastes (Wilson, 1969). It is postulated that when solid wastes are generated in the form that they are produced in the home or industry they have properties that give them a degree of availability which in the terms of thermodynamics is a measure of the maximum energy, in the form of work, obtainable by allowing matter to react with its environment and come into equilibrium with it. As soon as different categories of solid waste are mixed together, for example aluminium with steel, glass with plastics or clean paper with dirty paper the entropy increases greatly and the availability decreases. To bring mixed wastes back into a separated condition requires the expenditure of energy which may merely take the form of work for sorting but if the mixing

is accompanied by contamination or reaction it is very much greater. Obviously from this point of view it is desirable to prevent wastes becoming mixed and attempts in the U.S.A. are now being made whereby refuse is sorted at the point of production. The mixing of the wastes in refuse is the crux of the recycling problem, for if more use is to be made of the raw materials domestic waste must at some stage be sorted. The cheapest way of sorting would obviously be at the beginning of the process and if the householder could be persuaded to separate his rubbish into paper, vegetable and garden waste, metals, glass and plastics much reclamation would become economic and recycling would become a reality (Tinker, 1972). The advantages of home sorting are cheap labour, the householders effort being free of cost, simplicity because all the separation is done manually, the prevention of contamination of one waste by another which often makes recycling impossible, and finally present availability as there would be no need to wait for the development and perfection of an automatic sorting device (Goleuke, 1972(a); Abrahams, 1969). The disadvantages of this scheme are first the persuasion of the householder to cooperate, this could be done by legislation, rate rebates or small government subsidies on the wastes needed for recycling (Tinker, 1972) and secondly the need for the collection of the separate streams of waste could increase the costs of collection. Since 1956 the Borough of Worthing has used a system where the wastes have been kept separate at source by the householder (Fulbrook et al, 1973). At the moment in the U.S.A. and the U.K. recycling is already a part, albeit a small one, of present disposal systems and the aim for

the future should be to expand the recycling aspect from the merely incidental to where it becomes the major motivation (Goleuke, 1972b), the instigation of refuse separation at source would be one way of promoting this aim.

Recycling is needed for the conservation of resources and to reduce the waste load requiring final disposal (Goleuke, 1972a). The main barriers to full scale recycling or waste reclamation are of course economic ones, primarily the processing costs and the marketing of the salvaged products. It would be technologically possible to feed the separated elements of refuse into manufacturing processes for re-use but initially manufacturers would not be equipped to handle an increased input of recycled materials. Increased recycling of materials will not be practicable unless there is an acute shortage of the material concerned or if new markets or if new uses do not exist for the recovered material (Burch, 1971). If the above proposals for the collection of refuse become a reality even larger quantities of paper than are presently available would become available for recycling. Hanlon (1971) has estimated that in the U.K. only 25 per cent of the available waste paper is recycled by the paper making industry and it is clear, therefore that if the above proposals were adopted for the disposal of refuse substantial quantities of waste paper would become available for recycling in new ways.

In view of the findings of the Government's Working Party (H.M.S.O., 1971) that the composts produced from refuse could be toxic and that composting can make little

contribution to the disposal of refuse in this country; coupled with the misgivings revealed by other sources about the future of composting such as the poor demand for the product even when no charge is made for it, a decision was taken to alter the aims of the project. It was decided to study the biodegradation of the main component of refuse, which is cellulose in the form of waste paper, instead of the biodegradation of refuse as a whole as with composting. The decision was taken after research on the micro-organisms present in a refuse disposal system and the microbiology of the composting process had been completed, this was approximately eighteen months after the starting date of the project. During these eighteen months much valuable information had been gained about the cellulolytic mycoflora of refuse and it was considered that the most profitable way of continuing the project and utilising the results of the research already accomplished was in studying the biodegradation of waste paper with the aim of producing an animal feedstuff. The biodegradation of cellulose is the rate limiting step in the composting of refuse and from the predictions made about the increasing paper content of refuse it is likely to remain so in the foreseeable future. Thus the logical step for promoting the biodegradation of cellulose (in the form of waste paper) from refuse would be to isolate it from the remainder of the refuse by a separate collection system and treat it in a separate process where the conditions required for cellulose biodegradation could be made more nearly optimum.

The aims of the project thus became:-

(i) To develop a process (or processes) for the upgrading of waste paper to a protein and vitamin enriched animal feedstuff paying particular attention to the potential of the thermophilic fungi in such a process.

(ii) To conduct a preliminary economic evaluation of suggested processes and to study potential markets for the products of such processes.

The traditional method of recycling paper is through the waste-paper industry which re-pulps it and produces more paper from it. Recently this method of recycling has encountered some serious problems. In the U.S.A. the slump in the traditional waste paper recycling industry was caused by the advent of integrated paper mills which produce paper directly adjacent to the company owned forests which are situated far away from the sources of waste paper (other than their own mill-broke) and it is therefore much cheaper and more convenient for them to use their own supplies of virgin pulp produced from the timber of the neighbouring forests. Another problem for the waste paper recycling industry is that the supply of virgin pulp has been expanded much too quickly creating a surplus of pulp which has been holding down the price of paper produced from wastepaper pulp to the point where it has become uneconomical to continue production (Abrahams, 1969; Hanlon, 1971; Quimby, 1971). Paper produced from de-inked recycled fibre is not as aesthetically pleasing as that made from virgin fibres as it is invariably not as bright and contains specks from impurities. Scrap paper which is used to produce high quality papers

must be de-inked and this is an expensive process which causes its own form of pollution. Therefore it would appear that for the near future the main market for recylced fibres will be in the production of the lower grade paper products such as cardboard. In the United Kingdom the surplus of virgin pulp led to an increase in the importation of cheap pulp and paper products from the Scandanavian countries with the subsequent suppression in the demand for waste paper (Anon., 1972a). This low demand for waste paper led to a recession in the wastepaper industry and during 1971 consumption fell by over 110,000 tons, which was over 12 per cent of the production which usually runs at 1½ million tons/annum and stocks of waste paper rose by over 36,000 tons (Flintoff, 1970; Anon., 1972b; Hughes, 1973). This produced a situation where waste paper was a readily available source of material for any process capable of utilising it. The situation was best summarised by a statement from the Joint Waste Paper Advisory Council, (1971) in which it was stated that unless there was a reversal in the economic situation both in the U.K. and worldwide the demand for paper and board would continue to remain depressed and existing levels of supplies of waste paper were more than adequate to meet the demands of paper and board mills in the near future.

The lack of markets for scrap paper means that at the present time only a small proportion of the available scrap is recycled. The U.S.A. re-used only 19 per cent of the 58-60 million tons of paper it consumes annually, Japan re-uses 45 per cent, Austria 35 per cent and the U.K.

25 per cent (Burch, 1971; Hanlon, 1971; Quimby, 1971). In the U.S.A. the ration of reclaimed fibre input to annual paper and paperboard consumption is now the lowest it has been for 50 years (Quimby, 1971). Updegraff, (1971) has estimated that it costs the U.S.A. 1.5 billion dollars to collect and dispose of the waste paper which is present in the municipal solid wastes. The estimated revenue from the 11,400,000 tons of waste paper that the U.S.A. does recycle each year is 250 million dollars yet it continues to throw away at least 37,000,000 tons of scrap paper which it is thought would be worth recycling (Anon., 1972c), an estimated loss of resources of 800 million dollars. In the U.K. it has been estimated that 140 million pounds (sterling) worth of waste paper is destroyed each year by the local authorities when they do dispose of their refuse (Hughes, 1973). The need for salvaging and recycling paper through other ways than the traditional waste paper industry is urgent to stop the waste of a potentially useful resource and to reduce the costs of the disposal of solid wastes. In New York it has been estimated that a saving of 100 million dollars a year could be made on solid wastes management by the year 2000 if 80 per cent rather than the present 20 per cent of paper residuals were recycled rather than landfilled or incinerated. For the United Kingdom Flintoff (1970) has estimated that an annual increase of 10 per cent in the cost of refuse collection and disposal is unavoidable even without any increase in the present standards of operation. Expenditure on refuse, domestic and industrial, already represents nearly 1 per cent of the gross national product and it is predicted to continue to absorb an ever increasing proportion unless

the present pattern is changed (Flintoff, 1970). Paper accounts for 65 per cent by volume of all domestic refuse and by 1980 the proportion will be 70 per cent. Clearly the best way of substantially reducing the amount of refuse requiring disposal lies in the separate collection of as much as possible of this paper. In view of the recent failure (due to economic reasons beyond their control) of the board and paper mills to utilise their normal quota of this paper other methods of utilising it are urgently required.

The abundance of paper in refuse both in the U.S.A. and the U.K. has already been pointed out but a substantial proportion of this paper usually over 25 per cent by weight is newspaper (Hanlon, 1971) and recycling schemes for newspaper could make a significant contribution to reducing the load of solid wastes. Newspaper is and has always been a prominent material for recycling because newspapers are easy for the householder to keep apart from the rest of the refuse. This ease of separation combined with the volume of waste papers produced makes newspaper a natural target for recycling (Burch, 1971; Hanlon, 1971). New York City has to dispose of 350,000 tons of newsprint annually, much of which is at the present time incinerated adding to the problems of air pollution in that city (Hancock, 1971). In 1969 in the U.S.A. as a whole 8.8 million tons of newspapers were produced but of these only 2.1 million tons were recycled, the other 6.7 million tons (approximately 75 per cent of the consumption) were incinerated or used in landfills. Of the 2.1 million tons which were recycled

25 per cent were in the form of distribution residuals, i.e. unsold copies which means that only a very small percentage of the newspapers sold to the public are ever recycled (Quimby, 1971). Volunteer campaigns to recycle newspapers in the U.S.A. have been so successful with their collections that the paper industry cannot handle the large quantities of newspapers now available to them with the result that the price of waste newsprint has now fallen. Newsprint would therefore appear to be readily available in the U.S.A. for any process capable of utilising it, other than the traditional one of paper making. The situation would appear to be very similar in the U.K. where it has been estimaged that the majority of the 28,000 tons of newsprint used every week enters the refuse disposal system (Diamant, 1971). The average weekly consumption of waste paper in the U.K. is 36,700 tons (Anon, 1972b) of which only a small proportion is waste newsprint which means that a substantial proportion of the newsprint which now finds its way into the refuse disposal system would be available for recycling by any process other than paper making.

The quality of paper is determined primarily by the length of the cellulose fibres in it. Wood for high grade paper is broken down chemically to keep the fibres long, whereas lower grade papers such as newsprint are made from wood pulp produced by the cheaper process of mechanical grinding which breaks the fibres into shorter lengths. (Wenzl, 1970; Hanlon, 1971). This means that waste newsprint is only suitable for producing the lower grades of paper with similar short fibre lengths. In the U.K. the market for these products has been particularly depressed and most

predictions for a recovery in the British waste paper industry tend to be especially pessimistic about the demands for the lower grades such as newsprint and cardboard (Anon, 1972a). Another consideration concerning the availability of paper for new methods of recylcing is that usable wood fibres possessing sufficient strength for paper making can only survive the pulping processes a limited number of times (Hancock, 1971) and will then have to be disposed of, which means that they will eventually become available for alternative recycling schemes.

Upgrading Wastepaper

An alternative system to the traditional waste paper recycling schemes outlined previously could be developed from a process for the microbial upgrading of waste newsprint.

Waste newsprint as a source of cellulosic substrate for the production of an animal feedstuff has several advantages over other forms of wastepaper. Newsprint consists of ground and pulped softwoods having essentially the same composition as woods of this type, that is 40-50 per cent cellulose, 15-35 per cent lignin, and 25-35 per cent hemi-celluloses (Wenzl, 1970). It is free from additives and fillers commonly associated with higher quality papers which could possibly prove to be toxic to livestock, e.g. barium and cadmium sulphates and zinc, lead and titanium pigments. Printed newsprint has an ink content of less than 1 per cent of its total weight (Dinius and Oltjen, 1972) and the ink usually consists of a mixture of mineral

oil 86 per cent, carbon black 13 per cent and toners 1 per cent (Casey, 1960; Askew, 1969) and is not known to be toxic. This is borne out by the fact that feeding trials have successfully been completed using printed newsprint in substantial amounts in the diets of beef and dairy cattle. These feeding trials have shown that printed newsprint is non-toxic and nutritious not adversely affecting the yields of beef or milk respectively (Dinius and Oltjen, 1972; Anon, 1971). Other forms of waste paper have also been successfully used as feeds for beef and dairy cattle without causing any ill effects; these have included 'crumble' or mill broke a form of waste produced during the paper making process (Anderson, 1970) and in the U.S.A. newsprint, glossy magazines, coarse magazines, brown wrapping paper and cardboard have all successfully been used in the diets of dairy cattle (Mertens, Campbell, Matz and Marshall, 1971). Clearly a process which could make these waste cellulosic materials particularly newsprint more palatable, digestible and nutritious, by the addition of some microbial protein and vitamins and the breakdown of some of the recalcitrant carbon compounds increasing the availability of the carbohydrate fraction, should have a valid contribution to make in the develoment of animal feedstuffs in the future.

Cellulose is the most abundant of all naturally occurring organic compounds comprising at least one third of the vegetative matter on the earth and in the form of waste paper it has been shown to present a serious disposal problem. The development of a process for converting cellulosic wastes into a protein and vitamin enriched feed-

stuff could therefore have significant economic and environmental implications. Once a process for the production of an animal feedstuff from waste newsprint has been developed then there is no reason why it could not find application elsewhere. Many of the developing countries which have an acute deficiency of suitable animal feedstuffs produce large quantities of cellulosic wastes such as grain stalks hulls and bagasse which would be suitable substrates for a process of microbial upgrading (Han, Dunlap and Callihan, 1971). In recent years the demand for feedstuff containing a significant concentration of protein such as cereal crops and fish protein concentrates has increased and this is reflected in the higher costs of these materials. A considerable amount of the protein rich materials which were once used for animal feedstuffs are now being used more and more for human consumption (Spicer, 1971). The provision of adequate supplies of protein is now generally agreed to be the world's most serious nutritional problem (Thatcher, 1954; Bunker, 1963; Altschul, 1966; Gray and Abou-el Seoud, 1966; Brown, 1968; Postgate, 1969; Scrimshaw, 1969; Snyder, 1970; Han, Dunlap and Callihan, 1971) and an animal feedstuff that could be produced and used in place of high grade cereal crops, which could be more efficiently used in human nutrition, would readily find a market (Altschul, 1968). Animal feedstuffs produced from cellulose would be of particular value to ruminant animals supplying carbohydrates in the form of cellulose, which they are uniquely equipped to digest and biologically valuable protein and vitamins as opposed to a nitrogenous material such as urea with a low biological value. An opinion has been expressed that in the production of animal feeds biosynthesised

proteins would score because of their consistent quality which in modern compounding is of paramount importance (Spicer, 1971).

Newsprint is a source of cellulose which because of its high lignin content is relatively resistant to decay (Updegraff, 1971; Razzell, 1971) and previous attempts which have been made to ferment similar cellulosic substrates have usually involved complex steps of hydrolysis and neutralisation before the actual fermentation could take place (Thatcher, 1954; Rose, 1961). Dunlap and Callihan (1969) have stated that these expensive pretreatments are the most probable reasons why cellulose fermentations have failed to find any application in a peacetime economy. This point is illustrated by the more recent attempts to produce yeast from hydrolysed paper in refuse in the U.S.A., the economics of such a process have been described by Goleuke (1972b) as highly unfavourable. It was therefore an essential requirement for the present project to accomplish the fermentation of the cellulose substrate without resorting to hydrolysis or any other form of expensive chemical treatment. The breakdown of the cellulosic substrate was to be achieved by the use of cellulolytic organisms which could achieve a high rate and degree of decomposition.

The group of micro-organisms most commonly associated with the decomposition of cellulose are the fungi and in this connection they have been the subject of most intensive studies because of their high cellulase activity

(Han, Dunlap and Callihan, 1971; Rogers, Coleman Spino and Purcell, 1972). The fungi have been associated with the decomposition of cellulose from at least as early as 1926 when Waksman and Skinner found that the number of fungi, but not of bacteria, increased in soils amended with finely divided paper. Detailed accounts of the biodegradation of cellulose presented by Siu (1951) and Gascoigne and Gascoigne (1960) list many species of fungi which are prominent in the decomposition of cellulose. In a recent study of the utilisation of cellulose from waste paper by Updegraff (1971) it was reported that the fungi tested were greatly superior to the bacteria in their rate of breakdown of cellulose and that none of the bacteria isolated even Cellulomonas came near to the growth rates, as demonstrated by protein synthesis, obtained by fungi such as Myrothecium verrucaria on cellulosic substrates such as newsprint. In the present investigation many of the species of fungi isolated from the samples of refuse and the experimental windrow of refuse were found to be cellulolytic and were screened for the possibility of being used as the agents of cellulose breakdown and of biosynthesis of protein for the upgrading of waste newsprint. In particular the isolation of many of the species of thermophilic fungi described by Cooney and Emerson (1964) and the obvious cellulolytic activity that many of these fungi displayed on cellulose agar (Eggins and Pugh, 1962) was thought to be of significance and a special emphasis was placed on the screening of these fungi.

The thermophilic fungi are generally regarded as being ubiquitous and have not so far displayed any unusual

features of geographical distribution (Cooney and Emerson, 1968). Emerson (1968) has stated that the reason for their ubiquity and common occurrence is their capacity to occupy a temperature niche which most fungi and indeed most other micro-organisms cannot penetrate. The ability of the thermophilic fungi to rapidly breakdown cellulose and to exploit a unique ecological niche is of importance because few micro-organisms can exploit a cellulose-mineral salts environment (Han , Dunlap and Callihan, 1971) and fewer still can grow at the high temperature exploited by the thermophilic fungi. This presented an opportunity to develop a system of fermentation where sterilisation (and its resultant high costs) would not be required to provide an environment to promote the growth of the desired organism or organisms; this would be done instead by making use of the principle of ecological heterogeneity, when only a limited number of organisms, in this case thermophilic fungi, have the potential ability to exploit a given environment. These principles are expanded in the relevant chapter of this thesis.

Thermophily - literally a love of heat - was first described within the fungi by Lindt in 1886 who isolated Mucor pusillus from damp bread which had been incubated at 30°C (Cooney and Emerson, 1964). The thermophilic fungi may loosely be classified as those capable of growing above 40-50°C (Pounds and Lucas, 1972) with temperature maximum between 50-60°C. Most fungi grow between the limits of 10 and 40°C and have an optimum somewhere around 25-35°C (Cochrane, 1958), these can be thought of as mesophilic. Crisan (1959) defined thermophilic fungi as those whose

optimum temperature for normal growth lies at or above 40°C regardless of their minimum or maximum temperature limits for growth. The known fungal species able to grow above 45°C are few and can be divided into two categories thermotolerant and thermophilic. The definition proposed by Crisan (1959) does not differentiate between these two categories and was not accepted by Cooney and Emerson (1964) because of the large numbers of thermotolerant fungi it would include, Cooney and Emerson (1964) defined a thermophilic fungus as one that has a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C. Fungi such as Aspergillus fumigatus and Absidia ramosa with maxima near 50°C but with minima well below 20°C are considered to be thermotolerant and are excluded by this definition. Craveri et al (1964) and (1967) regarded a true thermophile as being unable to grow below 25°C and on this basis two of the fungi accepted by Cooney and Emerson would be excluded and relegated to the status of thermotolerants. Apinis (1963a, 1963b) has used the term thermophilous to describe those fungi which are isolated at soil plate incubation temperatures of 45°C-50°C and this definition therefore also includes both thermotolerant and thermophilic fungi. Apinis (1963a) divided the thermophilous fungi into three groups depending upon their temperature relationships. The three groups were:-

(i) the micro-thermophilic fungi with a temperature range above 40°C but not exceeding 48°C and an optimum range for growth of 25-35°C

(ii) the ortho-thermophilic fungi with optimum temperatures for growth between 40 and 50°C and maximum up to 60°C but unable to grow at temperatures below 20°C (Coincides closely with the true thermophiles as defined by Cooney and Emerson).

(iii) the psychro-tolerant or thermotolerant species with a wide temperature range for growth but also growing well at temperatures as high as 48°C.

Evans (1971) isolated thirty-two species of thermophilous fungi from coal-spoil tips which were capable of growth at elevated temperatures but some would also grow below 20°C and were therefore excluded by the definition of Cooney and Emerson (1964). The definition of the thermophilic fungi as given by Cooney and Emerson (1964) although an arbitrary working definition is at the present time the most precise way of defining these fungi in a standard manner and accordingly their definition will be accepted and used throughout this thesis. The known thermophilic fungi which satisfy the requirements of Cooney and Emerson's definition are listed in table 1.4 which gives the reference to their first known isolations and whether the fungus is known to be cellulolytic.

The generally world wide distribution of the thermophilic fungi is a result of the universal occurrence of self heating masses of organic debris. Thermophilic fungi are abundant in self-heating piles of organic matter (Ramabadran, 1967-1968) including hay and wheat straw (Chang and Hudson, 1967; Gregory and Lacey, 1963), wood chip piles

Table 1.4: The thermophilic fungi which are known to satisfy the requirements of Cooney and Emerson's definition.

Fungi	First References of Isolation and Occurrence	Cellulolytic Ability (2)
Cephalosporium (Allescheria terrestis)	Apinis, 1963a; Evans, 1969, 1971, 1972	Yes
Chaetomium brittanicum	Ames, 1961.	Unknown
Chaetomium thermophile var coprophile	Ames, 1961; Cooney and Emerson, 1964	Yes
Chaetomium thermophile var dissitum	Cooney and Emerson, 1964	Yes
Chaetomium thermophile var thermophile	La Touche, 1950	Yes
Didymostilbe coprophila	Mirza and Qureshi, 1970	Unknown
Humicola insolens	Cooney and Emerson, 1964	Yes
Humicola lanuginosa (1)	Tsiklinskya, 1899	No
Humicola grisea var thermoidea	Cooney and Emerson, 1964	Yes
Humicola stellata	Bunce, 1959	No
Malbranchea pulchella var sulfurea	Miehe, 1907b	Yes
Mucor miehei	Cooney and Emerson, 1964	No
Mucor pusillus	Lindt, 1886	No

Fungi	First References of Isolation and Occurrence	Cellul-olytic Ability
Myriococcum albomyces	Cooney and Emerson, 1964	Yes
Papulaspora thermophila	Fergus, 1971	Unknown
Sporotrichum thermophile	Henssen, 1957	Yes
Stilbella thermophila	Fergus, 1964	Yes
Talaromyces duponti	Griffon and Maublanc, 1911	No
Talaromyces emersonii	Stolk, 1965; Eggins and Coursey, 1964; Apinis and Eggins, 1966.	No
Thermoascus aurantiacus(3)	Miehe, 1907a	Yes
Thermomyces ibadanensis	Eggins and Coursey, 1964	Unknown
Thielavia thermophila	Fergus and Sinden, 1969; Hedger and Hudson, 1970.	Yes
Torula thermophila	Cooney and Emerson, 1964	Yes
Mycelia sterilia	Craveri et al, 1972	Unknown

(1) First identified as Thermomyces lanuginosus.

(2) Cellulolytic ability is taken as the ability to degrade filter paper or other sources of crystalline cellulose. References:- Eggins and Malik, 1969; Fergus, 1969; Malik, 1970; Chang,1967

(3) There are more than one variety of this organism, not all of them are cellulolytic.

(Tansey, 1970, 1971; Smith and Ofosu-Asiedu, 1972; Shields, 1969), stored grains (Flannigan, 1969; Mulinge and Apinis, 1969; Mulinge and Chesters, 1970), mushroom composts (Pope, Knaust and Knaust, 1962; Fergus, 1964; Hayes, 1969), town refuse composts (Klopotek, 1962; Muller, 1964; Mills and Eggins, 1970), agricultural composts (Cooney and Emerson, 1964; Emerson, 1968), peat piles (Kuster and Locci, 1964), fermentation of cocoa and curing of tobacco (Jensen, 1948; Pounds and Lucas, 1972), stocks of oil-palm kernels (Eggins and Coursey, 1964) and in the retting of guayale (Allen and Emerson, 1949). Cellulose is a major component of very nearly all these habitats and it has been found that a decrease in the cellulose content often occurs during self-heating (Stutzenberger, Kaufman and Lossin, 1970; Chang, 1967; Waksman, Umbreit and Cordon, 1939; Hayes, 1969). Waksman and Cordon (1939) reported that the decomposition of plant residues in composts was particularly extensive at the higher temperatures and that a number of thermophilic organisms including actinomycetes bacteria and fungi were found to be concerned in the decomposition process taking place at higher temperatures. Whilst studying these thermophilic micro-organisms in pure culture Waksman and Cordon (1939) also found that only one of those tested, a thermophilic fungus identified as Thermomyces (thought by Cooney and Emerson to be Humicola insolens), could equal the amount of decomposition brought about by the natural thermophilic micro-flora.

This association of the thermophilic fungi with the composting and humification processes indicated the

probable cellulolytic nature of this group of fungi. Cooney and Emerson's (1964) review of the humification and composting action of the thermophilic fungi showed that few species had been examined for their cellulolytic ability and that confusion over taxonomy and the naming of the species investigated had led to conflicting reports.

Humicola lanuginosa is now known not to decompose cellulose (Norman, 1930; Reese, 1946) whereas Humicola insolens has been found to be a vigorous cellulose decomposer (Reese, 1946). Sporotrichum thermophile has also been found to be able to utilise cellulose as the sole carbon source (Henssen, 1957) but in this case its growth was much slower than when either pectin or glucose was present with the cellulose. Recently much more attention has been given to studying the cellulolytic abilities of these fungi. Fergus (1969) assayed the cellulolytic activity of many of the known thermophilic fungi. The abilities of the fungi to degrade filter paper and carboxymethyl cellulose (C.M.C.) were tested and Fergus (1969) found that Chaetomium thermophile var coprophile, Chaetomium thermophile var dissitum, Humicola grisea var thermoidea, Humicola insolens, Malbranchea pulchella var sulfurea Sporotrichum thermophile and Torula thermophila were able to degrade both the filter paper and the C.M.C. showing them to possess the cellulase enzymes C_1 and Cx respectively. Other varieties of Malbrandea pulchella and Sporotrichum thermophile and a species of Talaromyces thermophilus could not degrade filter paper but could degrade C.M.C. showing them to possess only the Cx enzyme of the cellulase complex. Humicola lanuginosa

Humicola stellata, Mucor miehei, Mucor pusillus and Thermoascus aurantiacus could not degrade the filter paper nor attack the C.M.C. showing that they did not possess either of the cellulase enzymes. Chang (1967) tested the ability of the thermophilic fungi that she isolated from composts of wheat straw, to degrade cellulose, by using the method of Garrett (1962) using weight loss in filter papers as the criterion of degradation. Mucor pusillus, Talaromyces duponti and Humicola lanuginosa were found by Chang to be unable to utilise this form of cellulose and Malbranchea pulchella was found to be able to cause only slight degradation. However Chaetomium thermophile, Humicola insolens (2 varieties) Sporotrichum thermophile and the thermotolerant Aspergillus fumigatus could all cause significant degradation of this form of cellulose. Chang (1967) also reports that the thermophilic fungi generally and in particular Chaetomium thermophile were found to be more strongly cellulolytic than the mesophilic fungi which were tested, these included some species which have previously been reported (Siu, 1951) as being highly cellulolytic, e.g. Fusarium, Stysanus and several species of Coprinus. Eggins and Malik (1969) had also reported that most of the thermophilic fungi were cellulolytic producing clearing in the cellulose agar (Eggins and Pugh, 1962) used. This agar had a very pure form of cellulose as its base namely Whatman's Chromedia C.F.11 which had been ball milled in a fine suspension for 72 hours. Only Mucor pusillus and Talaromyces duponti of the thirteen thermophilic fungi tested did not produce any clearing while Malbranchea pulchella only produced slight clearing. Malik (1970) using a modification of Rautela and Cowlings (1966) technique in which the regenerated cellulose was replaced by the highly pure form of crystalline

cellulose described above, reported that Chaetomium thermophile, Sporotrichum thermophile and Cephalosporium sp. were the most cellulolytic of the thermophilic fungi tested. Humicola insolens, Humicola grisea var thermoidea, Torula thermophila and Aspergillus fumigatus were all found to be highly cellulolytic. Tansey (1970,1971) used the agar diffusion assay of Rautela and Cowling with regenerated cellulose to compare the cellulolytic abilities of the thermophilic fungi, and reported that the two varieties of Chaetomium thermophile, coprophile and dissitum, Sporotrichum thermophile, Talaromyces emersonii and Thermoascus aurantiacus produced the best clearing while Humicola grisea var thermoidea, Humicola insolens, Malbranchea pulchella, Myriococcum albomyces, Stilbella thermophila and Torula thermophila all produced some clearing unlike Humicola lanuginosa, Humicola stellata, Mucor pusillus, Talaromyces duponti and another variety of Thermoascus aurantiacus that all failed to clear the cellulose. Tansey (1971) also reported that several of the thermophilic species tested at 45°C had cellulolytic rates of degradation 2 or 3 times those of the mesophilic species Chaetomium globosum and Trichoderma viride both of which have previously been noted for their highly cellulolytic natures (Siu, 1951; Gascoigne and Gascoigne, 1960).

The results of the research outlined above clearly indicate that the majority of the species of thermophilic fungi are cellulolytic and that several species are highly cellulolytic degrading cellulose at a significantly quicker rate than some of the most cellulolytic mesophilic micro-fungi. The occurrence of these fungi, i.e. mesophiles and thermo-

philes, in samples of refuse and compost were investigated. In the past it has been assumed that the micro-organisms responsible for the composting of refuse are indigenous to the wastes. The literature revealed that very little was known of the microbiology of composting of refuse not even the identity of the micro-organisms present in refuse. Consequently samples of refuse and compost were obtained from a refuse disposal system, which employed composting as a means of treating the wastes, and the frequencies of occurrence of the mesophilic and thermophilic fungi present in each sample were determined, employing Warcup's (1950) isolation technique.and also an elective isolation technique. Isolations were made at a range of temperatures between 25 and 50^{o}C and subsequently the ranges of temperatures over which many of the fungi could grow were determined and were found to correlate with the temperatures at which they were isolated. Only the species of thermophilic and mesophilic fungi were identified as these have been shown to be the micro-organisms most likely to be involved in the decomposition of cellulose, which is now the rate limiting step in the process of composting of refuse. Actinomycetes have in the past been shown to be active in composts and their presence or absence in the samples was noted but at this stage no attempt at identification was made. Because of the widespread occurrence of bacterial species no attempt was made to record their presence or identities at this stage. After the identification of the fungi in the samples of refuse had been completed an experimental windrow of pulverised refuse (from the same source) was set up and the behaviour of the thermophilic and microbial populations

during the composting process was observed. Changes in the numbers of the populations of actinomycetes, bacteria and fungi were estimated and actively growing fungi and actinomycetes were isolated. Thus the micro-organisms which were responsible for thermogenesis and the decomposition of the refuse were identified.

After the decision had been taken to concentrate on the biodegradation of the main component of refuse, that is waste paper, a screening programme was set up for the selection of suitable micro-organisms which could rapidly break down cellulose. Previous research had indicated that in culture many of the fungi, particularly the thermophilic fungi, were amongst the most cellulolytic of all micro-organisms. In the present investigation many cellulolytic fungi had been isolated from the samples of refuse and the experimental windrow. A further selection of thermophilic and mesophilic fungi was made by means of an elective isolation technique in which fungi were isolated from soil amended with powdered newsprint. The linear growth rates of fungi selected by means of the isolation technique and from references given in the literature were compared by the criterion of colony diameters obtained on cellulose and newsprint agars. The results of this screening programme indicated the faster growth rates and strong cellulolytic activity of many of the thermophilic fungi and these were the fungi chosen for use in the development of a process for the microbial upgrading of waste newsprint.

The growth rates of the thermophilic fungi selected were observed on suspensions of newsprint with two different

nitrogen sources in shake flask cultures. On the basis of the results from these growth tests two species of thermophilic fungi were selected for further work and two systems of fermentation for waste newsprint were developed.

The first process of fermentation developed involved the use of the technique of solid substrate fermentation. A simple, low cost process for the production of a protein and vitamin enriched feedstuff was developed where the need for conditions of complete sterility were eliminated. Growth of the desired fungus being obtained by heavy inoculation, manipulation of the environmental conditions for rapid growth of the desired organisms and the confinement of infection characteristics of solid substrate fermentations. The effects of the moisture content of the newsprint and the C:N ratio on the fermentation process were observed.

Using the same thermophilic fungi a second process of fermentation was developed using a technique of submerged fermentation with suspensions of newsprint. This technique presented an opportunity to study the growth of the thermophilic fungi in more optimal conditions providing an idea of the maximum yields which could be hoped for in other methods of fermentation. The effect of increasing nitrogen concentrations on the growth of the fungi in submerged fermentations were examined and the differences in the growth rates of the fungi on pure cellulose and newsprint were observed to estimate the effect of lignin, in the newsprint, on the fermentation. Analyses of the newsprint and the degree of breakdown obtained by two of the species of

thermophilic fungi on this substrate indicated that it may have lignolytic ability.

The results from both systems of fermentation suggested that the thermophilic fungi have an important role to play in the microbial upgrading of cellulosic wastes. The absence of toxicity noted in the feeding trials, carried out with the product of the solid substrate fermentations, suggest that in the future the thermophilic fungi because of their extremely rapid growth rates may become important industrial micro-organisms.

CHAPTER 2 Pages

Chapter 2

THE FUNGI OF A REFUSE DISPOSAL SYSTEM

Introduction

The assumption has been made by many researchers that the micro-organisms which effect the decay of organic matter in the composting of refuse are indigenous in great numbers to the wastes present in refuse (Berkeley Project, 1953; Goleuke, Card and McGauhey, 1954; Wylie, 1960; McCalla, 1960; Obrist, 1963; White, 1965; Kershaw, 1968; Gray and Sherman, 1969; Gray, Sherman and Biddlestone, 1971). The main source of evidence supporting this hypothesis comes from the research carried out on the effectiveness of inoculums in the composting of refuse from which it has generally been accepted that the use of inocula such as soil, actively composting refuse and horse manure do not produce any signigicant effect on the composting process (Berkeley Project, 1953; Goleuke, Card and McGauhey, 1954; Burman, 1961). The American Public Works Association (1961) reported that special starters or inoculants are unnecessary in composting because if the conditions for composting are not right then added organisms will not grow and multiply, if they are right the organisms already present will grow and multiply. In several cases experience has shown that the recycling of small amounts of actively composting materials shortens the lag periods required for large numbers of the right organisms to develop but that this does not appreciably shorten the duration of the composting process (A.P.W.A., 1961).

The findings reported above can be criticised because the activities of the micro-organisms in composts of refuse are not fully known or understood and have not yet been widely investigated. Gray and Sherman, (1969) reported that the complex ecological process of composting involving the activities of mixed microbial populations is unlikely to succumb to rigorous scientific analysis for many years. Thus, if the nature of the micro-organisms active in composting is not yet known then it is surely more difficult to make any meaningful decisions about the use of inoculums in the composting of refuse.

In the literature there is almost a total lack of any reports concerning the microbiology of refuse presumably because most researchers have assumed that the micro-organisms responsible for effecting the decay or organic wastes in composts of refuse are indigenous to the refuse and have consequently refrained from further investigation concentrating instead on the manipulation of environmental conditions in mechanised composting systems. Von Klopotek, (1962, 1963) carried out one of the rare investigations into the occurrence and condition of mould fungi in the composting of municipal wastes. The aim of her investigation was to study the action and appearance of fungi in the composting process and not the nature of those present in the refuse disposal system. Von Klopotek found that in the initial samples of refuse and throughout the composting process the number and variety of mesophilic fungi was far in excess of that of the thermophilic fungi; which represented only 0.02% of the total population. Apart from Von Klopotek's inves-

tigation and very recent work by Stutzenberger, Kaufman and Lossin (1970) implicating the thermophilic actinomycetes in the degradation of cellulose in composts of refuse, there is a complete lack of any meaningful qualitative data concerning the fungi and microbiology as a whole, of refuse before, during or after composting. The microbiology of composting will be fully discussed in the next chapter which is concerned with the microbiological changes which occur in the composting of refuse.

Although it is commonly assumed that the micro-organisms essential to composting are indigeneous to the wastes in refuse the source of these micro-organisms if they are present is not known. The most likely source of micro-organisms in refuse at the present time would appear to be the vegetable and putrescible content, the remainder of the refuse consists of ashes, paper, rags, glassware and plastics (H.M.S.O., 1971) all of which are unlikely to contain great numbers of micro-organisms. The weight of vegetable and putrescible matter in refuse has not increased since 1935/36 and since that time most vegetable matter has been washed and prepacked before distribution to the consumer. This means that any soil adhering to the vegetable matter will be removed before distribution and is therefore unlikely to be a major source of micro-organisms in household refuse. If the micro-organisms essential to composting are indigeneous to the refuse when it is composted but are not present in the refuse when it enters the disposal system then the disposal system itself must be the means of inoculation. It is possible that dirty dustbins, refuse collection wagons

or refuse treatment plants might contain a thriving microflora which could inoculate the refuse with the organisms which are essential for composting.

The purpose of the present investigation is to determine the nature of the micro-organisms concerned in the composting of refuse and whether they are in fact indigeneous to the refuse or the disposal system. Accordingly samples of refuse and compost were obtained from a refuse disposal system, which utilises composting as the means of treating the refuse and isolations were made of the mesophilic and thermophilic fungi present in these samples as these are the organisms most frequently associated with cellulose decomposition, the rate limiting step in composting (Regan and Jeris, 1970). Research by Waksman, Umbreit and Cordon, (1939) and Hayes (1969) had indicated that thermophilic actinomycetes played an active role in the composting of materials such as straw and horse manure and so in this investigation the presence of thermophilic actinomycetes was noted but no attempts at identification were made. Burman (1961) considers that in the composting of refuse the bacteria may be responsible for the utilisation of the more amenable carbohydrates such as sugar and starches and of the proteins in the initial stage of composting, playing an important role in thermogenesis. It is unlikely that the bacteria will be involved in the decomposition of cellulose in composting and because of this and the widespread distribution of the bacteria their presence in the samples of refuse and compost was not recorded. This is not a reflection on the importance of the bacteria in

composting and in the chapter of this thesis concerned with the microbiology of the composting process careful consideration is given to the behaviour of the bacterial populations in conjunction with the populations of the actinomycetes and fungi.

Materials and Methods

The samples of refuse necessary for the present microbiological investigation were obtained from Chesterfield Rural District Council which operates a refuse composting system based on a pulverisation plant developed by the Swiss firm Buhler Brothers Limited (Wilson, 1965; H.M.S.O. 1971). The composting plant treats the refuse from half of the district. After collection the refuse is unloaded into a reception hopper at the plant and from this point the refuse is conveyed to a horizontal rotor hammer-mill for coarse milling and is then fed following magnetic separation of ferrous metal to a twin rotor hammer-mill for finer milling. The pulverised refuse is screened and provision has been made for the addition of sewage sludge to the product which is then composted by windrowing.

The following samples of refuse and compost were collected from the refuse disposal system operated by Chesterfield R.D.C.:-

i) refuse from household dustbins
ii) scrapings from the bottom and sides of the same household dustbins
iii) refuse from the reception hopper of the composting plant
iv) pulverised refuse
v) pulverised refuse and sewage sludge
vi) immature compost
vii) mature compost

The refuse and scrapings were collected from 20 household dustbins in the Chesterfield district, samples were obtained from dustbins in two separate locations within this district. The refuse from the dustbins was collected on a weekly basis. The first location was a residential area and the second one a council estate. The scrapings were taken from the bottom and sides of the dustbins and were removed with a spatula which was sterilised between each sampling by flaming in alcohol. The scrapings were collected in sterile universal bottles and the sample of refuse removed from these bins were collected in clean plastic bags.

Samples of refuse (from the reception hopper of the plant), pulverised refuse, pulverised refuse and sewage sludge, immature compost and mature compost were collected in large clean plastic bags which had been sterilised by wiping the inner surfaces with 70% alcohol. The sample of refuse taken from the reception hopper of the composting plant consisted mainly of ash, small pieces of vegetable matter and paper, the larger inert pieces of refuse such as plastic and metal objects were not collected. The sample of immature compost was taken from the central region of a two-week old windrow, the temperature of compost at the time of collection was 59^{o}C. The sample of mature compost was collected from a windrow which was eight-months old. The samples of pulverised refuse and pulverised refuse and sewage sludge were collected immediately they were discharged from the pulverisation plant. The sample of pulverised refuse was obtained when the supply of sewage and sludge to

the pulverised product was temporarily stopped.

Isolations were made from all of these samples at the following temperatures, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C. This range of temperatures was chosen becuase composting involves the decomposition of organic wastes over a similiar range of temperatures with the greatest decomposition occurring in the range of 45°-60°C. This range includes the optimum temperatures for growth of the thermophilic fungi and actinomycetes which are the micro-organisms thought to be responsible for the breakdown of the cellulose, hemi-cellulose and lignin fractions in the composting process (Burman, 1961). The importance of the effect of temperature upon the activities of the microbial populations of composts has been demonstrated by Waksman, Cordon and Hulpoi, (1939). In the present investigation the purpose of making isolations at a range of temperatures between 25 and 50°C was to find out the effect of temperatures upon micro-organisms in a microbial population and therefore reveal their importance in the composting process.

The major part of decomposition in composting processes is thought to be brought about by aerobic thermophilic micro-organisms and the most successful mechanical composting systems for the treatment of refuse have all promoted conditions suitable for aerobic thermophilic composting, (this point is discussed in greater detail in the chapter on the microbiology of composting). The isolation of aerobic thermophilic micro-organisms from the samples of refuse was therefore especially significant.

Some of the first studies on the isolation of the thermophilic fungi by conventional techniques (Crisan, 1959) had indicated a relative rarity of these fungi in soil. Subsequently Eggins and Malik (1969) were successful in isolating many of the confirmed thermophilic fungi from one pastureland soil by the use of soil enrichment methods and selective isolation media. In the present investigation it was expected that the thermophilic fungi would probably be widespread throughout the samples of mature and immature compost but that in the other samples of refuse it was expected that they would only be rarely isolated by conventional techniques. In order to try and ensure that any thermophilic fungi present in the samples were isolated it was decided to adopt the soil enrichment methods used by Eggins and Malik (1969). The soil enrichment method has been one of the most common procedures used for the isolation of specific micro-organisms (Waksman and Schatz, 1945).

The enrichment technique was not used with the samples obtained from the household dustbins because the nature and quantities of these samples made it impracticable. Isolations were made from these samples with the technique developed by Warcup (1950) for the isolation of fungi from the soil. The soil plate method was developed by Warcup to eliminate some of the disadvantages inherent in the isolation of fungi with the dilution plate technique. With the dilution plate method some of the heavier soil particles present in the suspension were inevitably left behind when the dilution plates were prepared and therefore any mycelium

present in these particles was also lost. This method also conferred an advantage on the heavily sporing fungi by dispersing their spores throughout the suspension ensuring their subsequent domination of the dilution plates. By deliberately reducing the degree of soil dispersion Warcup's method minimises the advantages of the heavily sporing fungi. The degree of soil dispersion was reduced by retaining the whole of the soil for isolation thus making it possible to culture any fungus mycelium embedded in the soil particles. The advantages, in numerical terms, of the heavily sporing species was further reduced by Warcup's method of recording the frequency of species not as numbers of colonies per plate but as simply present or absent on the individual isolation plate. Garrett (1951) described the soil plate method of Warcup as the best compromise so far evolved as a non-selective method for the general isolation of soil fungi.

In the present investigation the scrapings from the bins were plated out in the normal way as for soils. However, this procedure could not be used with the large pieces of refuse which were plated out by removing small pieces of paper and vegetable matter with sterile forceps and scissors and placing them aseptically into a petri dish. A small quantity of any particulate matter present in the refuse such as ash was also added to each petri dish. The plating out procedure was then completed in the normal way using two isolation media. These were a cellulose agar developed by Eggins and Pugh (1962) for the selective isolation of cellulolytic fungi and a glucose-starch agar containing the same mineral salts as the cellulose medium and

substituting an equivalent concentration of glucose and starch for the cellulose. Rose Bengal at a concentration of 1 part in 15,000 (0.066g/litre) was added to both media to suppress the growth of bacteria and also to limit the spread of fast growing fungi over the whole isolation plate thus giving slower growing fungi a chance to develop, (Smith and Dawson, 1944; Garrett, 1963; Ottow, 1972). The plates were incubated at the following temperatures, 25, 30, 35, 40, 45 and 50°C.

With the remaining five samples four methods of isolation were used at each temperature. The first method was the Warcup isolation technique as described above; the remaining three methods involved an adaption of this technique. Three separate 100g. portions of each sample were taken, the moisture content of each sample was adjusted to 25-30% of the total weight and then one portion was amended with powdered cellulose (Whatman's Chromedia CF11) another with glucose and the third portion was left unamended. The cellulose and glucose added amounted to 4% of the total weight of the sample. Each of the three portions was placed in a clean beaker which was covered with a watch glass and incubated at one of the temperatures listed above for 7 days. At the end of this time 40 Warcup isolation plates were prepared from each incubated portion, 20 with cellulose agar and 20 with glucose starch agar. The isolation plates were incubated at the same temperature as the amended and unamended portions for 7 days after which time they were examined and the presence of fungi noted. For the plates incubated at 40, 45 and 50°C the presence of

any actinomycetes was also noted. The frequency of occurrence of a particular species on either the cellulose or glucose-starch plates was calculated as a percentage from the total number of plates on which it appeared. The procedure was repeated for all five samples for each of the six temperatures mentioned.

After the sample of immature compost had been collected from the two-week old windrow ten screened substrate tubes (Eggins and Lloyd, 1968) were buried in the central region of the same windrows so that isolations could be made of the actively growing mycoflora and compared with the results obtained from the other isolation techniques. (A full description of the screened substrate tube technique is given in Chapter 3 which is concerned with the microbiology of composting). The screened substrate tubes were left in the windrow for 7 days after which time isolations were made from the screened cellulose strip on cellulose and glucose-starch agar plates. The plates were incubated at 50°C and after 7 days the fungal species present were noted and their percentage frequency of occurrence calculated. The identifications of the fungi were carried out with reference to the work of Gilman, (1957); Barnett, (1960); Ames (1961); Cooney and Emerson, (1964) and Barron, (1968).

After the isolation studies outlined above had been completed the effect of temperature on the growth of ten thermophilic and eleven mesophilic fungi all isolated from the refuse disposal system with the exception of Rhizoctonia

solani, a member of the mycelia sterilia, was investigated.

A widely used method for measuring the linear growth of fungi was developed by Brancato and Golding (1953) who found that the changes in the diameter of a fungal colony with incubation time gave a reliable measure of growth and consequently this method has been used in the present study. The medium used was yeast, peptone and soluble starch agar (Cooney and Emerson, 1964) which was found to be an acceptable general growth medium for the twenty test species. Using an 8mm cork borer, inocula were cut out from 7 day old cultures of the twenty fungi required, these were also grown on YPSS agar. The inocula were aseptically transferred to the centre of the petri dishes each of which contained 15ml of YPSS; three replicates were prepared for each test species. The inoculated plates were incubated at the following temperatures, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65^{o}C. The colony diameter was measured every 24 hours for the mesophilic fungi and every 12 hours for the thermophilic fungi. Accurate measurements of the diameters of the colonies were obtained by removing the lid of the petri dish and opening out the points of a pair of dividers till they were touching the edges of the colony across a diameter, the distance between the points of the dividers was then read off against a rule. Two measurements were taken for each colony, the second measurement being taken at right angles from the first. The points of the dividers were sterilised between each measurement by flaming them in alcohol. The usual precautions of aseptic procedure were observed at all times.

Results

The average percentage frequencies of occurrence of the fungi isolated from the samples of refuse and scrapings taken from the household dustbins are given in Tables 2.1(a) and 2.1(b). The frequencies of occurrence of the fungi isolated from the samples from each dustbin at 25, 30, 35, 40, 45 and 50°C are given in Tables 1-12, Appendix II.

The fungi were well represented in both the samples of refuse and scrapings taken from the household dustbins. Ten of the thirteen thermophilic fungi described by Cooney and Emerson (1964) were isolated plus a thermophilic species of Cephalosporium. Ten thermophilic fungi were present in the refuse and nine in the scrapings. Thirty-three different species of mesophilic fungi were isolated and of these all were present in the refuse and twenty-five in the scrapings. The thermophilic and mesophilic fungi generally had a higher frequency of occurrence in the refuse than in the bin scrapings, thus showing that the refuse had a richer and more varied mycoflora than the bin scrapings.

The location of the dustbins did not in any way appear to affect either the nature or the frequency of occurrence of the fungi isolated. The results of the isolations made from the dustbins sampled in the residential area are given in Tables 1-12 Appendix II, under the columns numbered 1-10 and the results of the isolations made from the dustbins from the council estate are given in the same tables under the columns numbered 11-20. The

Table 2.1(a) Average percentage frequency of occurrence of the fungi isolated from samples of refuse and bin scrapings on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 50°C, 45°C and 40°C.

FUNGI	REFUSE						SCRAPINGS					
	50°C		45°C		40°C		50°C		45°C		40°C	
	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	25.5	64.75	80	86.75	74.75	86.75	33	28.25	47	58.25	45.75	55.5
Aspergillus fumigatus (O.V.)						3.75						
Aspergillus niger					0.25	0.5						
Cephalosprium sp.	4	6.25	15.75	9.75	10.25	15.25			0.5	2.5	60	57.75
Chaetomium thermophile	14.25	13.5	7.0	4.75	4.25	4.75	7.75	4.5	2.5		2.25	
Coprinus sp.					0.25							
Eurotium sp.					1.25	0.25						
Humicola grisea	0.5	0.75		1								
Humicola insolens	8.0	45	2	0.25	6	0.75	4	0.5	0.75			
Humicola lanuginosa	12	11.5	5.25	0.5	2.5		3.5	5.75	1			
Malbranchea pulchella	3	2	2.25				6.25	1.25	10.75	2.5	2.75	1.25
Mucor pusillus	0.25		4.75	10.25	10.75	14.25		0.5	0.5	4.25	0.75	5.25
Penicillium funiculosum				3.75	4.75	2						
Sporotrichum thermophile	1											
Talaromyces duponti	14.75	8.25	0.5	0.25	0.25	3.25	2					
Thermoascus aurantiacus							1.5					
Torula thermophila	18.75	7	9.25	6.25	12	3	1.25	2.25				
Actinomycetes	23.5	10	29.75	24	28.25	8.5	8.25	4.25	12.25	4	7.5	4.75

Table 2.1(b) Average percentage frequency of occurrence of the fungi isolated from samples of refuse and bin scrapings on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 35°C, 30°C and 25°C.

FUNGI	REFUSE						SCRAPINGS					
	35°C		30°C		25°C		35°C		30°C		25°C	
	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS
Alternaria			2.25	2	7	6			5	3	8.5	1.75
Arthrobotrys sp.				1								
Aspergillus fumigatus	79.75	79.25	25.5	26.25	10.75	4.5	63.75	73	7	12.75	10.75	11.5
Aspergillus fumigatus (O.V.)*	7.25	3										
Aspergillus niger	10.75	4.25	0.75	16.75	0.75	4.75	0.5	0.75	5.75	8		8.25
Aspergillus sp.			2.25	2	1.25	0			8	2.25	3.25	0.25
Aureobasidium pullulans	1.25	0.75							5.75	0.5	4.25	6.75
Cephalosporium sp. +	14.25	9.5					5.5	10.5				
Cephalosporium sp.			34.25	30.75	27.25	13.75			36.5	33	23.5	
Cerastomella sp.			1						1.75	0.25	5.75	0.75
Chaetomium globosum			1		3.5				2.5	1.5	6.25	0.5
Eurotium sp.				1						1		0.75
Eurotium vermiculatum			1.25	0.25						1		0.25
Fusarium sp.			54.25	18.75	64.25	27			21.75	13.5	30.25	10.75
Geotrichum candidum					1.75	7.25					1.25	1.25
Gliocladium roseum			58.5	17.25	69	42			7.25	2	16.75	1
Gliomastix sp.			3.75		10	0.5						1
Graphium sp.					2							
Humicola grisea			10	4	14	5.25			7.25	1	16.25	2

Cont'd/...

Table 2.1(b) Cont'd.

FUNGI	REFUSE						SCRAPINGS					
	35°C		30°C		25°C		35°C		30°C		25°C	
	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS
Malbranchea pulchella +	0.25	2					0.75					
Monila brunnea		1.5	0.5		5.25	2.25			24.5	4	29.75	2.25
Mucor globosus			76	64.25	62.5	75			37	54.5	39	50.25
Mucor pusillus +	18.5	35					1					
Paecilomyces varioti		1.75	3.75	1.25	4.75	1.25		1.75	o.25	0.5		0.5
Penicillium funiculosum	23	35.5	73	91	67.75	84	1.25	2.25	73.5	77.75	84.5	85.5
Penicillium sp.			5.75	22.25	6.25	6.5			16	26.25	12.25	25.25
Periconia sp.			0.75	0.25	8	9.5						
Rhizopus nigricans			5	20	12.25	15			1.75	10	14.75	20.5
Sporotrichum pruinosum			1		3.75				1.5	0.25	10	2.5
Stachybotrys atra			6.25		6.25	0.5						
Stysanus sp.			19.5	2	17	1.5					0.25	
Torula thermophila +	5.75	1.75	0.25									
Trichoderma viride			28	21	41	55			27.25	13.75	14.75	17.5
Ulocladium sp.			3.75		1.5				3.75	2.75	5	1.5
Verticillium lateritium			8.5	4.75	2.25	1.5						
Verticillium sp.					1							
Zygorhyncus moelleri				0.25		0.5						

* O.V. - Orange Variant

\+ Denotes Thermophile

results presented in Tables 1-12, Appendix II would seem to indicate a lack of correlation between the mycoflora of the refuse and the scrapings removed from the same bin.

The temperature of incubation of the isolation plates had a marked effect on the nature of the fungi isolated from these samples.

At 50°C the fungi isolated were, with the exception of the thermotolerant Aspergillus fumigatus, all thermophilic. In the refuse samples the most commonly occurring thermophilic fungi were Torula thermophila, Talaromyces duponti, Chaetomium thermophile and Humicola lanuginosa, but the most commonly occurring fungus was the thermotolerant Aspergillus fumigatus. The remaining thermophilic fungi all had a frequency of occurrence of less than 10%. From the scrapings removed from the bins Aspergillus fumigatus was again the most frequently isolated fungus, all of the thermophilic fungi isolated from these samples had a frequency of occurrence less than 10% and of these the most common were Chaetomium thermophile, Malbranchea pulchella and Humicola lanuginosa. Tables 1 and 2 presented in Appendix II show that at 50°C the distribution of the thermophilic fungi was spread evenly over the dustbins sampled and was not limited to one or two heavily infected bins. Aspergillus fumigatus was present in every sample of refuse. Fifteen of the twenty samples of refuse contained two or more species of thermophilic fungi and five samples contained at least four different species of these fungi. The most widespread thermophile was Chaetomium

thermophile which appeared in eleven of the samples of refuse, Humicola lanuginosa and Talaromyces duponti each appeared in eight of these samples. The thermophilic actinomycetes were also widespread appearing in eighteen of the samples of refuse with an average frequency of occurrence of 23.5%. Only two of the samples of scrapings did not contain any thermophilic or thermotolerant fungi, four samples contained only Aspergillus fumigatus and some thermophilic actinomycetes and five samples contained at least two species of thermophilic fungi. In the samples of scrapings Chaetomium thermophile and Malbranchea pulchella were the most commonly occurring thermophiles appearing in six and four samples respectively. Thermophilic actinomycetes were present in six samples.

At 45°C the situation was similiar to that described above, apart from Penicillium funiculosum and the thermotolerant Aspergillus fumigatus which had a greatly increased frequency of occurrence at this temperature, all the fungi isolated were thermophilic. Penicillium funiculosum usually accepted as a mesophilic fungi was isolated from two of the samples of refuse on the glucose-starch agar, repeated attempts to sub-culture it at this temperature were unsuccessful demonstrating that it was not a true thermophile. In both the samples of refuse and scrapings the frequency of occurrence of Chaetomium thermophile, Humicola grisea, Humicola insolens, Humicola lanuginosa, Talaromyces duponti and Torula thermophila all decreased compared with the results obtained at 50°C. However, the frequencies of occurrence of Malbranchea

pulchella, Mucor pusillus and Cephalosporium sp. all increased. The distribution of the fungi throughout the sample was similiar to that at 50°C, in the samples of refuse Chaetomium thermophile and Cephalosporium sp. were the most widespread fungi appearing in seven of the samples whereas in the scrapings Malbranchea pulchella and Mucor pusillus were the most widespread fungi appearing in four and six of the samples respectively. The thermophilic actinomycetes remained widespread appearing in sixteen of the samples of refuse and ten of the samples of scrapings.

At 40°C more fungi, usually known as mesophiles were isolated albeit with low frequencies of occurrence from the samples of refuse but not from the scrapings. These fungi were Aspergillus niger, Coprinus sp., Eurotium sp. and Penicillium funiculosum, in addition an orange variation of Aspergillus fumigatus was also isolated from two of the samples of refuse. In all seven species of thermophilic fungi were isolated from the samples of refuse at this temperature compared with the eleven species isolated at 50°C. Only four species were isolated from the samples of bin scrapings compared with ten species isolated at 50°C. Of all the thermophilic fungi isolated at this temperature only Mucor pusillus showed an increased frequency of occurrence in both sets of samples. The frequency of occurrence of Cephalosporium sp. in the samples of scrapings increased dramatically but in the samples of refuse it decreased slightly. In both sets of samples Aspergillus fumigatus remained the most frequently occurring fungus, in the samples of refuse Cephalosporium sp. and

Mucor pusillus were the most frequently occurring thermophiles whereas in the samples of scrapings Cephalosporium sp. and Malbranchea pulchella were most frequent. The thermophilic actinomycetes remained abundant at 40°C especially in the samples of refuse where their average frequency of occurrence on the cellulose plates was 28.25%. This compared with an average frequency of occurrence of 7.5% from the samples of bin scrapings.

At 35°C a mixture of thermophilic and mesophilic fungi were isolated from both sets of samples. The mesophilic fungi isolated were Aspergillus niger, Aureobasidium pullulans, Paecilomyces varioti and Penicillium funiculosum. Only three thermophilic fungi were isolated namely, Cephalosporium sp., Malbranchea pulchella and Mucor pusillus, compared with the eleven species isolated at 50°C. The most frequently isolated fungus was again Aspergillus fumigatus which appeared in every sample. The most frequently occurring thermophilic fungus in the samples of refuse was Mucor pusillus which had its highest frequency of occurrence at this temperature. In the samples of bin scrapings the most commonly occurring thermophilic fungus was Cephalosporium sp. Apart from Penicillium funiculosum in the samples of refuse the appearance of the mesophilic fungi at this temperature was not widespread their average frequency of occurrence being limited to less than 5% in all cases.

At 25°C and 30°C the mesophilic fungi dominated the isolation plates the only thermophile appearing at these temperatures was Torula thermophila which was iso-

lated from one sample of refuse at 30°C with a very low frequency of occurrence. There appeared to be very little difference between the fungi isolated at 30°C and 25°C, most of the fungi appearing at both temperatures with similiar frequencies of occurrence. The samples of refuse contained a greater variety of fungi than the samples of scrapings and these fungi were usually present with a larger frequency of occurrence than those in the scrapings. At 25°C and 30°C the most frequently isolated fungi in both sets of samples were Aspergillus fumigatus, a mesophilic Cephalosporium sp., Fusarium sp., Gliocladium roseum, Mucor globosus, Penicillium funiculosum, Penicillium sp., Rhizupus nigricans and Trichoderma viride. In the samples of refuse Stysanus sp. was also frequently isolated as was Monilia brunnea from the samples of bin scrapings. Aspergillus fumigatus (O.V.), Arthrobotrys sp., Graphium sp., Periconia sp., Stachybotrys atra, Verticillium lateritium, Verticillium sp. and Zygorhyncus moelleri were only isolated from the samples of refuse.

The fungi isolated from the samples of:-

(i) refuse from the reception hopper of the pulverisation plant

(ii) pulverised refuse

(iii) pulverised refuse and sewage sludge

(iv) immature compost

and (v) mature compost

by the normal and modified Warcup's techniques are listed in Tables 2.2(a) and 2.2(b). The frequencies of occurrence of the fungi isolated from the five samples, listed above,

Table 2.2(a)

The thermophilic and thermotolerant fungi isolated from the samples of refuse, pulverised refuse, pulverised refuse and sewage sludge, mature and immature compost.

Aspergillus fumigatus	Fresenius
Aspergillus fumigatus (O.V.)*	Fresenius
Cephalosporium sp.	
Chaetomium thermophile	La Touche
Humicola grisea	Traaen
Humicola insolens	Cooney et Emerson
Humicola lanuginosa	(Griffon et Maublanc)Bunce
Malbranchea pulchella	Saccardo et Penzig
Mucor pusillus	Lindt
Sporotrichum thermophile	Apinis
Talaromyces duponti	(Griffon et Maublanc emend Emerson) Apinis
Talaromyces emersonii	Stolk
Thermoascus aurantiacus	Miehe
Torula thermophila	Cooney et Emerson

* Orange variant

Table 2.2(b)

Mesophilic fungi isolated from the samples of refuse, pulverised refuse, pulverised refuse and sewage sludge, mature and immature compost.

Absidia sp.	
Arthrobotrys sp.	
Aspergillus niger	van Tieghem
Aspergillus sp.	
Cephalosporium sp.	
Chaetomium aterrimum	Ellis and Everhart
Chaetomium globosum	Kunze
Cerastomella sp.	
Cunninghamella sp.	
Dicoccum asperum	Corda
Didymocladium sp.	
Eurotium sp.	
Fusarium sp.	
Gliocladium deliquescens	Olsen-Sopp
Gliocladium roseum	(Link) Thom.
Graphium sp.	
Humicola grisea	Traaen
Hyaloflorea sp.	
Mucor globosus	Fischer
Oidodendron sp.	
Paecilomyces varioti	Bainier
Penicillium funiculosum	Thom.
Penicillium sp.2	
Penicillium sp.3	
Penicillium sp.4	
Periconia sp.	
Rhizopus nigricans	Ehrenberg
Sporotrichum pruinosum	Gilman and Abbott
Stachybotrys atra	Corda
Stysanus sp.	
Trichoderma viride	Pers. ex Fr.
Verticillium sp.	
Zygorhyncus moelleri	Namyslowski

by the normal Warcup's technique are presented in Tables 2.3(a) - 2.3(f). These results indicate that all of the five samples contain a relative abundance of thermophilic fungi and actinomycetes, particularly the samples of immature and mature compost. However, the distribution of the mesophilic fungi was resticted mainly to the samples of refuse and pulverised refuse only ten species being isolated from the samples of mature and immature compost.

At 50°C, Table 2.3(a), a total of twelve different species of thermophilic fungi were isolated from the five samples, of these ten species had been previously described by Cooney and Emerson (1964). The other thermophilic fungi were Talaromyces emersonii and this was first described by Stolk (1965) and Cephalosporium sp. Eight species of thermophilic fungi were present in the sample of refuse taken from the reception hopper of the pulverisation plant. This sample of refuse also contained the thermotolerant Aspergillus fumigatus and an abundance of thermophilic actinomycetes. In this sample the most frequently occurring fungi were Chaetomium thermophile, Aspergillus fumigatus, Humicola lanuginosa, Mucor pusillus and Talaromyces duponti. With the samples of pulverised refuse, sewage sludge, immature and mature compost, the fungi with the greatest frequency of occurrence were Aspergillus fumigatus, Chaetomium thermophile, Humicola lanuginosa and Mucor pusillus. The frequencies of occurrence of these four fungi were smallest in the samples of pulverised refuse and sewage sludge and greatest in the sample of mature compost. The percentage frequencies of occurrence of Chaetomium thermophile, Humicola

Table 2.3(a) Percentage frequency of occurrence of fungi isolated from samples from a refuse disposal system, on cellulose (C) and glucose starch (GS) agars by a normal Warcup's technique at 50°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	40	40	40	55		5	20	10	10	20
Cephalosporium sp.				5	5	25				
Chaetomium thermophile	85	75	30	35	25	25	70	35	100	40
Humicola grisea	10	10	20		5					
Humicola insolens	5		5		5			5	100	45
Humicola lanuginosa	40	35	85	80	35	5	85	70	100	75
Malbranchea pulchella		5			5					
Mucor pusillus	10	25	10	50	5	15	10	40	65	100
Sporotrichum thermophile				10						
Thermoascus aurantiacus				5				10		
Talaromyces duponti	10	20					5	5		
Talaromyces emersonii		5								
Torula thermophila							5			
Actinomycetes	20	15	40	25	50	60	85	75	100	85

Table 2.3(b) Percentage frequency of occurrence of fungi isolated from samples from a refuse disposal system, on cellulose (C) and glucose-starch (GS) agars by a normal Warcup's technique at 45°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	85	90	100	100	100	100	100	100	100	100
Cephalosporium sp.		10		5				5		15
Chaetomium thermophile	35	15	70	20	10	20	65	40	65	30
Humicola grisea	25	15	5	-						
Humicola insolens	5	-		5					70	15
Humicola lanuginosa	25	-	60	40			75	80	85	70
Malbranchea pulchella			45	40	5					
Mucor pusillus	5	50	5	30	20	60	20	90		100
Sporotrichum thermophile			15	10						
Thermoascus aurantiacus			25	10					50	
Talaromyces duponti		10								
Talaromyces emersonii		10				5				
Torula thermophila								10		
Actinomycetes										

Table 2.3(c) Percentage frequency of occurrence of fungi isolated from samples from a refuse disposal system, on cellulose (C) and glucose-starch (GS) agars by a normal Warcup's technique at 40°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	100	100	85	100	40	100	100	100	85	100
Cephalosporium sp.	20	25	15	20						
Chaetomium thermophile	15		15				20	15	100	40
Humicola grisea	5		5							
Humicola insolens	5		10						90	90
Humicola lanuginosa	10		5	15			30	25	60	85
Malbranchea pulchella			40	15					25	45
Mucor pusillus	45	75	5	40	25	80	60	100	60	100
Sporotrichum thermophile										
Thermoascus aurantiacus										
Talaromyces duponti			5							
Talaromyces emersonii				15						
Torula thermophila			5							
Actinomycetes	25	15	45	15	15	5	85	60	45	40

Table 2.3(d) Percentage frequency of occurrence of fungi isolated from samples from a refuse disposal system, on cellulose (C) and glucose-starch (GS) agars by a normal Warcup's technique at 35°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Absidia sp.		15								
Aspergillus fumigatus	90	70		80	20		80			
Aspergillus niger		30							30	50
Aspergillus fumigatus*(O.V.)							25			
Arthrobotrys sp.	35		25	20			60			
Cephalosporium sp. +	20		25				25			
Chaetomium thermophile	20	20	10						5	
Chaetomium globosum							15			
Cunninghamella sp.	35	30	35	35						
Cerastomella sp.			5							
Didymocladium sp.	10									
Eurotium sp.									15	20
Graphium sp.			25				60			
Humicola grisea var.thermoidea									5	

Cont'd/...

Table 2.3(d) Cont'd.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Humicola insolens +			10						15	
Malbranchea pulchella +	20		10						20	25
Mucor globusus	35	35	15	25						
Mucor pusillus	20	40		55	20	100	25	100	40	60
Paecilomyces varioti				40						
Penicillium funiculosum		35	10	20						
Penicillium sp.2		10								
Penicillium sp.3		20								
Penicillium sp.4		35								
Rhizopus nigricans	20	10		5						
Talaromyces emersonii			5	5					15	5
Trichoderma viride	20	15	25	20	100	100	60	100	30	15
Verticillium sp.			5	35						

* O.V. - Orange Variant

\+ Denotes Thermophile

Table 2.3(e) Percentage frequency of occurrence of fungi isolated from samples from a refuse disposal system, on cellulose (C) and glucose-starch (GS) agars by a normal Warcup's technique at 30°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	15	25		20	10	65	100	70	100	100
Aspergillus niger	15	5	15	45						30
Arthrobotrys sp.					60	10	50	35	100	
Cephalsoproum sp.	45	40	40	30	40					
Chaetomium aterrimum										
Chaetomium globosum	30	5	10		10				70	
Cunninghamella sp.	50	65	25	40						100
Cerastomella sp.			5		15					
Dicoccum asperum	5		20							
Didymocladium										
Fusarium sp.	40	20	65	25	40	10	45	10	60	30
Gliocladium roseum	5	15	30	50						
Graphium sp.					10		45	45	30	
Humicola grisea	10	10	5	10	5				70	

Cont'd/...

Table 2.3(e) Cont'd.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Hyaloflorea sp.			5							
Mucor globosus	60	100	50	100	85	70				
Mucor pusillus +	10	15	20	20	15	25	35	90	100	30
Paecilomyces varioti	55	50	50	30						
Penicillium funiculosum	75	65	45	55	10	20	20		90	100
Penicillium sp.2	55	30	10	40						
Penicillium sp.3		5								
Penicillium sp.4	10	25	15							
Periconia sp.			15		25					
Rhizopus nigricans		40		5	5					
Sporotrichum pruinosum				5						
Stachybotrys atra					55					
Stysanus sp.	25		65	30	20		40	5	70	20
Trichoderma viride	60	55	40	15	35	35	35	35	100	45
Verticillium sp.		20								
Zygorhyncus moelleri		15								

+ Denotes Thermophile

Table 2.3(f) Percentage frequency of occurrence of fungi isolated from samples from a refuse disposal system, on cellulose (C) and glucose-starch (GS) agars by a normal Warcup's technique at 25°C.

Fungi	Refuse		Pulversied Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Arthrobotrys sp.	5		45		100		100		95	
Aspergillus fumigatus									100	100
Aspergillus fumigatus (O.V.)										5
Aspergillus niger				35						35
Cephalosporium sp.	50	50	45		35		20			
Chaetomium aterrimum			5							
Chaetomium globosum	25	5	10						25	5
Cunninghamella sp.	45	25	60	85					10	100
Cerastomella sp.	25		90							
Dicoccum asperum	5	10								
Didymocladium sp.	5		10	10						
Fusarium sp.	25	20	30	100	45	20	40		35	30
Gliocladium deliquescens	5		80							
Gliocladium roseum	45	30	75	100						

Cont'd/...

Table 2.3(f) Cont'd.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Humicola grisea	10	10	5						100	20
Mucor globosus	30	50		100	100	100				
Mucor pusillus +							100	100	40	30
Oidodendron sp.	25	10	35							
Paecilomyces varioti	60	50	55	20						
Penicillium funiculosum	45	55	60	75	15		20		45	100
Penicillium sp.2	10	40	30	30						
Penicillium sp.4			5							
Periconia sp.										
Rhizopus nigricans	5	65								
Stysanus sp.	10		95	50	10	10	60	10	100	25
Trichoderma viride	55	50	10	40	100	20	100	100	100	
Verticillium sp.			10							
Zygorhyncus moelleri	5	15								

+ Denotes Thermophile

insolens, Humicola lanuginosa, Mucor pusillus and the thermophilic actinomycetes all reached a maximum in the sample of mature compost indicating that they may have been active in the composting process. In the sample of immature compost which was collected from a two-week old windrow at a temperature of 59°C the most frequently isolated organisms were the thermophilic actinomycetes, Humicola lanuginosa and Chaetomium thermophile.

At 45°C, Table 2.3(b) the results obtained were very similiar to those described for 50°C, the most frequently isolated fungi from the five samples were again Aspergillus fumigatus, Chaetomium thermophile, Humicola lanuginosa and Mucor pusillus and the thermophilic actinomycetes. The percentage frequencies of occurrence of these fungi were again greatest in the sample of mature compost. The percentage frequency of occurrence of Aspergillus fumigatus had increased in all the samples at this lower temperature. Similiarly the frequencies of occurrence of Humicola grisea, Malbranchea pulchella and Mucor pusillus had all slightly increased in some of the samples.

At 40°C, Table 2.3(c) the most frequently isolated organisms from the five samples were Aspergillus fumigatus, Mucor pusillus and the thermophilic actinomycetes. The frequencies of occurrence of Chaetomium thermophile and Humicola lanuginosa were much lower than at the isolation temperatures of 45 and 50°C throughout the five samples, however, their frequencies of occurrence were again much greater in the sample of mature compost. Aspergillus

fumigatus and Mucor pusillus were isolated from all five samples with increased frequencies of occurrence at this lower temperature whereas the increase in the frequency of occurrence of Cephalosporium sp was restricted to the samples of refuse and pulverised refuse. Malbranchea pulchella and Humicola insolens both showed increased frequencies of occurrence in the samples of pulversied refuse and mature compost respectively.

With the three isolation temperatures of 50°C, 45°C and 40°C (when the development of the thermophilic fungi was greatest) the frequency of occurrence of Humicola insolens remained very low in the samples of refuse, pulverised refuse, pulverised refuse and sewage sludge and immature compost, but in the sample of mature compost it was consistently isolated with a very high frequency of occurrence of between 70 and 100% at these three temperatures. This may indicate that Humicola insolens plays an active role in the composting process.

At 35°C, Table 2.3(d) a mixture of thermophilic and mesophilic fungi were isolated. The thermophilic fungi isolated were Cephalosporium sp, Chaetomium thermophile, Humicola grisea, Humicola insolens , Mucor pusillus, Malbranchea pulchella and Talaromyces emersonii. With the exception of Mucor pusillus the frequencies of occurrence of these fungi were greatly reduced when compared with the results obtained at the isolation temperatures of 45°C and 50°C. The most frequently isolated fungi at this temperature from all of the five samples were Aspergillus

fumigatus (of which two varieties were isolated), Mucor pusillus and Trichoderma viride. Cunninghamella sp. was frequently isolated from the samples of refuse, pulverised refuse and immature compost. Chaetomium globosum, Eurotium sp., Graphium sp., Penicillium funiculosum, Penicillium sp. and Rhizopus nigricans were all frequently isolated from the sample of refuse.

At 25°C and 30°C, Tables 2.3(e) and 2.3(f), the fungi isolated were almost exclusively mesophilic, the only exceptions were the thermophilic Mucor pusillus, which was isolated at 25°C and 30°C (from all five samples at 30°C yet at 25°C it was only isolated from the samples of compost) and the two thermotolerant variations of Aspergillus fumigatus. Thirty-one different species of mesophilic fungi were isolated and of these the ones most frequently occurring throughout the five samples at the temperatures of 25°C and 30°C were Fusarium sp., Penicillium funiculosum, Stysanus sp. and Trichoderma viride. Aspergillus fumigatus was frequently isolated at 30°C from all five samples, particularly the two samples of compost, but at 25°C it was only isolated from the sample of mature compost. Only ten species of mesophilic fungi were isolated from the samples of mature and immature compost which is less than half of the number isolated from the samples of refuse and pulverised refuse. This would indicate that the mesophilic mycoflora is in some way changed and reduced in numbers during the composting process, i.e. these results support the hypothesis that many of the mesophilic species of fungi are indeed killed off during the peak heating phase of the

composting process and that subsequent recolonisation occurs. Cephalosproium sp., Cerastomella sp., Dicoccum asperum, Didymocladium sp., Gliocladium deliquescens, Gliocladium roseum, Hyaloflorea sp., Mucor globosus, Paecilomyces varioti, Penicillium spp. 2, 3 and 4, Periconia sp., Rhizopus nigricans, Verticillium sp. and Zygorhyncus moelleri were all isolated from one or more of the samples of refuse, pulverised refuse, pulverised refuse and sewage sludge, but not from either of the samples of compost.

With this normal Warcups technique the number of species of fungi (and their frequencies of occurrence) isolated from the sample of pulverised refuse and sewage sludge were consistently lower than the corresponding results obtained from the samples of refuse and pulverised refuse. The isolation plates prepared with samples of pulverised refuse and sewage sludge were often covered in a film of bacteria and it appears that this has inhibited the development of the fungi resulting in their lower frequencies of occurrence.

The frequencies of occurrence of the fungi isolated from the portions of the five samples which had been incubated for 7 days before plating out at that temperature of incubation are presented in Tables 2.4(a) - 2.4(f).

At 50°C, Table 2.4(a), a total of nine species of thermophilic fungi were isolated compared with the twelve species isolated by the normal Warcup technique. The most frequently isolated species with the modified Warcup

Table 2.4(a) Percentage frequency of occurrence of those fungi, isolated, from samples of refuse etc. which had previously been incubated for 7 days at 50°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 50°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	20	40	40	55			30	70		100
Cephalosporium sp.									100	
Chaetomium thermophile	100	20	100	100	100	100	100	100	100	90
Humicola grisea		5								
Humicola insolens	45	15	40	20	15	5	25	30	45	15
Humicola lanuginosa	100	70	100	100	100	100	100	70	100	75
Malbranchea pulchella	10		80							
Mucor pusillus	25	70	20	45	5	25		65	15	55
Sporotrichum thermophile										
Thermoascus aurantiacus										
Talaromyces duponti										
Talaromyces emersonii		5								
Torula thermophila			10							
Actinomycetes	100	85	95	60	75	75	85	70	95	80

Table 2.4(b) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been incubated for 7 days at 45°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 45°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	100	90	100	100	100	100	100	90	100	85
Cephalosporium sp.	5	-					35			40
Chaetomium thermophile	90	20	80	75	100	95	65	40	100	80
Humicola grisea										
Humicola insolens	25		35		60	5	45	10	100	
Humicola lanuginosa	90	90	80	30	40	50			100	85
Malbranchea pulchella				40	40					40
Mucor pusillus	30	45	15	55	15	65	5	90	10	100
Sporotrichum thermophile										
Thermoascus aurantiacus										
Talaromyces duponti										
Talaromyces emersonii										
Torula thermophia			5	5						
Actinomycetes	40	50	45	45	40	30	60	55	70	70

Table 2.4(c) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been incubated for 7 days at 40°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 40°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	100	100	100	65	100	95	100	100	100	100
Cephalosporium sp.			100	65		5	10		40	
Chaetomium thermophile				5	35		5			
Humicola grisea										
Humicola insolens	15		10		5		20	5	40	
Humicola lanuginosa	20	25	15	5						
Malbranchea pulchella										
Mucor pusillus	30	50	10	65	20	40	45	100		
Sporotrichum thermophile										
Thermoascus aurantiacus										
Talaromyces duponti										
Talaromyces emersonii										
Torula thermophila										
Actinomycetes	25	15	30	45	5		45	35	60	50

Table 2.4(d) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been incubated for 7 days at 35°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 35°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	100	100	75	60	100	90	100	100	100	100
Aspergillus niger			20	45					40	60
Chaetomium globosum	80									
Cunninghamella sp.	60	100	100	100			40	65		
Eurotium sp.	85								20	25
Graphium sp.	35		100							
Humicola insolens			25						15	
Hyaloflorea sp.			5							
Malbranchea pulchella									40	55
Mucor pusillus	100	100	100	10	100	90	100	100	75	80
Penicillium funiculosum	40	100		5						
Penicillium sp.2	15	40								
Paecilomyces varioti			45							
Rhizopus nigricans		100								
Trichoderma viride	45		55	35	100	80	100	100	20	5
Verticillium sp.				35		40				

Table 2.4(e) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been incubated for 7 days at 30°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 30°C.

Fungi	Refuse		Pulversied Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	35	40	40	45	20	45	25	100	60	75
Aspergillus niger				35						60
Arthrobotrys sp.			55		35	80	100		90	
Cephalosporium sp.			10	10						
Cerastomella sp.									10	
Chaetomium globosum	40								95	
Cunninghamella sp.	80	65	65	75	20	20		35		85
Eurotium sp.	5									15
Fusarium sp.	100	60	80	100	50	45	85		60	25
Gliocladium roseum	10		20							
Graphium sp.			15				55			
Mucor globosus	100	100	100	100	80	100				

Cont'd/...

Table 2.4(e) Cont'd.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Mucor pusillus +	5	20		30			90	100	70	65
Penicillium funiculosum	45	80	15	25	10	30	15	45		70
Penicillium sp.2	40	55		20		30				60
Periconia sp.									45	
Rhizopus nigricans	15	75						80		
Stachybotrys atra									5	
Stysanus sp.	5		100						50	
Trichoderma viride	100	100	60	45	100	100	100	100	45	50

+ Denotes Thermophile

Table 2.4(f) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been incubated for 7 days at 25°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 25°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus				10				25		
Aspergillus fumigatus (O.V.)			5	15					95	40
Aspergillus niger				5						
Arthrobotrys sp.			100		80		100		25	
Chaetomium globosum	40				20				55	
Cunninghamella sp.	55	60	85	20		10		10	40	60
Fusarium sp.	100	40	100	100	70	50	20		65	30
Geotrichum candidum				100		25				
Gliocladium roseum	40	15	90							
Graphium sp.		5								
Humicola grisea					10				40	10
Mucor globosus	100	100	100	100	100	100				
Mucor pusillus		5		10			60	85	70	75
Penicillium funiculosum	60	100	45	100	80	55	15	10	35	80
Penicillium sp.				30						
Rhizopus nigricans	80	85						5		
Stachybotrys atra			60							
Stysanus sp.	45		100				25		85	40

technique were in order of frequency, Chaetomium thermophile, Humicola lanuginosa, the thermophilic actinomycetes, Humicola insolens, Mucor pusillus and Aspergillus fumigatus. These were, with the addition of Humicola insolens the same species that were isolated by the normal Warcup's technique but in this instance the frequency of occurrence of each of the fungi was much greater in all the samples, except for that of the mature compost in which the frequencies of occurrence were approximately the same.

At 45°C, Table 2.4(b), Aspergillus fumigatus, Chaetomium thermophile, Humicola insolens, Humicola lanuginosa, Mucor pusillus and the thermophilic actinomycetes remained the most frequently isolated organisms. With the exception of Aspergillus fumigatus these organisms were all isolated with increased frequencies of occurrence compared with those obtained by the normal Warcup technique. At this temperature with the incubated samples only seven species of thermophilic fungi were isolated.

At 40°C, Table 2.4(c), the most frequently isolated species from all the samples were, Aspergillus fumigatus, Humicola insolens and the thermophilic actinomycetes. Cephalosporium sp. was frequently isolated from the samples of pulverised refuse, pulverised refuse and sewage sludge, mature and immature compost, whereas Mucor pusillus was frequently isolated from the samples of refuse, pulverised refuse, pulverised refuse and sewage sludge and immature compost. With this modified Warcup technique the most frequently occurring species were the same as those isolated

by the normal technique but the number of species isolated from the five samples was reduced from ten to five.

With this modified Warcup technique the differences in the frequencies of occurrence of the fungi isolated at 50, 40 and 45°C from the two samples of compost and of the fungi isolated from the three remaining samples was not so marked as with the normal technique.

At 35°C, Table 2.4(d), a mixture of thermophilic, thermotolerant and mesophilic fungi were isolated but in this case the number of species was reduced from the twenty-six recorded at the corresponding temperature by the normal technique to sixteen. The thermophilic fungi isolated were Humicola insolens, Malbranchea pulchella and Mucor pusillus. The most frequently isolated fungi from all five samples were Aspergillus fumigatus, Mucor pusillus, Trichoderma viride, the same as with the normal technique except that in this case the frequencies of occurrence were generally higher.

At 30°C, Table 2.4(e) and 25°C, Table 2.4(f) the only thermophilic fungus isolated was Mucor pusillus which was frequently isolated from all the samples except for pulverised refuse and sewage sludge. The most frequently isolated fungi from all five samples were Aspergillus fumigatus (only at 30°C), Cunninghamella sp., Penicillium funiculosum, Fusarium sp. and Trichoderma viride. Arthrobotrys sp., a nematode trapping fungus was frequently isolated from all of the samples except for the refuse.

Mucor globosus was isolated with an extremely high frequency of occurrence from the samples of refuse, pulverised refuse and sewage sludge, but as with the normal technique it was not present in either of the samples of compost. Only twenty species of fungi were isolated from the incubated samples at 30°C and only nineteen species at 25°C compared with the thirty species and twenty-seven species isolated by the normal Warcup technique at 30 and 25°C respectively. The frequencies of occurrence of the fungi isolated from the incubated samples were much greater than those of the fungi isolated by the normal Warcup technique.

The results described above indicate that the technique of incubating the sample at the temperature of isolation for a period of 7 days before plating out reduces the number of species isolated but increases the frequency of occurrence of the quicker growing and heavily sporulating species. These type of fungi such as Chaetomium thermophile, Aspergillus fumigatus, Trichoderma viride, Mucor pusillus and Fusarium sp. were particularly prevalent in the samples of mature compost indicating that they may be active in the composting process.

The frequencies of occurrence of the fungi isolated from the portions of the samples which were amended with cellulose and incubated at the temperature of isolation for 7 days before plating out are presented in Table 2.5(a) - 2.5(f).

Table 2.5(a) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been amended with cellulose and incubated for 7 days at 50°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 50°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus						15		5		
Cephalosporium sp.									60	100
Chaetomium thermophile	100	60	95	60	45	30	100	95	100	100
Humicola grisea	20	25	20							
Humicola insolens	80	75	40	15	25		35	5	45	15
Humicola lanuginosa	100	100	100	100	5	30	90	95	100	100
Malbranchea pulchella			40	35						
Mucor pusillus	65	65	70	80	40	75	100	55	5	10
Sporotrichum thermophile			35	10						
Thermoascus aurantiacus										
Talaromyces duponti										
Talaromyces emersonii										
Torula thermophila							5			
Actinomycetes	100	100	100	100	100	100	100	100	100	100

Table 2.5(b) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been amended with cellulose and incubated for 7 days at 45°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 45°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	100	80	50	70	100	100	100	40		100
Cephalosporium sp.					5	-			15	80
Chaetomium thermophile	100	90	100	100	100	100	100	95	100	
Humicola grisea		10	15	5		5				
Humicola insolens	100	10	40	10	40	5	40		100	
Humicola lanuginosa	100	100	100	100	25	10	85	80	100	
Malbranchea pulchella			100	100	85	35				
Mucor pusillus	10	90			100	100	75	100	·10	20
Sporotrichum thermophile			45	20						
Thermoascus aurantiacus										
Talaromyces duponti					10					
Talaromyces emersonii										
Torula thermophila		10	100	30	80	15	100	45		
Actinomycetes	95	60	100	55	65	40	95	95	100	100

Table 2.5(c) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been amended with cellulose and incubated for 7 days at 40°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 40°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	100	100	100	100	95	70	100	100	100	100
Cephalosporium sp.			45	15			15	45	35	20
Chaetomium thermophile										
Humicola grisea	15	5	10							
Humicola insolens	40	25	35	15	85	25				
Humicola lanuginosa	60	40	25	40						
Malbranchea pulchella			10							
Mucor pusillus				10	80	100	10	65	10	60
Sporotrichum thermophile			40						40	
Thermoascus aurantiacus										
Talaromyces duponti										
Talaromyces emersonii										
Torula thermophila										
Actinomycetes	40	10	55	40	35	50	45	45	50	45

Table 2.5(d) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been amended with cellulose and incubated for 7 days at 35°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 35°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Absidia sp.	10	100					100	100		
Arthrobotrys sp.										
Aspergillus fumigatus	95		20	85	65	100	100	95	85	70
Aspergillus niger	10	60		10				25		
Chaetomium globosum	70	10								
Chaetomium thermophile +	10									
Eurotium sp.	35									
Graphium sp.	45		100		10					
Humicola insolens									15	
Hyaloflorea sp.			25	15						

Cont'd/...

Table 2.5(d) Cont'd.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Malbranchea pulchella +			15							
Mucor pusillus +	45	40	80	10	55	35	15	100	15	65
Paecilomyces varioti			35	25						
Penicillium funiculosum	55	50			30	30	15	25	5	35
Penicillium sp.2	30	25	50	50	25		20			
Penicillium sp.3		60								
Stachybotrys atra			20							
Rhizopus nigricans	30	75	25	40						
Trichoderma viride	15	5	35	5	55	15	15	5	45	15
Verticillium sp.				30						
Cunninghamella sp.	100	95	100	90	20					

+ Denotes thermophiles

Table 2.5(e) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been amended with cellulose and incubated for 7 days at 30°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 30°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Arthrobotrys sp.			25		40		100		75	
Aspergillus fumigatus				10	65	70			40	
Aspergillus niger	15	40								100
Aspergillus sp.								100		
Cephalosporium sp.		5								
Chaetomium atterrimum	10									
Chaetomium globosum	50	15			10	5				
Cunninghamella sp.	10	25	60						70	
Dicoccum asperum	10									
Eurotium sp.	5								15	
Fusarium sp.	100	20	100	100	60	30	20		85	30
Gliocladium roseum	20	20	100	25	40	20				

Cont'd/...

Table 2.5(e) Cont'd.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Graphium sp.	50		50							
Mucor globosus	65	70	45	100						
Mucor pusillus +					10	5	30	20	20	40
Oidodendron sp.	40	5	35							
Paecilomyces varioti	50	95	50	80						
Penicillium funiculosum	100	75	40	90	15	5	45	65	85	100
Penicillium sp.2	30	25	40	85				15	50	
Penicillium sp.3		30								
Rhizopus nigricans	25	60	15	5				25		
Stachybotrys atra			55							
Stysanus sp.	95	15	100	30	40	5	35	15	90	30
Trichoderma viride	100	95	80	45	70	30	100	100	90	10
Verticillium sp.		20		50						

+ Denotes Thermophiles

Table 2.5(f) Percentage frequency of occurrence of those fungi isolated from samples of refuse etc., which had previously been amended with cellulose and incubated for 7 days at 25°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 25°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Arthrobotrys sp.			100		30		85	5	60	
Aspergillus fumigatus							55		40	
Aspergillus niger										30
Aspergillus sp.										
Cephalosporium sp.	40		55							
Chaetomium aterimum			15							
Chaetomium globosum	55	10			5					
Cerastomella sp.			100							
Cunninghamella sp.				20						
Dicoccum asperum	15									
Eurotium sp.										
Fusarium sp.	75	70	60	100	50	40	40	10	70	40
Gliocladium deliquescens			60							
Gliocladium roseum	50	10	85	100	80	75				

Cont'd/...

Table 2.5(f) Cont'd.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Graphium sp.	35		30							
Humicola grisea			20							
Mucor globosus	50	65	50	75						
Mucor pusillus +					15	25	10	15	5	20
Oidodendron sp.	80		35							
Paecilomyces sp.	95	100	90	100						
Penicillium funiculosum	60	100	60	90	15	5	40	70	65	80
Penicillium sp.2	45	20	35	30				30	5	20
Penicillium sp.3										
Penicillium sp.4			5							
Rhizopus nigricans	20	100	5	30						
Stachybotrys atra										
Stysanus ap.	75	10	90	20	40	15	15	5	45	20
Trichoderma viride	80	65	95	100	100	35	100	95	100	80
Verticillium sp.			5							
Zygorhyncus moelleri		100								

+ Denotes Thermophile

At 50°C, Table 2.5(a), a total of nine species of thermophilic fungi plus the thermophilic actinomycetes and the thermotolerant Aspergillus fumigatus were isolated. The thermophilic actinomycetes were isolated more frequently than any one of the fungi, having an average frequency of occurrence from all five samples of 100%. The most frequently isolated fungi from all of the samples were Chaetomium thermophile, Humicola lanuginosa, Humicola insolens and Mucor pusillus. The frequencies of occurrence of these fungi and the thermophilic actinomycetes were much greater than those obtained from the isolations made with the normal Warcup's technique, but were not greatly different from those obtained with the unamended and incubated samples with the exception of the increase in the frequency of occurrence of the thermophilic actinomycetes. The frequency of occurrence of Aspergillus fumigatus was substantially reduced in comparison with the results of the other techniques of isolation.

At 45°C Table 2.5(b), the most frequently isolated fungi were again Chaetomium thermophile, Humicola lanuginosa and Humicola insolens. The frequency of occurrence of Aspergillus fumigatus increased at this lower temperature for isolation. The frequency of occurrence of the thermophilic actinomycetes remained high in all of the five samples, particularly the two samples of compost. With the exception of the sample of pulverised refuse Mucor pusillus was found to be widespread and was frequently isolated. Torula thermophila appeared quite frequently on the plates prepared from the samples of refuse, pulverised refuse,

and sewage sludge and immature compost. Malbranchea pulchella was prevalent in the samples of pulverised refuse and sewage sludge.

At 40°C, Table 2.5(c), the most frequently isolated fungus from all five samples was Aspergillus fumigatus. The frequencies of occurrence of the seven species of thermophilic fungi which were isolated had decreased from those obtained at the higher temperature of incubation and were isolated only infrequently. The frequency of occurrence of the thermophilic actinomycetes also decreased with the lower temperature of incubation but were still isolated from each sample.

At 35°C, Table 2.5(d), a mixture of thermophilic and mesophilic fungi were isolated. The thermophilic fungi isolated were Chaetomium thermophile, Humicola insolens, Malbranchea pulchella and Mucor pusillus, with the exception of Mucor pusillus these fungi were only isolated from one sample and usually had a very low frequency of occurrence. The number of species isolated was reduced from twenty-six with the normal Warcup technique to twenty-one, the most frequently isolated fungi from all five samples were Aspergillus fumigatus, Mucor pusillus and Trichoderma viride. At this temperature the mesophilic fungi were mainly isolated from the samples of refuse and pulverised refuse, only seven species were isolated from the sample of immature compost and only five from the sample of mature compost. The most frequently occurring mesophilic fungi were Trichoderma viride and Penicillium funiculosum.

At 30°C, Table 2.5(e), and 25°C, Table 2.5(f), the only thermophilic species isolated was Mucor pusillus which only appeared in the samples of pulverised refuse and sewage sludge and mature and immature compost. With this technique of amending the samples with cellulose twenty-five species of fungi were isolated at 30°C and twenty-six species at 25°C. These figures are greater than those obtained with the isolations made from the unamended and incubated samples at 30°C and 25°C, but less than those obtained by the normal Warcup's technique. The most frequently isolated fungi from the five samples at these two temperatures were Fusarium sp., Penicillium funiculosum, Stysanus sp. and Trichoderma viride. The remaining species were once again mainly isolated from the samples of refuse and pulverised refuse.

The effect of amending the samples with cellulose and incubating them at the temperature of isolation for 7 days was similiar to that produced by just incubating the samples, in that the number of fungal species recorded at any temperature was less than that recorded by the normal Warcup technique. The frequency of occurrence of some of the known cellulolytic fungi, mesophilic and thermophilc, such as Humicola insolens, Chaetomium thermophile, Aspergillus fumigatus, Chaetomium globosum, Fusarium sp. Trichoderma viride, Stysanus sp. and to a lesser extent Gliocladium roseum, increased in the isolations made from the samples incubated with an amendment of cellulose. This was particularly noticeable with the sample of refuse and pulverised refuse. The frequency of occurrence of some sugar fungi notably the thermophiles Humicola lanuginosa,

Mucor pusillus and the mesophiles Mucor globosus and Paecilomyces varioti also increased in the samples of refuse and pulverised refuse. They were probably fulfilling the role of a secondary sugar fungus defined by Garrett (1951, 1963). These changes in the populations of the fungi were not apparent in the two samples of compost, most probably because of the effect of the composting process on which an increase in the population of cellulose decomposing organisms is almost certain to occur and any subsequent additions of cellulose to such samples would be unlikely to markedly affect a microbial population already containing an abundance of cellulose decomposing organisms.

The frequencies of occurrence of the fungi isolated from the portions of the samples which were amended with glucose and incubated at the temperature of isolation for 7 days before plating out are presented in Tables 2.6(a) - 2.6(f).

At 50°C, Table 2.6(a), a total of seven species of thermophilic fungi were isolated of which the most frequently occurring from all five samples were Chaetomium thermophile, Humicola lanuginosa and Mucor pusillus. The thermophilic actinomycetes remained widespread throughout all the samples. The number of species of thermophilic fungi isolated from all samples amended with glucose was similiar to the number isolated from the samples which had been either amended with cellulose or just incubated and was much lower than the number isolated by the normal Warcup's technique. The frequencies of occurrence of the sugar fungi, i.e. Humicola

Table 2.6(a) Percentage frequency of occurrence of those fungi isolated from the samples of refuse etc., which had previously been amended with glucose and incubated for 7 days at 50°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 50°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	85	90		5	10	15				
Cephalosporium sp.										
Chaetomium thermophile	75	60	100	55	20	20	100	65	100	40
Humicola grisea										
Humicola insolens						5				
Humicola lanuginosa	85	85	100	100	65	95	100	95	100	85
Malbranchea pulchella	5	15	20	25						
Mucor pusillus	100	100	80	70	100	100	80	85	100	100
Talaromyces duponti					5					
Talaromyces emersonii										
Thermoascus aurantiacus				5						
Sporotrichum thermophile										
Torula thermophila										
Actinomycetes	45	5	55	15	40	10	35	20	55	40

Table 2.6(b) Percentage frequency of occurrence of those fungi isolated from the samples of refuse etc., which had previously been amended with glucose and incubated for 7 days at 45°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 45°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	100	100	100	100	100	100	100	100	90	100
Cephalosporium sp.	40	15	50	20			15			
Chaetomium thermophile	20		30		65	5	40		100	45
Humicola grisea										
Humicola insolens									80	
Humicola lanuginosa					45	40			85	65
Malbranchea pulchella	40	55	30	45						
Mucor pusillus	100	100	80	50	70	45	20	100	45	100
Sporotrichum thermophile										
Thermoascus aurantiacus				5						
Talaromyces duponti										
Talaromyces emersonii										
Torula thermophila									75	
Actinomycetes	40	30	35	5	30		25	15	50	30

Table 2.6(c) Percentage frequency of occurrence of those fungi isolated from the samples of refuse etc., which had previously been amended with glucose and incubated for 7 days at 40°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 40°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	100	100	100	100	100	100	100	100	100	100
Cephalosporium	20	5								
Chaetomium thermophile										
Humicola grisea										
Humicola insolens									100	
Humicola lanuginosa										
Malbranchea pulchella	25	25	25	30						
Mucor pusillus	80	95	55	75	10	60	70	100	50	95
Sporotrichum thermophile										
Thermoascus aurantiacus										
Talaromyces duponti										
Talaromyces emersonii										
Torula thermophila										
Actinomycetes	20	5	35	15	15	5	40	25	60	45

Table 2.6(d) Percentage frequency of occurrence of those fungi isolated from the samples of refuse etc., which had previously been amended with glucose and incubated for 7 days at 35°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 35°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	100	100	90	85	80	85	100	100	70	85
Aspergillus niger	60	100	55	90	45	80				100
Aspergillus sp.								15		
Cephalosporium sp.+								5		
Cunninghamella	100	20	100	45	65	10	85		100	
Graphium sp.			5							
Malbranchea pulchella		5		10						
Mucor pusillus	30	50	10	55	15	45	100	100	25	100
Penicillium funiculosum	100	100	10	30	5	25	15	30	10	25
Penicillium sp.2	100	100	90		100	100	100	45		
Penicillium sp.3					20					
Stachybotrys atra										
Stysanus sp.										
Trichoderma viride	15	5								20

+ Denotes Thermophiles

Table 2.6(e) Percentage frequency of occurrence of those fungi isolated from the samples of refuse etc., which had previously been amended with glucose and incubated for 7 days at 30°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 30°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Arthrobotrys sp.			100		20		15		45	5
Aspergillus fumigatus	60	65	70	55	25	50	25	40	60	70
Aspergillus niger	5	15	65	40	5	15	10	40	25	100
Aspergillus sp.				35						
Cephalosporium sp.				15						
Cunninghamella sp.	100	100	65	60	40	45		45	100	100
Fusarium sp.	100	10	100	100	35	25	20	20	15	
Geotrichum candidum				60						
Gliocladium roseum			20							
Graphium sp.			5							
Mucor globosus	100	75	100	100	80	100				
Penicillium funiculosum	85	100	25	45	85	60	60	40		100
Penicillium sp.2	80	15	40	15	20					
Rhizopus nigricans	65	100	65	95						
Stachybotrys atra			60							
Stysanus sp.			100							
Trichoderma viride	100	100		40	80	100	100	100	25	20

Table 2.6(f) Percentage frequency of occurrence of those fungi isolated from the samples of refuse etc., which had previously been amended with glucose and incubated for 7 days at 25°C, on cellulose (C) and glucose-starch (GS) agars by Warcup's technique at 25°C.

Fungi	Refuse		Pulverised Refuse		Pulverised Refuse and Sewage Sludge		Immature Compost		Mature Compost	
	C	GS	C	GS	C	GS	C	GS	C	GS
Arthrobotrys sp.			50	10	15		25			
Aspergillus fumigatus					10	5	10		60	85
Aspergillus niger		5	45	10			30	30	15	70
Aspergillus sp.				10						
Cephalosporium sp.				5						
Cunninghamella sp.	100	100	65	70	30	45	40	60	100	100
Fusarium sp.	50	25	50	50	45	30	30	30	15	
Geotrichum candidum				60	80	70				
Gliocladium roseum			15							
Graphium sp.							5			
Mucor globosus	100	100	100	100	100	100	40	80	50	55
Penicillium funiculosum	100	100	100	100	95	80	45	50	30	55
Penicillium sp.2	100	100	90	85	55	85				
Rhizopus nigricans	100	100	60	40						
Stachybotrys atra			40							
Stysanus sp.			70							
Trichoderma viride	100	100		40	80	90	100	95	15	40

lanuginosa and Mucor pusillus were much greater from the samples amended with glucose than from the samples used in the normal Warcup technique. The frequency of occurrence of Mucor pusillus from the samples amended with glucose was also much greater than from either the samples which were amended with cellulose or from the samples which were unamended but incubated. The frequency of occurrence of Humicola lanuginosa remained approximately the same with all three modified Warcup's techniques. The frequency of occurrence of Chaetomium thermophile remained unaffected by the amendment of the samples with glucose.

At 45°C, Table 2.6(b), the most frequently isolated species from all five samples were Aspergillus fumigatus, Chaetomium thermophile, Mucor pusillus and the thermophilic actinomycetes. The frequency of occurrence of Aspergillus fumigatus was much greater at this temperature than at 50°C and averaged approximately 100% from all of the samples. The occurrence of thermophilic fungi was infrequent at this temperature, Humicola lanuginosa was only isolated from two of the samples, Humicola insolens was isolated from the sample of mature compost and Malbranchea pulchella and Cephalosporium sp. were only isolated from the samples of refuse and pulverised refuse. Chaetomium thermophile was the most frequently isolated thermophile from each sample, but its frequency of occurrence was lower than that obtained with the normal Warcup technique.

At 40°C, Table 2.6(c), only four species of thermophilic fungi were isolated and only one of these

Mucor pusillus was isolated from every sample. The thermotolerant Aspergillus fumigatus was also isolated from every sample with an average frequency of occurrence of 100% which was slightly higher than that obtained with the normal Warcup technique. The thermophilic actinomycetes were also isolated from every sample but with lower frequencies of occurrence than at the temperatures of 45 and 50°C.

At 35°C, Table 2.6(d), only three species of thermophilic fungi were isolated namely Cephalosporium sp., Malbranchea pulchella and Mucor pusillus which was frequently isolated from all five samples. The thermotolerant Aspergillus fumigatus was frequently isolated from all five samples. Ten mesophilic fungi were isolated and of these Cunninghamella sp. and Penicillium funiculosum were the only species to be isolated from all five samples. Asperillus niger was isolated with an increased frequency of occurrence (compared with the results from the other isolation techniques) from the samples of refuse, pulverised refuse, pulverised refuse and sewage sludge and mature compost. The effect of amending the samples with glucose had been to reduce the frequencies of occurrence of the cellulolytic fungi such as Trichoderma viride, increasing the frequencies of occurrence of the sugar fungi such as Aspergillus niger, Cunninghamella sp. and Penicillium sp.2 and also to reduce the number of species isolated when compared with the normal Warcup's technique.

At 25°C and 30°C, Tables 2.6(f) and 2.6(e) res-

pectively, the reduction in the number of species in the number of species isolated was most marked. At both temperatures only the same seventeen species were isolated compared with the thirty species isolated at 30° and the twenty-seven species isolated at 25°C by the normal Warcup technique. The most frequently occurring species in all five samples were Cunninghamella sp., Fusarium sp., Mucor globosus, Penicillium funiculosum and Trichoderma viride. Aspergillus niger was frequently isolated from all five samples at 30°C but not at 25°C. The frequency of occurrence of the cellulolytic fungus Trichoderma viride had been depressed at 35°C but at 25°C and 30°C it was again found to be widespread throughout all five samples with frequencies of occurrence equal to those obtained by the other isolation techniques.

The main effect of amending the samples with glucose was as in the cases of amending them with cellulose or simply incubating them at the temperatures of isolation for 7 days, to reduce the number of species of fungi isolated at all temperatures. The other effects of amending the samples with glucose were, to reduce the frequencies of the cellulolytic fungi and to increase the frequencies of occurrence of the sugar fungi, these latter effects were not so pronounced at the higher isolation temperatures, i.e. 40, 45 and 50°C.

The frequencies of occurrence of the fungi isolated by the screened substrate tube from the two-week old windrow at the Chesterfield plant are presented in Table 2.7.

Table 2.7 Percentage frequency of occurrence of the actively growing fungi, isolated by the ten screened substrate tubes placed in a two-week old windrow at the Chesterfield plant, on cellulose (C) and glucose-starch (GS) agars.

FUNGI	C	GS
Chaetomium thermophile	70	50
Humicola lanuginosa	80	60
Humicola insolens	10	
Actinomycetes	100	70

Chaetomium thermophile, Humicola lanuginosa, Humicola insolens and the thermophilic actinomycètes were also fre-isolated by Warcup's technique from the samples of mature compost thus indicating that these organisms are active in the composting process. Chaetomium thermophile, Humicola lanuginosa and the thermophilic actinomycetes were also frequently isolated from the samples of refuse and pulverised refuse.

The results of the effect of temperature on the linear growth rate of ten thermophilic and eleven mesophilic fungi are presented in the graphs in Figs. 2.1 and 2.2 respectively.

The results in Fig. 2.1 show that the majority of the thermophilic fungi examined have an optimal temperature for growth between 40 and 50°C. In the isolation studies previously described the majority of the thermophilic fungi were isolated in this temperature range. Chaetomium thermophile, Humicola lanuginosa, Talaromyces emersonii and Thermoascus aurantiacus were the only four fungi capable of growth at 60°C. Thermoascus aurantiacus was the most rapidly growing species covering the whole plate in 6-7 days at 60°C, none of the other three species reached this maximum diameter, their growth at 60°C being very slow. Humicola grisea, Humicola insolens, Mucor pusillus and the two varieties of Sporotrichum thermophile were the only fungi examined which could grow at 25°C, the lowest temperature of incubation used. Mucor pusillus and the two varieties of Sporotrichum thermophile were the fastest

FIGURE 2.1 THE EFFECT OF TEMPERATURE ON THE GROWTH OF SOME THERMOPHILIC FUNGI

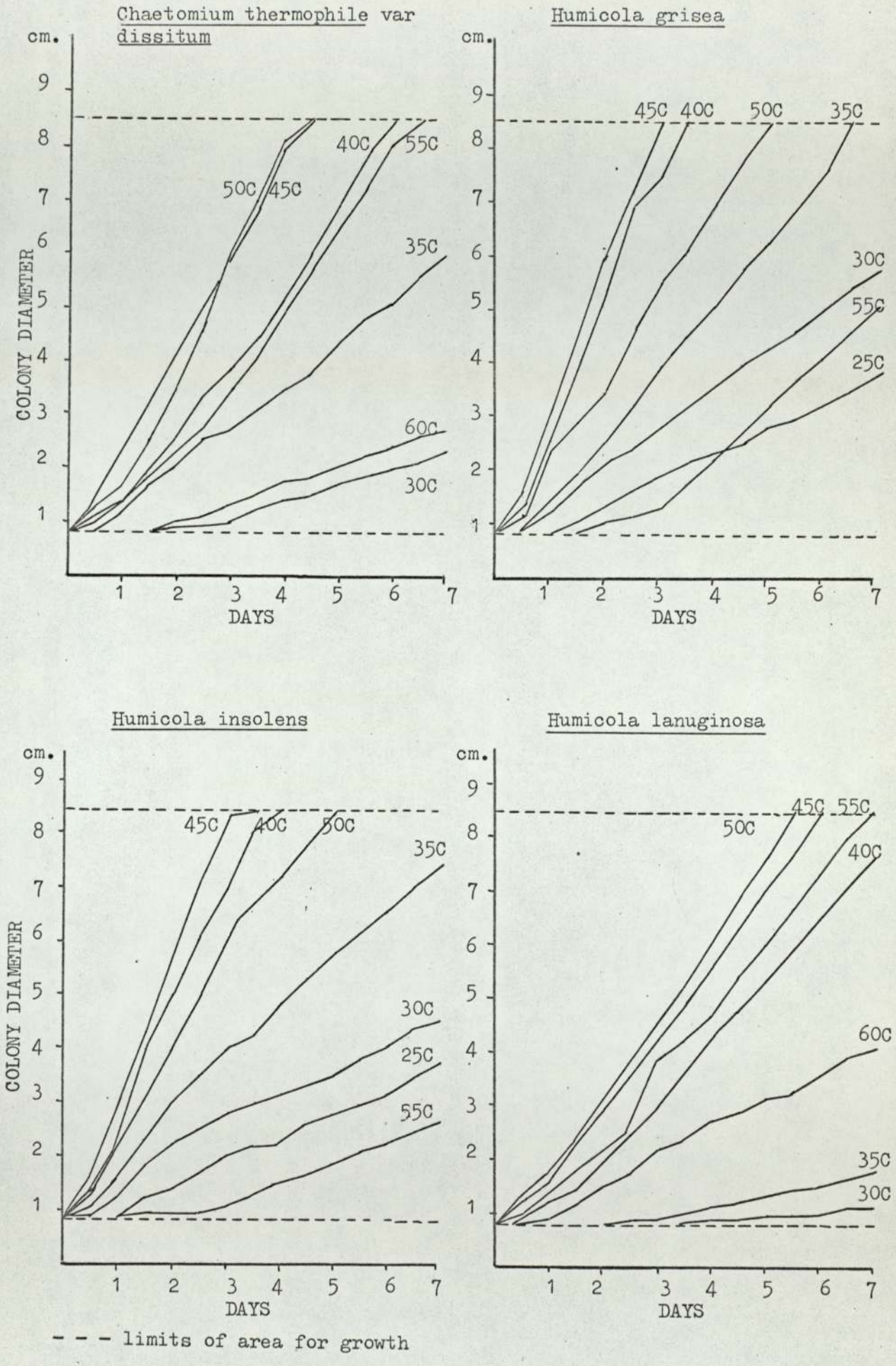


FIGURE 2.1 continued

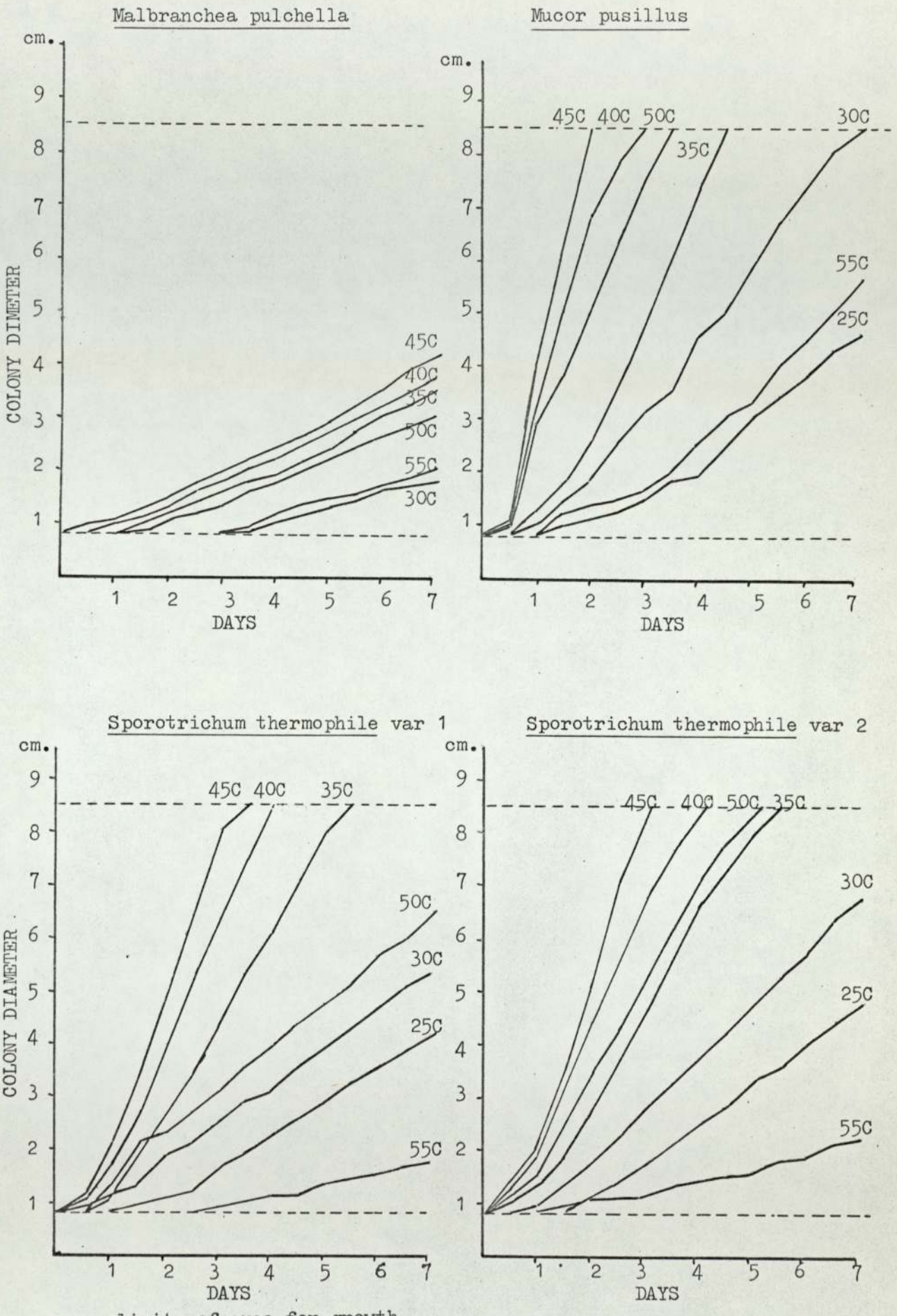
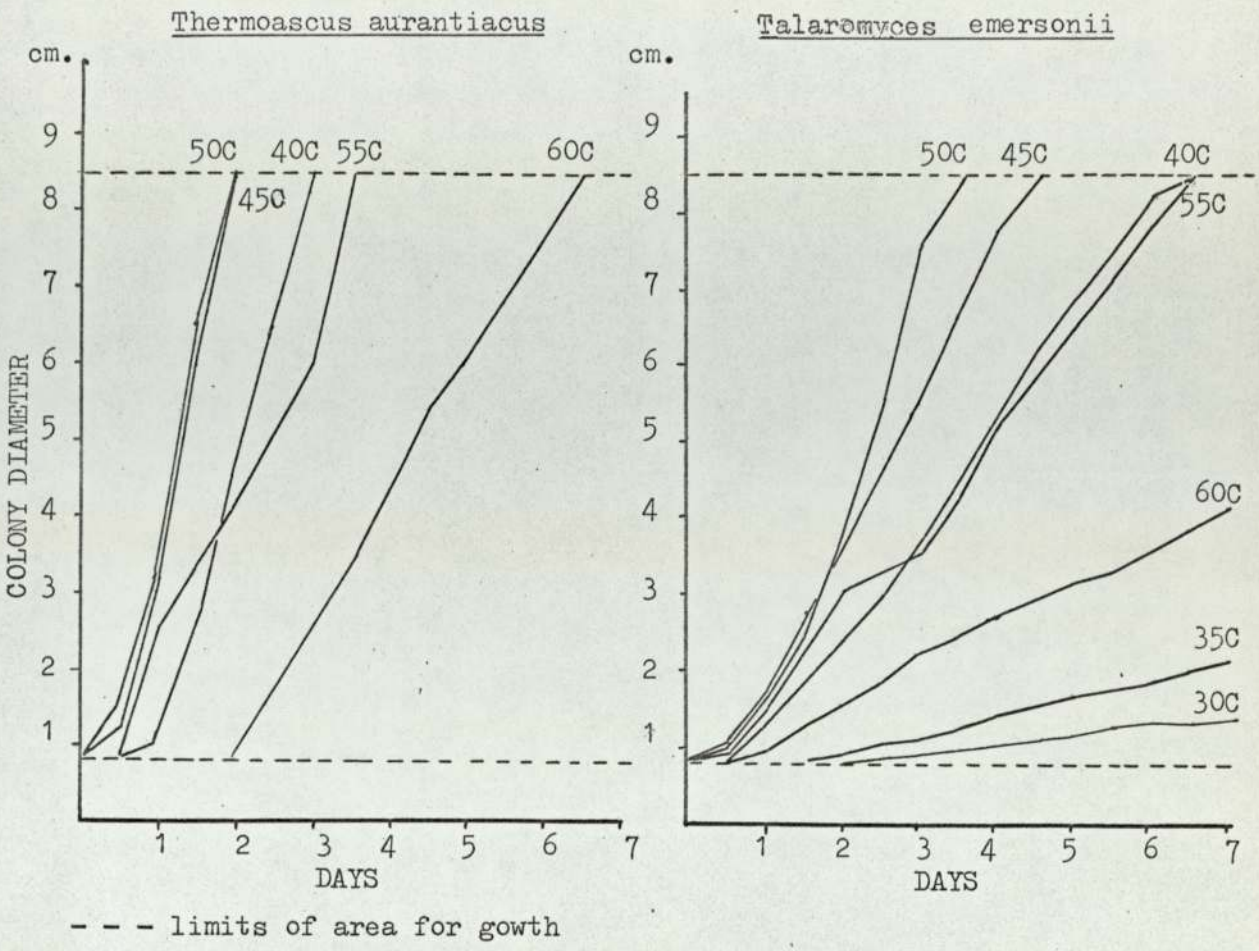


FIGURE 2.1 continued



growing at 25°C which probably explains the frequent isolation of Mucor pusillus at 25 and 30°C. The most frequently isolated thermophilic fungi from all of the samples examined were Humicola lanuginosa and Chaetomium thermophile. These two fungi were both found to have an optimum temperature for growth of between 45 and 50°C, below 45°C their growth rates decreased quickly. This probably explains why Chaetomium thermophile and Humicola lanuginosa were mainly isolated at 45 and 50°C. The slowest growing of all the thermophilic fungi tested was Malbranchea pulchella. All of the test thermophilic fungi except for Thermoascus aurantiacus were capable of growth at 35°C and it is perhaps surprising that so few species were regularly isolated at this temperature.

The graphs of the linear growth rate of the eleven mesophilic fungi tested are presented in Fig. 2.2. These graphs show that only Aspergillus niger and Penicillium funiculosum, the two most frequently occurring 'mesophilic' fungi at the high temperatures of isolation, could grow above 35°C. Aspergillus niger was found to have an optimum temperature for growth of 30-35°C but was capable of growing at 45°C. Penicillium funiculosum's maximum temperature for growth was 40°C, explaining its frequent isolations at this temperature. On one occasion Penicillium funiculosum was isolated from a sample of refuse taken from a household dustbin, at 45°C, although this temperature is apparently above its maximum temperature for growth, repeated attempts to culture Penicillium funiculosum at this temperature failed. Fusarium sp. and Humicola grisea were the only two fungi

FIGURE 2.2 THE EFFECT OF TEMPERATURE ON THE GROWTH OF SOME MESOPHILIC FUNGI

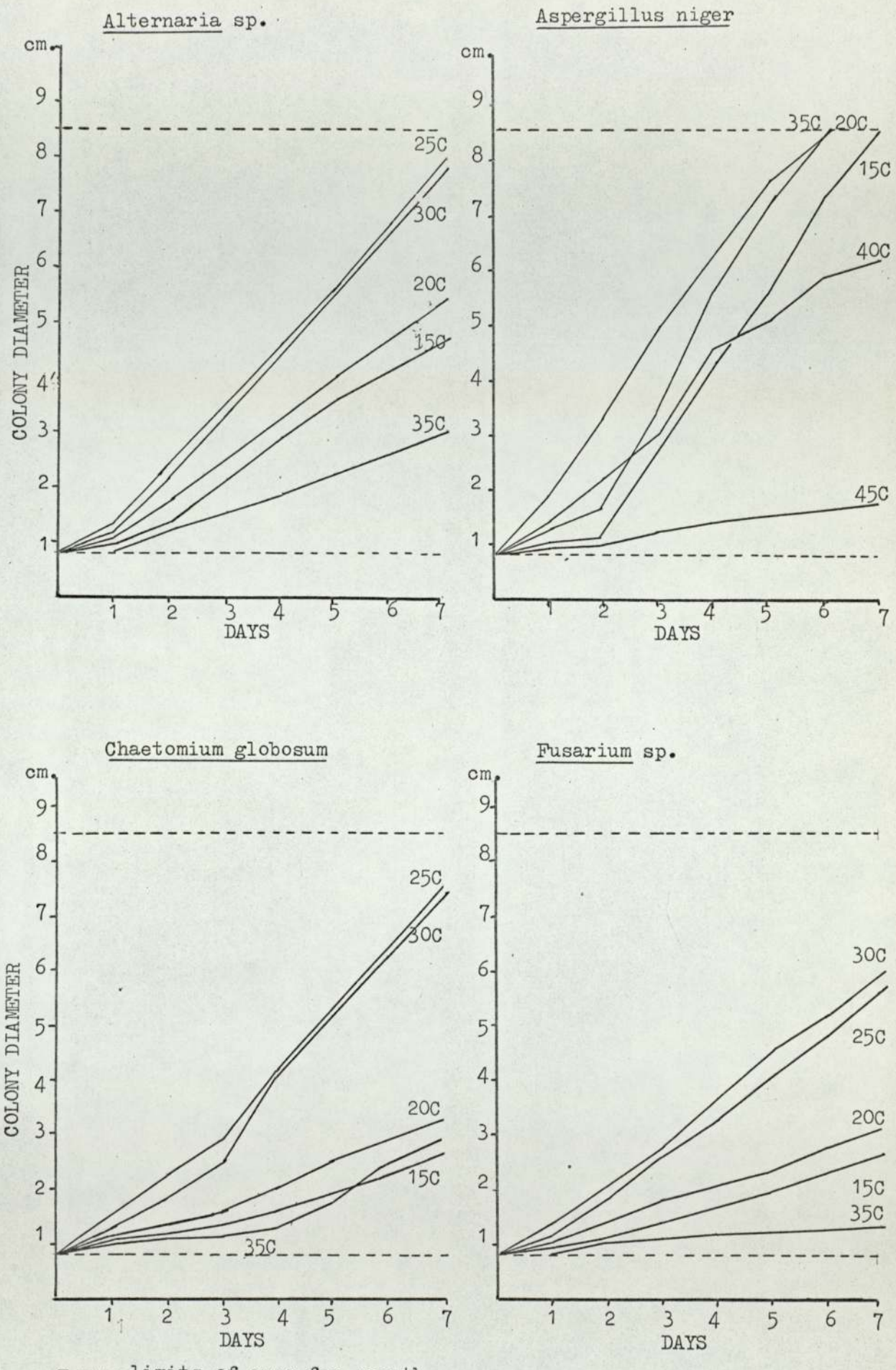


FIGURE 2.2 continued

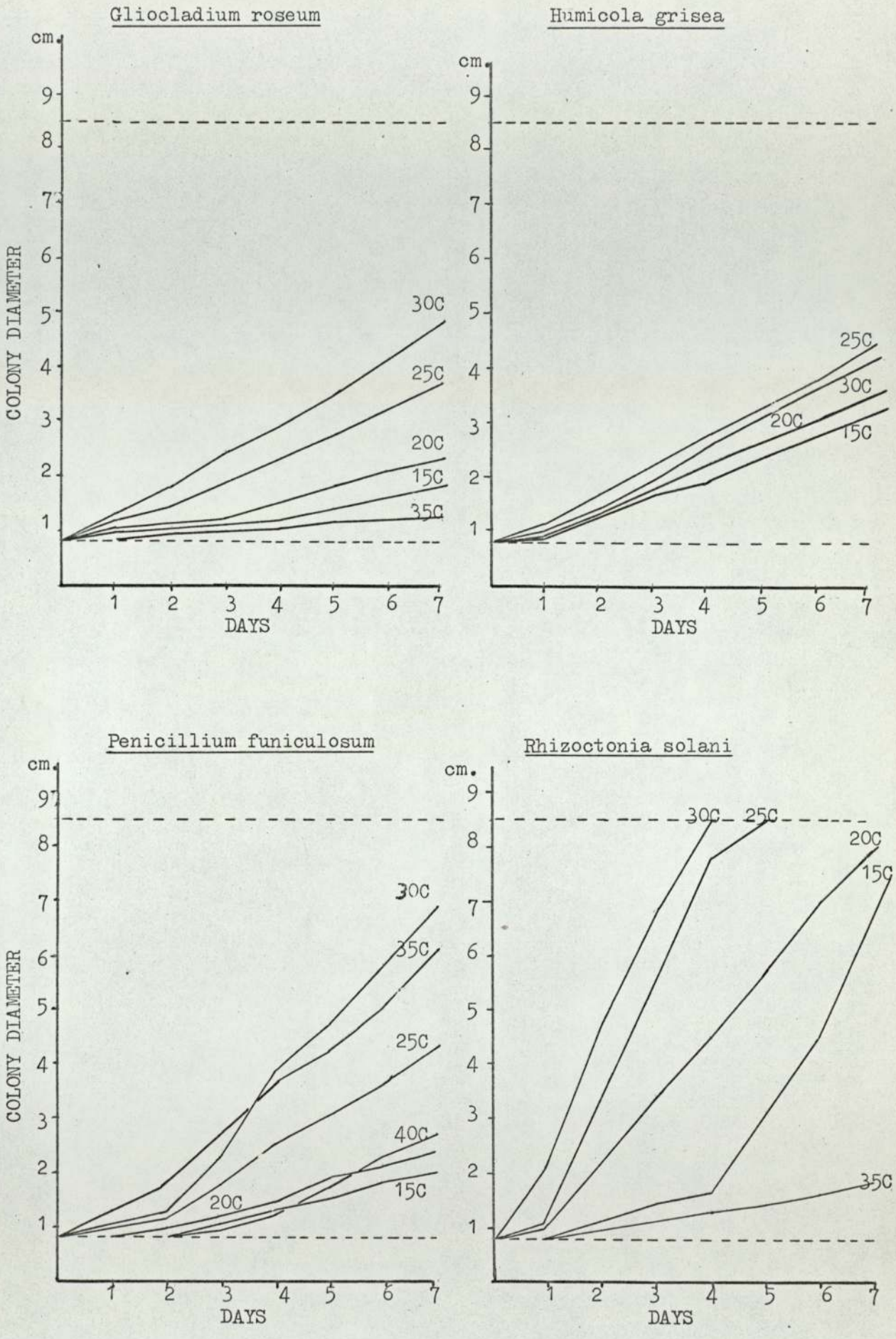
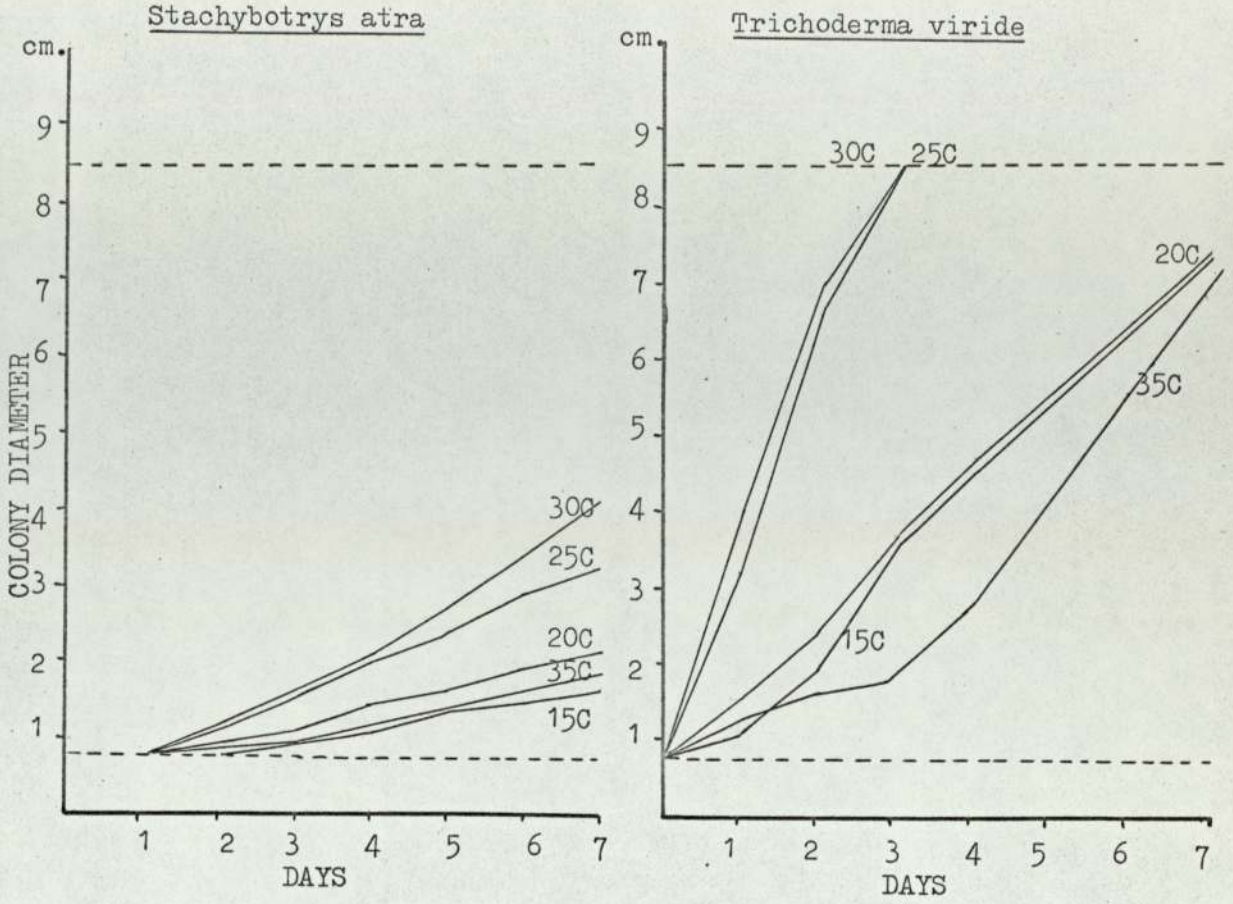
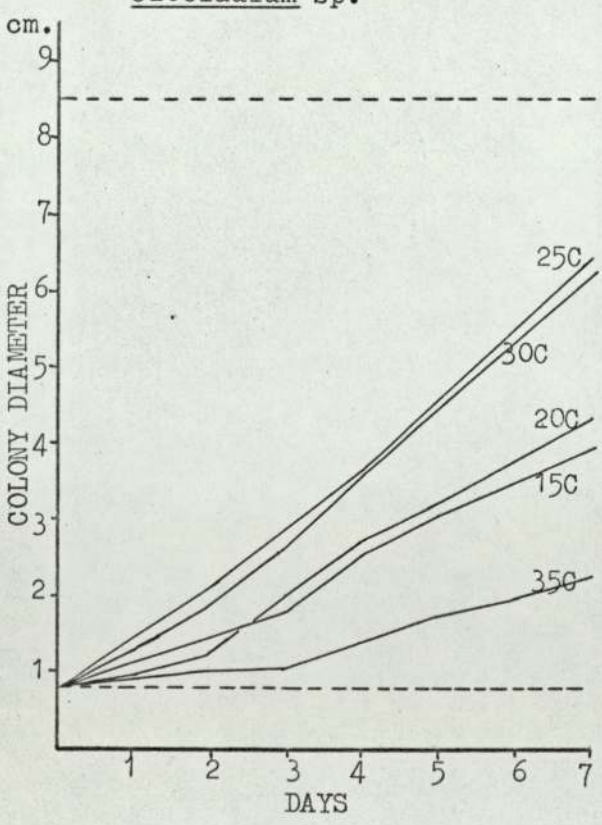


FIGURE 2.2 continued





- - - limits of area for growth

which failed to grow at 35°C thus explaining why they were both only isolated at 25 and 30°C. The optimum temperature range for the growth of ten of the eleven test fungi (Penicillium funiculosum was the exception) was 25-30°C. The difference between the growth rate of a particular fungus at 30°C and 25°C was usually only slight thus explaining the similarity between the results of the isolations performed at these two temperatures.

At 35°C with each of the isolation techniques a mixture of thermophilic and mesophilic fungi were isolated which from the results of the studies on the effect of temperature on the growth of mesophilic and thermophilic fungi would not be unexpected because the majority of these organisms can grow at 35°C.

Discussion

A total of eleven thermophilic, forty mesophilic and two thermotolerant fungi were isolated in the present investigation. Talaromyces emersonii and Cephalosporium sp. were the thermophilic fungi isolated not described by Cooney and Emerson (1964). Talaromyces emersonii was first isolated from Italian compost by Stolk (1965). Since that time it has been isolated from several locations where cellulosic wastes have been undergoing thermophilic biodegradation, i.e. from stacks of the palm kernel Elaeis guineensis Jacq., in Nigeria (Eggins and Coursey, 1964; Apinis and Eggins, 1966), from bagasse, compost and horse dung in India, (Ramabadran, 1967), from self-heated wood chip piles in California, (Tansey, 1971), from coal spoil tips in England, (Evans, 1971) and from samples of soil and refuse compost in England, (Mills, Barnes and Eggins, 1971). The two thermotolerants isolated were both variants of Aspergillus fumigatus, the second and least common of the two variations isolated was orange in colour. Considerable strain variation has been reported in Aspergillus fumigatus (Raper and Fennell, 1965), resulting in the occurrence of these mutants or coloured variations. Rai, Tewari, Agarwal and Wadwari (1968) and Evans (1971), have previously described the occurrence of such orange and buff coloured variants from soils in India and from coal spoil tips in England, respectively. Cephalosporium sp. is now known to be the conidial stage of the cleistothecial Ascomycete Allescheria terrestis, Apinis; which was first isolated from soil in Nottingham, England (Apinis, 1963).

The isolation studies of the present investigation have shown that refuse, even on entry into the refuse disposal system is a rich source of micro-organisms and therefore it is unlikely that inoculation with soil, manures or other composts will make any signigicant difference to the composting of refuse. The isolations made from the samples of refuse and scrapings taken from the household dustbins show that the refuse generally contains a more varied microflora than the scrapings removed from the bottoms and sides of the dustbins. It is therefore most unlikely that this stage of the refuse disposal system could act as an inoculating agent for the refuse. The samples of refuse and pulverised refuse taken from the plant at Chesterfield proved to contain an abundance of thermophilic and mesophilic fungi and thermophilic actinomycetes (these were not identified). The microflora's of the samples of refuse from the household dustbins and from the Chesterfield pulverisation plant were very similar, with most of the fungal species isolated common to both samples, e.g. only six species of mesophilic fungi were isolated from the refuse from the dustbins which were not present in the refuse taken from the pulverisation plant, and likewise only six species of mesophilic fungi were present in the refuse from the pulverisation plant and not in the refuse from the household dustbins. The thermophilic microflora's were practically identical only Talaromyces emersonii being isolated from the samples from the refuse pulverisation plant and not from the samples from the household dustbins. These results indicate that a representative collection of dustbins were sampled and that the microflora is consistent throughout the refuse

disposal system before composting occurs.

The samples of refuse (from the household dustbins), refuse from the pulverisation plant and pulverised refuse were found to contain eleven of the thirteen thermophilic fungi described by Cooney and Emerson (1964) plus Talaromyces emersonii, Stolk, when previous studies (Crisan, 1959, 1964) had indicated their relative rarity even in soils. Many of the mesophilic cellulolytic fungi isolated from these samples have also been frequently isolated from soils (Malik, 1970). The addition of sewage sludge to the pulverised refuse appears to have inhibited the isolation of many fungi for with all of the isolation techniques used at each of the six temperatures there was a general tendency for a reduction in the number of species isolated from this sample and for a reduction in the frequencies of occurrence of those fungi which were isolated. On many of the isolation plates prepared for the sample of pulverised refuse and sewage sludge a bacterial slime was found covering the surface of the media, despite the addition of rose bengal, inhibiting the growth of the fungi.

The microflora of the samples of mature and immature compost differed from the microflora of the other three samples particularly the samples of refuse and pulverised refuse. From the samples of compost, Chaetomium thermophile, Humicola lanuginosa, Humicola insolens (mature compost only) and thermophilic actinomycetes were all isolated at the temperatures of 45 and 50°C with much higher frequencies of occurrence than from the samples of refuse and pulverised

refuse. Humicola insolens appeared only infrequently on the isolation plates prepared from the samples of refuse, pulverised refuse, pulverised refuse and sewage sludge and immature compost but when isolated from the sample of mature compost it always had a higher frequency of occurrence. Chaetomium thermophile, Humicola insolens, Humicola lanuginosa and the thermophilic actinomycetes were also all isolated by the screened substrate tube from the windrow at the pulverisation plant. This finding coupled with the prevalence of the same organisms in the samples of compost would indicate that those organisms are active in the composting process for refuse. Humicola lanuginosa, Chaetomium thermophile and the thermophilic actinomycetes were all frequently isolated from the samples of refuse etc. indicating that some of the micro-organisms essential for the composting of refuse are indigenous to the wastes. The mesophilic microflora of the two samples of compost was very much different to that of the samples of refuse and pulverised refuse only ten species of mesophilic fungi being isolated from the composts by the normal Warcup technique at 25 and 30°C compared with over thirty species isolated by the same technique from the samples of refuse and pulverised refuse. This difference between the two types of samples is almost certainly due to the large numbers of mesophilic fungi which are killed off during the period of peak heating in the composting process.

From all of the samples examined the isolation technique which yielded the largest number of fungal species was the standard Warcup technique. The technique of incu-

bating or amending the samples with either cellulose or glucose reduced the number of species isolated but increased the frequencies of occurrence of the heavily sporulating and quicker growing fungi which could utilise the added carbon source. In the case of the cellulose amendments these fungi were the cellulolytic fungi and the secondary sugar fungi and with the glucose amendment the sugar fungi. Eggins and Malik (1969), found that, by incubating portions of unamended soil or soil amended with either glucose or cellulose, the number of thermophilic fungi isolated was increased. This differs from the findings of the present investigation but it must be remembered that the fungi in the soil may be subject to different ecological conditions to those fungi present in refuse. Eggins and Malik (1969) also made a series of sequential isolations from their soil samples whereas in the present investigation isolations were only made after 7 days of incubation of the samples. The maximum number of thermophilic fungi isolated by Eggins and Malik (1969) at any one time from any of their soil samples was (excluding the Streptomyces spp and Aspergillus fumigatus) seven which is comparable with the numbers isolated from the samples of refuse etc.

The effects of the different isolation techniques have been fully reported in the section on the results which serve to demonstrate the advantages of enrichment and incubation of the samples to enhance the isolation of selected groups of fungi. For instance, amending the samples with cellulose and then incubating them at 50°C provided favourable conditions for the fungi active in the decomposition

of cellulose and accordingly the frequencies of occurrence of Chaetomium thermophile, Humicola lanuginosa, Mucor pusillus, Humicola insolens and the thermophilic actinomycetes were substantially increased indicating that in the composting process these organisms may well be active in the decomposition of cellulose.

The range of temperatures over which the isolations have been made has shown that the best temperatures for the isolation of the thermophilic fungi are 40, 45 and 50°C with the highest frequencies of occurrence and the highest number of species being recorded at 50°C. Above 48°C it was found that the development of Aspergillus fumigatus is restricted enabling the isolation of some of the slower growing thermophiles. The mesophilic fungi were mainly isolated at 25 and 30°C and there was a close similarity between the results obtained at these temperatures. At 35°C much fewer species of either the thermophiles or the mesophilic fungi were isolated although it has been shown from the investigations on the effect of temperature on the growth of these fungi that the majority of mesophilic and thermophilic fungi are able to grow quite well at these temperatures. The frequencies of occurrence of the fungi isolated at 35°C were usually less than those obtained at the other temperatures.

The thermophilic actinomycetes were present throughout the refuse disposal system with the highest frequencies of occurrence obtained from the samples of compost particularly the samples amended with cellulose. The isolation of

thermophilic actinomycetes by the screened substrate tube from the windrow at Chesterfield would indicate that they are active in and essential for the composting process. This hypothesis and the recent work by Stutzenberger Kaufman and Lossin, (1970) is discussed fully in the chapter on the microbiology of composting.

The results of the present investigation have shown that there is good reason to believe that some of the micro-organisms essential for the composting of refuse are indigenous to the wastes (many of the fungi isolated from these samples of refuse were active in the experimental windrow used in Chapter 3) but they do not reveal the source of these micro-organisms. The report of the British Government's working party on refuse disposal (H.M.S.O., 1971) reveals that in many areas there has been an increase in the quantities of garden refuse collected. This situation has been brought about by the lack of facilities for burning refuse in homes and it is believed that this trend will continue. Clearly such material as garden refuse will contain a wealth of micro-organisms which could be active in the composting of refuse. When the plant was opened at Chesterfield, the Rural District Council responsible for its operation actively encouraged householders to put their garden refuse in their dustbins to provide materials suitable for the encouragement of the composting process. Their campaign has succeeded in introducing some of the micro-organisms, essential for composting, into the refuse disposal system.

CHAPTER 3 Pages

Chapter 3

MICROBIOLOGICAL CHANGES IN THE COMPOSTING OF REFUSE

Introduction

Composting is a term used to describe a controlled process of decay of accreted organic wastes effected by an indigenous population of micro-organisms (Wylie,1960; White, 1965; Raisbeck,1967). Under natural conditions the decay of the large quantities of organic material produced annually by plants takes place slowly on the surface of the ground yielding humus which is stored in the soil thus completing a natural cycle (Gray, Sherman and Biddlestone, 1971). Composting is the application of this natural process by man as a means for the production of organic fertilisers (humus) from vegetable wastes and animal manures and more recently also as a method of disposal for wastes, such as refuse, containing large quantities of organic matter (Wylie, 1960; Gray, 1966).

Composting has been variously defined as: a process of fermentation (Berkeley Project, 1953; Gotaas, 1956; Wylie, 1960; Rao and Block, 1963; Kershaw, 1968), a biological or biochemical process of decomposition (Jann, Howard and Salle, 1959; American Public Works Association (A.P.W.A.), 1961), a process of aerobic thermophilic decomposition (Schulze, 1960; Wuest, 1968; Kochtitzky, Seaman and Wiley, 1969; Gilbertson,1969; Bell and Pos, 1971;) and a process of

selective incineration (Snell, 1960). These descriptions highlight one or more of the essential phases of the complete composting process which is now understood to involve the heaping together of the organic matter to effect its decomposition by micro-organisms. The purpose of heaping the material is to provide the conditions of temperature and moisture favourable to these micro-organisms (Rao and Block, 1963). Once the material has been heaped together composting may be either aerobic or anaerobic, this usually depends on the particle size and moisture content of the material (Schulze, 1960) which can markedly affect the volume of air present in the interstices between the particles. Aerobic composting is characterised by high temperatures, rapid changes in the C:N ratio with the production of humus. With anaerobic composting there is very little temperature elevation and it is characterised by the production of a strong offensive odour. The transformation of organic matter to humus in anaerobic composts is very slow. Composting can also be divided into two other general classifications of biological decomposition, thermophilic and mesophilic. Mesophilic decomposition occurs in the initial stages of composting quickly giving way to thermophilic decomposition as the heat released by microbial metabolism is retained by the insulating properties of the compost heap. The role of the mesophilic fungi in thermogenesis has been demonstrated in the self heating of hay in Dewar flasks by Norman (1930).

Aerobic composting is the type dealt with in the present investigation and is also the type on which most

systems of mechanised composting are based. In the modern sense composting could be defined as a process in which, under suitable environmental conditions, facultatively aerobic micro-organisms, principally thermophilic, break down organic matter to a stable humus. It is thought that the organisms involved are widely distributed in nature (Berkeley Project, 1953) and to a great degree have been found to be indigenous to the refuse.

Composting in its most primitive form, i.e. the piling up of animal or vegetable wastes, has been practised by man for centuries. The first advance in the practice of large scale composting was made about fifty years ago in India by Sir Albert Howard who developed the 'Indore Process' which involved the piling up of alternate layers of readily putrescible material such as garbage, nightsoil, animal manure or sewage sludge with stable organic matter such as straw and leaves. The material was turned periodically and the process was a combination of aerobic and anaerobic phases with the aerobic phases following each turning (Berkeley Project, 1953; Henderson, 1960).

Composting processes in which the principal objective was to dispose of refuse apart from producing a humus were started from 1920 to 1930. These processes were designed for the rapid production of compost by the mechanised handling of materials and also by less objectionable methods. The most important of these early processes was the Beccari

Process which relied mainly on the basis of anaerobic fermentation in air tight cells, provision being made for periodic aerobic fermentation by opening apertures in the cell wall. Anaerobic composting of this type never proved to be entirely satisfactory and after the limitations of these early plants had been revealed rapid progress towards systems of production on a large scale, were made by concentrating on the problem of creating and maintaining aerobic conditions within the mass of wastes being treated.

The major attempts to provide these conditions for the composting of refuse can be broadly split into four categories of mechanised composting (Wylie, 1960). These are:-

i. Mass composting
ii. Batch composting
iii. Multi stage composting
iv. Continuous composting.

Mass composting involves the tipping of refuse into cells, spraying with water between each layer of refuse and leaving it to compost for 4 - 8 months when it is dug out and screened. This system, known as the van Mannen system, was put into operation in the Netherlands in 1932 but the quality of the composts produced was very poor and it has not been repeated elsewhere.

The other three methods of mechanised composting depend on the careful selection and preparation of the materials

to be processed before they are subjected to controlled composting conditions. The preparation of the refuse for composting involves the removal of unsuitable material such as glass, plastics and metals, followed by the mechanical breaking up of the refuse into small particles to present a large surface area for microbial attack. The moisture content of the refuse is then adjusted to 40 - 60 percent of the total weight by the addition of water and sometimes sewage sludge.

The main method of batch composting is windrowing, in which the refuse, prepared as described above, is stacked in piles, approximately 6 feet high, which are periodically turned. This method takes 4 - 6 months to produce a mature compost and is currently used at plants designed by Buhler Brothers Ltd. of Switzerland.

All the methods of multi-stage composting involve the principle of intermittent disturbance of the compost to ensure the thorough introduction of air into the mass during the early stages of composting. The most well known process of this type is the John Thompson Fermentation Cell, which has been used in plants in Jersey and Bangkok. In this process pulverised refuse is hoisted into the upper of a series of layers of 'fermentation cells' and every 24 hours the layers of cells are inverted in sequence and the refuse is thus moved down one layer every day remaining in the system of fermentation cells for a total of 6 days. The refuse emerging from the bottom layer of cells is usually stacked and matured for 8 - 10 weeks. Another system operating in a similar fashion is the Simon-Lawden Process which involves

mechanically sweeping the refuse from the top floor of a circular tower via several intermediate floors to the bottom of the tower. One of the more recent developments involving the principle of intermittent disturbance is the Fermascreen designed by John Thompson Ltd. which consists of a hexagonal drum, in which three sides of the hexagon are formed by perforated plates covered on the outside by a hinged door, the other three sides are of plain steel, one of which is in the form of a door to facilitate loading of crude or pulverised refuse. The drum is rotated periodically to thoroughly mix the mass of refuse and aerobic fermentation takes place when the vessel is stationary and the doors are opened.

The only method of continuous composting is the Dano Bio-stabiliser which is unique because the wastes are kept in continuous motion throughout the composting process. This method of composting has become widespread, installations being operative at one time or another in Denmark, Sweden, Switzerland, Germany, Holland, France, Norway, Finland, U.S.A. and the United Kingdom. The Bio-stabiliser combines the three essential operations of mechanised composting, that is: reduction, mixing and fermentation, so that they all take place in a rotating cylinder. The course of the fermentation is controlled by controlling the moisture content of the substrate and the injection of air under pressure. The refuse takes 3 - 5 days to pass through the cylinder, after which time the large inert materials are removed and the product is matured by windrowing for 3 - 4 weeks (Berkeley

Project, 1953; Gotaas, 1956; Wylie, 1960; Gothard, 1961; A.P.W.A., 1961; White, 1965; Hughes, 1967; Raisbeck, 1967; Kershaw, 1968; H.M.S.O., 1971).

In the past much effort has been put into developing the mechanised composting plants, described above, for the achievement of rapid composting of the ever increasing volume of refuse that civilisations now produce (Gilbertson, 1969). However, apart from monitoring the physical and chemical parameters of the refuse as it is broken down to compost there is almost a complete absence of any meaningful data on what happens in the composting process, certainly very little work has been published on the microbiological changes which occur in this process. The opinion has been expressed (Gray, K. R., 1966) that the development of a better composting process must start with an assessment of the behaviour of the microbial population during composting. It has also been stated (Gray, K.R., 1969) that in the composting of a heterogeneous organic material such as refuse by a mixed microbial population, so many inter-related factors are involved that the complex ecological process is unlikely to succumb to rigorous scientific analysis for many years. Unfortunately the attitude of many research workers in the past has been typified by that given in a publication of the American Public Works Association (1961) in which it is stated, "There are many micro-organisms involved in composting and the roles of each are complex, but an extensive knowledge of their characteristics is not necessary for a compost plant operator.". Thus it is not

surprising that when attitudes such as this are common composting has progressed so little since the days of Sir Albert Howard and has fallen so widely into disrepute.

The aerobic composting of refuse is thought to consist of four main phases (A.P.W.A., 1961; Snell, 1960; Glathe, 1962; White, 1965; Raisbeck, 1967; Burman, 1961, 1967; Arditti, 1967; Gray and Sherman, 1969; Regan and Jeris, 1970; Gray, Sherman and Biddlestone, 1971).

The initial stage is known as the mesophilic phase which in the windrowing method can last 2 - 7 days. The mesophilic micro-organisms,especially the bacteria, are reported to be extremely active causing the oxidation of simple carbohydrates and thereby generating heat which is retained within the insulation of the windrow causing a rapid rise in temperature to 35 - 40^{o}C. After the temperature rises above 40^{o}C the mesophilic micro-organisms die out or become inactive giving way to the thermophilic species which become prominent during the thermophilic phase.

In the thermophilic phase the growth of the micro-organisms and the breakdown of organic matter is reported to be very rapid. Peak temperatures of 65 - 70^{o}C are usually observed, the refuse becoming alkaline with a pH of 8 - 9, and this is the period when most of the pathogenic micro-organisms are killed off (Berkeley Project, 1953).

The major breakdown of cellulose is thought to

occur in the post thermophilic stage (the cooling phase) and be brought about by the thermophilic actinomycetes and fungi. In the cooling phase the temperature falls slowly from its peak to ambient and it is thought that the rate of reaction and therefore heat production, becomes progressively slower as the micro-organisms exhaust all the readily available nutrients leaving mainly the more resistant celluloses and lignin as the sole carbon sources. When the temperature falls to 30 - 40^{o}C there is once more a rapid increase in the numbers of the mesophilic micro-organisms, which is maintained until the material cools down to ambient.

At the end of the cooling phase the compost is said to be stable and will, therefore, no longer develop the objectionable odours of decaying wastes. The compost is not yet thought of as being mature, for a mature compost will no longer display the phenomenon of thermogenesis when kept moist in a heap and whereas a stable compost has a C:N of 10:1. A stable compost is converted to a mature compost by stacking it at ambient temperatures for 6 - 8 weeks.

In the introduction to this thesis it was mentioned that in the United Kingdom and most other industrialised nations the character of refuse has changed since the development of mechanised composting 30 - 40 years ago, the greatest change being the rapid increase in the paper content of the refuse. In composting, paper has generally been considered to be a relatively stable material

that would be degraded only if extended treatment were made available (Jeris and Regan, 1968; Regan and Jeris, 1970; H.M.S.O., 1971). The abundance of paper in refuse and its resistance to rapid degradation in composting means that cellulose degradation is now the rate controlling step in the composting of refuse and Stutzenberger Kaufman and Lossin (1970) have reported that undegraded cellulosic wastes have been found in otherwise mature composts. Thus if composting is to develop any further as a means of refuse disposal then the conditions suitable for the degradation of cellulose within the composting process must be identified and promoted. At the present time the exact means by which cellulose degradation occurs in composting is not fully understood.

The majority of cellulose degradation was once thought to occur in the post-thermophilic stage and be brought about by the thermophilic fungi (Burman, 1961). The importance of the fungi in composting of organic wastes has been stressed by Gray, W.D. (1959) who was of the opinion that, as the majority of cellulose decomposing organisms were amongst the fungi, conditions in composts should be so arranged as to favour their growth. Burman (1961) reported that the region of activity of the thermophilic fungi is very restricted in maturing static heaps because of their critical growth requirements and it would, therefore, be doubtful whether they are the only major cellulose decomposers in composts of refuse. However, Snell (1960) and Schulze (1960) found respectively that

the maximum oxygen uptake in composts of refuse occurred at temperatures between 45 and 50°C and that out of ten temperatures examined, within the range 25 - 70°C, the optimum for composting was found to be 45°C. The optimum temperatures for the growth of the thermophilic fungi usually range between 45 and 50°C (Cooney and Emerson, 1964). Von Klopotek (1962) carried out an investigation into the occurrence and condition of fungi in refuse and found that the thermophilic fungi constituted only 0.02% of the mycoflora isolated from composts of refuse, which would suggest that the thermophilic fungi do not play a significant part in the composting process. In a subsequent publication Von Klopotek (1963) reported that the thermophilic fungi do not appear during heating but only during cooling when their numbers increase rapidly. She attributed the appearance of fungi in compost piles to the type of material used, its moisture content and its temperature.

Recent research investigating the types of micro-organisms capable of cellulolytic activity in the composting of refuse has revealed that the pH and temperature optima for C_x cellulase enzyme extracted from compost samples were identical with those subsequently observed for the C_x cellulase component of a thermophilic actinomycete, Thermomonospora curvata, isolated from the original compost (Stutzenberger, Kaufman and Lossin, 1970). Thermophilic actinomycetes have been found in abundance in high temperature well aerated composts and some species are thought to be active in cellulose degradation. Species of Thermopolyspora

and Thermomonospora are reported to be cellulolytic and predominate in composts at temperatures of 60 - 66°C (Waksman, 1967). Stutzenberger, Kaufman and Lossin (1970) came to the conclusion that the prevalence of Thermomonospora curvata in the refuse compost samples coupled with the correlation of the pH and temperature optima of the C_x cellulase enzymes indicated that Thermomonospora curvata may occupy a major role in cellulose degradation during the composting of refuse.

Although little work of any significance has been carried out into the microbiology of refuse composting considerable information is available regarding the microbiology of composts of other materials. Much of this work was done by Waksman and his co-workers as early as 1939. From these early studies it was found with composts of horse manure straw and urine that the thermophilic microbial populations were capable of bringing about much greater decomposition than the mesophilic microbial populations (Waksman, Cordon and Hulpoi, 1939). It was also discovered that, by controlling the temperature of the compost, it was possible to control the rate of decomposition and that on the whole degradation proceeded much faster at 50°C than any other temperature i.e. 28, 65 and 75°C. At the higher temperatures the thermophilic actinomycetes and bacteria prevailed but at 50°C the fungi were very active in association with the actinomycetes. At 28°C decomposition was limited and only occurred to any extent when the mesophilic fungi developed in the later stages, the lignin

fraction was hardly attacked supporting earlier work by Waksman and Gerrettsten (1931) which had shown that the resistance of lignin to decomposition was greater at lower temperatures. In an investigation into the thermophilic actinomycetes and fungi of composts Waksman, Umbreit and Cordon (1939) described a fungus they called Thermomyces, (thought by Cooney and Emerson, 1964, to be Humicola insolens), which was found to be widespread in composts of horse manure at 50°C and which in a subsequent investigation by Waksman and Cordon (1939) was the only organism tested, including bacteria and actinomycetes, which could compare with the total microbial population of the compost in bringing about decomposition of the hemicelluloses, cellulose and lignin components.

Investigations into the microbiological changes occurring in the composting of mixtures of wheat straw and horse manure for the production of mushroom compost have revealed carefully ordered changes in the populations of micro-organisms throughout the composting process (Hayes, 1969). In 'Stage I' where the mixture is windrowed in stacks the early increases and decreases in temperature were found to be paralleled with similar increases and decreases in the population of the thermophilic bacteria but not of actinomycetes and fungi. This finding is supported by the work of Webley (1947a, 1947b, 1948) who found, with composts of grass cuttings and barley straw that during the early stages of thermogenesis there was a rapid increase in the numbers of aerobic bacteria which were generally found to

be very sensitive to temperature changes. Hayes (1969) found in Stage II of the mushroom composting process, where the compost is incubated at 50 - 60°C, that the thermophilic actinomycetes became more numerous than either the bacteria or fungi but when the compost was supplemented with sucrose the bacterial population increased and the actinomycete population decreased. The supplementation of the compost with sucrose was found, by suppressing the actinomycetes, to inhibit the decomposition of cellulose and the hemicelluloses. This evidence supports the hypothesis of Stutzenberger (1971) that in refuse composting the major cellulose decomposers are thermophilic actinomycetes. In an earlier investigation into the thermophilic actinomycetes and fungi of mushroom composting (Fergus, 1964) found that Thermomonospora curvata was the most frequently isolated actinomycete and Humicola grisea var. thermoidea and Humicola insolens the most frequently isolated fungi. Fergus (1964) isolated eight species of thermophilic fungi and eleven species of thermophilic actinomycetes and observed that the thermophilic fungi were found to be restricted to the very outer layers of the compost whereas the actinomycetes were isolated from all regions of the compost.

The behaviour of the mycoflora of composts of wheat and barley straw and grass cuttings has been investigated by Eastwood (1952), Chang and Hudson (1967) and Chang (1967). Although the composts described by Eastwood (1952) both had thermophilic phases she failed to isolate any thermophilic

fungi, probably because her incubation temperature of 60°C was too high for their growth. She found that the bacteria were far more abundant in the composts of grass cuttings than in the composts of barley straw indicating the importance of the effect of the nature of the substrate on the course of composting. Eastwood (1952) found that mesophilic cellulose decomposing fungi were found to be abundant in straw compost at 25°C, the activities of the actinomycetes in these composts were not reported. Chang and Hudson (1967) isolated seven species of thermophilic fungi from their composts of wheat straw and these were reported to appear in carefully ordered succession. The thermophilic actinomycetes and bacteria were observed in large numbers before and during the period of peak heating when the thermophilic fungi had been suppressed. Chang (1967) in a subsequent study of the biochemistry and physiology of the fungi isolated from these composts revealed that some thermophiles notably Chaetomium thermophile could rapidly utilise both cellulose and hemicelluloses at a level and rate comparable with the total microbial population. The results of Chang (1967) indicate that the thermophilic fungi may also occupy a major role in the decomposition of cellulose in composts of organic wastes.

The evidence presented above concerning the microbiology of composting indicates that the greater part of decomposition is brought about by the activities of thermophilic actinomycetes or fungi or a combination of both their activities. Data comparing the cellulolytic capabilities

of the thermophilic actinomycetes and fungi are scarce but it has been generally regarded that the actinomycetes develop much less rapidly than the fungi and are, therefore, rather ineffective competitors when nutrient levels are high. Fergus (1969) compared the cellulolytic capabilities of some twenty varieties of thermophilic fungi and ten species of thermophilic actinomycetes and came to the conclusion that the majority of the fungi had greater cellulolytic capabilities than the actinomycetes. The validity of the results obtained by Fergus (1969) can be criticised because the cultural conditions used were the same for the actinomycetes and the fungi and were in fact better suited to the fungi. Thermomonospora curvata was one of the thermophilic actinomycetes examined by Fergus (1969) but the cultural conditions he used differed significantly from those found to be optimal for it by Stutzenberger (1971).

From the evidence of previous research there is little doubt that the thermophilic microbial populations of composts bring about an enhanced rate of decomposition compared with the mesophilic populations. Waksman, Cordon and Hulpoi (1939), Webley (1947a, 1947b, 1948) Eastwood (1952), Chang and Hudson (1967), and Hayes (1969) all noted the abundance of bacteria in composts especially during the initial stages of thermogenesis and peak heating. Hayes (1969) noted the utilisation of simple carbohydrates by the bacteria and along with Burman (1961) thinks that they may well be important in the initial stages of composting. The

abundance of the thermophilic actinomycetes has been noted by Waksman, Umbreit and Cordon (1939) and Hayes (1969) and recent research by Stutzenberger, Kaufman and Lossin (1970) and Stutzenberger (1971) has indicated that they could be major cellulose decomposers in composts. The strong cellulolytic nature and prevalence of some thermophilic fungi, found throughout composts, has been reported by Waksman and Cordon (1939) and Chang (1967) whereas other reports have indicated only a limited growth of such fungi in composts (Fergus, 1969; Burman, 1961). These references indicate an absence of evidence concerning the relative importance of the actinomycetes, bacteria and fungi in the composting process. Certainly in the literature there is a complete absence of any meaningful references concerned with the microbiological changes occurring during the composting of refuse and if composting is to progress as a means of refuse disposal it is an area where research must be carried out.

Consequently in the present investigation the activities of the bacteria, actinomycetes and fungi in the composting of refuse in a small experimental windrow have been studied. References to the abilities of the thermophilic microbial populations in bringing about an enhanced rate and degree of decomposition have been discussed and accordingly extra attention was given to the activities of such populations.

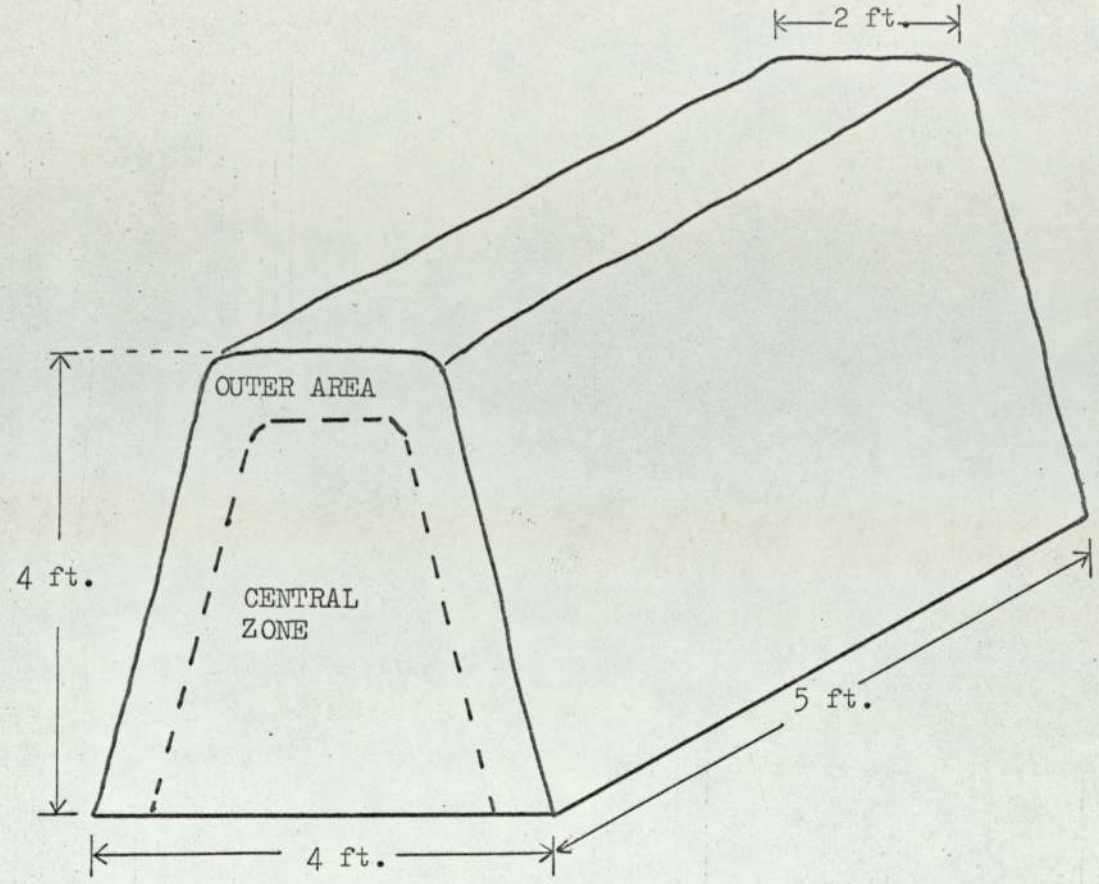
Materials and Methods

The refuse for the experimental windrow was again

obtained from the composting plant at Chesterfield. The refuse used had been pulverised, screened for ferrous metals and sieved to remove any remaining pieces of refuse larger than 1½ inches diameter. The finely pulverised refuse was collected as it was 'freshly' discharged from the plant ready for windrowing. Before the experimental windrow was constructed the moisture content of the refuse was adjusted to 40 - 50 percent of the total weight, the optimum level for composting, (A.P.W.A., 1961; Wylie, 1960; Gray et al., 1971; Berkeley Report, 1953).

The experimental windrow was set up in the open and it was approximately 4 feet wide at the base, 2 feet wide at the top, 5 feet long and 4 feet high in the centre, see Figure 3.1. During the daylight hours the windrow was left exposed, except when it was raining, and at night when it was covered with a polythene sheet. In all other aspects the composting process employed was completely comparable with that used by Buhler Brothers the designers of the plant at Chesterfield.

The refuse was windrowed for fifty days being turned once in this time. Previous workers studying the biochemical and ecological changes in composts (Chang and Hudson, 1967; Chang, 1967; Mills, 1973) did not turn or disturb their composts because they feared that this would complicate and interrupt their studies. However, when pulverised refuse is stacked as in a windrow then compaction will quickly occur which has the effect of



FIGRE 3.1 DIAGRAM OF EXPERIMENTAL WINDROW.

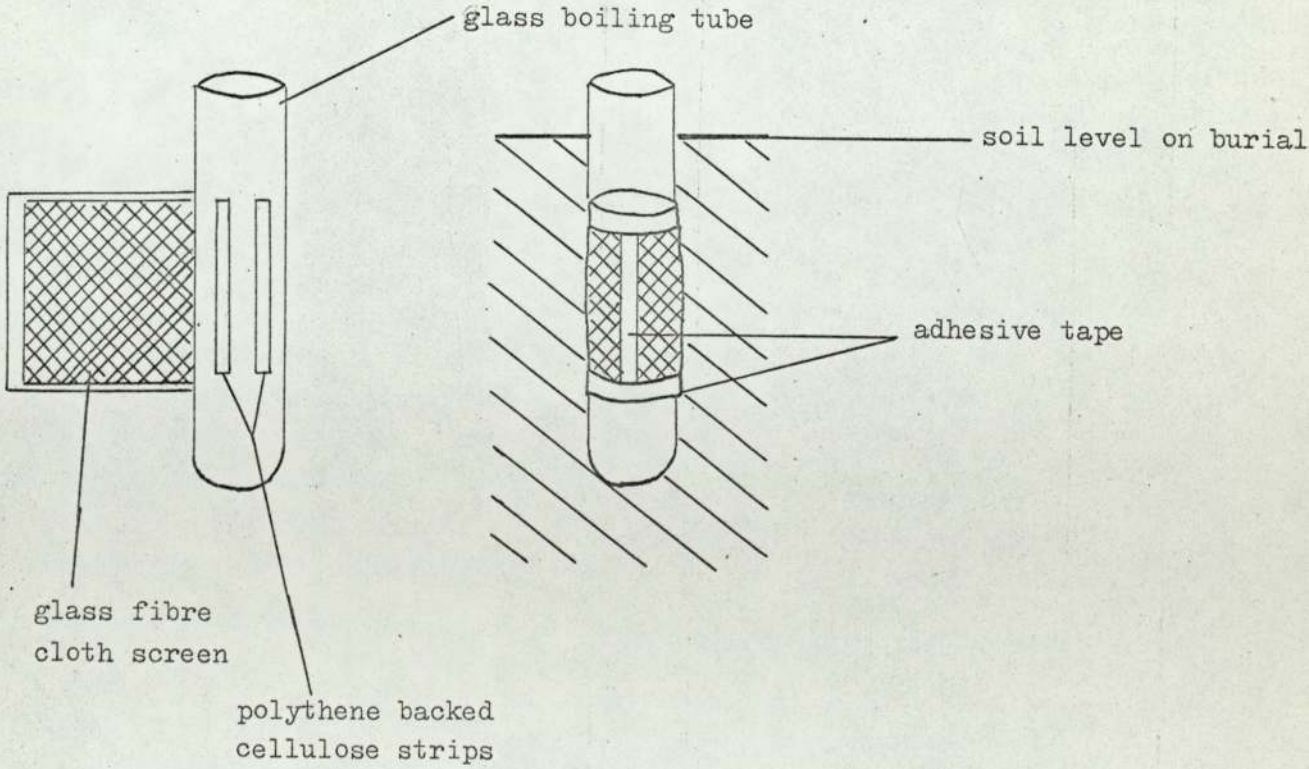


FIGURE 3.2 SCREENED SUBSTRATE ISOLATION TUBE.

closing the interstitial air spaces, causing water logging and generally promoting conditions of anaerobiosis. These conditions are atypical because the composting of organic wastes by windrowing is essentially an aerobic process and so it was decided to turn the windrow at least once and this was done on day 11, after the completion of the initial heating phase. Hayes (1969) while studying the microbiological changes in the composting of mixtures of wheat straw and horse manure turned his windrows three times during the first ten days which did not appear to upset the consistency of his results.

For the purposes of this study the windrow was theoretically divided into two regions, the outermost 6 inches of the windrow were designated the 'outer region' and the volume inside this 6 inch outer layer was designated the 'central region'. For the determination of the microbiological changes within the windrow, samples were taken from four sites in each region. The samples were removed with a pair of sterile forceps and a sterile spatula and collected in a clean sterile beaker covered with aluminium foil. Approximately 50 gms of compost were removed from each site at every sampling time giving a total of 200 g. of compost for each region. The compost from each region was thoroughly mixed to give a composite and representative sample which was used to determine the numbers of the microbial populations and the carbon and nitrogen contents of the compost in each region. Samples were taken at 2, 3, 4 or 5 day intervals over the period of fifty days, the time between samples being increased

with the age of the windrow.

The micro-flora of the windrow was examined by three methods:-

i. Warcup's soil plate method
ii. The screened substrate tube
iii. The dilution plate method.

Warcup's soil plate method was used for the general isolation of the fungi. A total of 40 Warcup isolation plates were prepared at every sampling time for each region of the windrow, 20 with glucose-starch agar and 20 with cellulose agar (Eggins and Pugh, 1962). Half of these plates (10 cellulose and 10 glucose-starch) were incubated at 50°C for the isolation of the thermophilic fungi and actinomycetes and the other half at 25°C for the isolation of the mesophilic fungi and actinomycetes. Rose bengal at a concentration of 1 part in 15,000 (0.066g/litre) was added to both of the media to suppress the growth of the bacteria and also to limit the spread of fast growing fungi over the whole plate thus giving the slower growing fungi a chance to develop (Smith and Dawson, 1944; Garrett, 1963; Ottow, 1972). In the preparation of the plates approximately 0.2 - 0.3g of compost were used per plate and the larger pieces of refuse such as paper and wood chips, broken glass etc. were excluded from the isolation plates, isolations being made from the smaller soil-like particles. The plates were examined after 7 days incubation and the

percentage frequency of occurrence of a particular species was determined by recording its presence or absence on each of the plates.

The fungi and actinomycetes active in the windrow were isolated by the screened substrate tube method of Eggins and Lloyd (1968). This method is one of the more recent developments of a series of techniques (Rossi, 1928; Cholodny, 1930; Chesters, 1940; Thornton, 1952) designed to isolate only the actively growing fungi present in soils and it successfully overcomes many of the criticisms levelled at the earlier techniques by Garrett (1951) and Chesters and Thornton (1956). The substrate used by Eggins and Lloyd (1968) was polythene coated 3.M.M. Whatman chromatography paper. The main advantages of this substrate are that it consists of a relatively undegraded fibrous cellulose which is manufactured with the total exclusion of extraneous soluble substances and secondly, the ease of handling facilitated by the inert polythene backing after the cellulose has been degraded. In the screened substrate tube the polythene backed chromatography papers are installed in the soil wound around test tubes, polythene side innermost. Each strip is then covered with glass fabric tape 2 inches wide by 0.003 inches thick which is then fastened by an adhesive tape of resin backed glass fibre which is heat and water resistant. The covering of glass fibre tape avoids direct contamination of the substrate by soil particles yet easily allows colonising growing hyphae to penetrate to the cellulosic

substrate. The screened substrate tubes are sterilised by autoclaving in covered beakers containing a few ml. of water.

Allsopp and Eggins (1972), using cotton textile strips, demonstrated the effectiveness of the glass fibre fabric screen by comparing the colonisation of screened and unscreened strips. It was found that there was an initial delay in the colonisation of the screened strips and the fungi colonising them were predominantly cellulolytic species. In comparison a greater variety of fungi were isolated from the unscreened strips and the spectrum obtained was similar to that obtained by the Warcup soil plate technique. These results demonstrated the success of the screened substrate tube in isolating only the active mycoflora of a soil.

The screened substrate tube was chosen for use in the present investigation because of:-

i. the ease of construction and, therefore, replication of the tubes,

ii. the easy handling of the substrate even after colonisation and degradation have taken place,

iii. it is not selective for fungi able to grow rapidly under conditions of low oxygen tension

and iv. it has been shown to be selective for fungi involved in the breakdown of cellulose which is the major fraction of town refuse.

The screened substrate tubes constructed for burial in the windrow had two strips of chromatography paper 0.5cm wide and 5cm long attached as shown in Figure 3.2. The tubes were autoclaved for 20 minutes at a pressure of 15 lbs/sq. in. in the manner previously described. The screened substrate tubes were buried in four sites in the windrow, two sites in the central region and two in the outer region, with 8 tubes being buried at each site. The tubes in the outer region were buried to a depth of 4 - 6 inches. Two tubes were sacrificed from each site at 2, 3, 4, or 5 day intervals, the time between each sacrifice increasing with the age of the windrow. Fresh sets of tubes were placed in the windrow on day 11, after the windrow was turned, and on day 27.

After the tubes were removed from the windrow any compost adhering to the outside of the glass fibre fabric screen was carefully removed before the eventual removal of the screen itself. The cellulose strips were removed with sterile forceps and cut into four pieces with scissors sterilised (by flaming with alcohol) between each cut. The pieces from each strip were plated out, two pieces to each plate of (a) cellulose and (b) glucose-starch agars. The plates prepared from one strip of a tube were incubated at 50°C and the plates prepared from the other strip, of the same tube, were incubated at 25°C. By this method it was possible to compare the activities, at a given time, of the mesophilic and thermophilic fungi and actinomycetes in the windrow. The plates were examined after 7 days of incubation and the species present recorded.

The quantitative changes in the microbial populations in the windrow, i.e. the numbers of actinomycetes, bacteria and fungi, were monitored by means of the dilution plate technique. This technique was originally developed for the estimation of the numbers of bacteria in soils and Garrett (1963) reported that the method could be used with some accuracy for this purpose and also for the quantitative estimation of the actinomycetes because the bacterial population consists of individual cells and the hyphae of the actinomycetes will readily fragment to give such individual cells. However, the dilution technique has been extensively criticised in regard to its use for the quantitative estimation of fungus propagules within the soil (Brierley, 1927; Chesters, 1948; Chesters and Thornton, 1956; Garrett, 1951, 1963; Warcup, 1950, 1951, 1955) because of the advantage it gives to abundantly sporing species and the mycelium present more often than not remains embedded in fragments of organic material which are left behind in the suspension.

Meiklejohn (1957) found whilst making quantitative estimations of bacteria and actinomycetes in soil with the dilution plate technique that the more dilute the suspension from which a plate count was made, the higher the estimate of numbers obtained from the plates. This discrepancy made it impossible to estimate which of the values had any real meaning, thus restricting the usefulness of the dilution plate to comparative purposes, similar to those of the present investigation.

The dilution plate technique has been successfully used to monitor the quantitative changes in the microbial populations of compost heaps of various materials. Two of the more recent studies being by Hayes (1969) and Chang and Hudson (1967) who worked with mushroom composts and wheat straw composts respectively. Hayes (1969) concluded that the dilution plate method proved to be a convenient and workable method for comparative estimations of populations where only gross changes were considered to be of significance. In composts the differences in abundance and composition of microbial populations are more discernible than in soils because of the rapid changes which occur in both population and species composition (Hayes, 1969). The dilution plate technique is, therefore, the most practical and meaningful one to use in monitoring numerical changes in the microbial populations of compost heaps.

In the present investigation the dilution plates were prepared in accordance with the precautions outlined by Collins and Lyme (1970). Approximately 10g., fresh weight, of compost was weighed accurately and placed in 100 ml. of sterile distilled water containing 0.1% yeast extract and shaken for 20 minutes on a reciprocal shaker.*

*Footnote The 0.1% yeast extract was added to minimise the loss in viability of the bacterial population (Straka and Stokes, 1957) since it has been found (Nelson, Macquillan and Campbell, 1957) that a rapid and extensive loss in viability occurs in the commonly used diluting fluids with as much as 40 - 60 percent of the entire population dying within 20 minutes and 90 percent in 1 hour.

Serial tenfold dilutions were prepared for the initial dilutions from the samples from the central and outer regions until final dilutions of $1:10^{10}$ had been obtained. The same dilutions were used for the estimation of the actinomycetes, bacteria and fungi and in the preparation of the plates 1ml. aliquots of the appropriate dilutions were used.

Nutrient agar was used for the enumeration of the bacteria, counts of both the thermophilic and mesophilic bacteria were carried out after 24 hours and checked after 48 hours of incubation. Plates were counted which had between 1 and 200 colonies, as recommended by Hayes (1969).

The actinomycetes, in the dilutions, were isolated on half-strength nutrient agar (the concentration of agar was made up to 2 percent by the addition of Oxoid Agar No.3) containing 25 ug/ml of the antibiotic pimafucin, which was added when the medium had cooled to below $50^{\circ}C$. Half-strength nutrient agar had previously been used for the isolation of mesophilic and thermophilic actinomycetes from hay and wheat straw composts by Gregory and Lacey (1962), and Chang and Hudson (1967) when used with the antibiotic actidione. Actidione was, however, found not to be effective at thermophilic temperatures by Latham (1969), who in an assessment of the different isolation techniques for thermophilic actinomycetes found that pimafucin was easily the most effective antibiotic tested achieving 100 percent suppression of fungi on dilution plates when

incorporated into the medium at a concentration of 25 ug/ml. Plates prepared in this investigation were counted after 48 hours and checked after 4 days of incubation, only those plates having between 1 - 200 colonies per plate were counted.

The dilution plates for the enumeration of the fungi were prepared with potato-dextrose agar which was found by Evans (1969) to isolate a general selection of fungi not favouring any specific group. Plates were counted with between 1 and 50 colonies, after 24 hours of incubation for the thermophiles and after 48 hours for the mesophiles; after these times spreading colonies made counting impossible. James and Sutherland (1939) reported that fifty colonies of fungi per plate was sufficient for accurate quantitative estimations preventing interference between developing colonies through competition and antagonism.

The remainder of the samples taken from the two regions of the windrow were used for the determination of moisture contents, carbon contents and nitrogen contents, samples for the determination of the pH being taken separately. The fresh samples were weighed, dried to constant weight at 60^{o}C, reweighed and the moisture content calculated as a percentage of the original weight. After drying the samples were reduced to a fibrous powder by a high speed laboratory grinder. In this manner it was possible to obtain samples of a representative

nature suitable for use with the relatively small scale analytical techniques required for the determination of the carbon and nitrogen contents of the samples.

The carbon contents of the samples were estimated from the percentage ash by the following equation:-

$$\text{Percentage Carbon} = \left(\frac{100 - \%\text{Ash}}{1.8} \right)$$

This method, commonly known as the New Zealand Method, has been reported to approximate more accurate methods within 2 - 10 percent and is recommended as sufficiently accurate for practical composting (Berkeley Report, 1953). The ash contents of the samples were determined by the method outlined in Tappi Standard T15m-58 for the determination of ash in wood. The samples were stored in a dessicator and approximately 2g. of each sample were accurately weighed out into a crucible of known weight and then reduced to ash in an electric muffle furnace at a temperature of 575^{o}C. The mean of two such determinations was used to calculate the carbon content for each sample.

The total nitrogen contents of the samples were determined by the micro-Kjeldahl method, as described by Leggat-Bailey (1967), apart from the following exceptions. The weight of sample used was 0.05 - 0.1g. and the period of refluxing after the digest had cleared was reduced from eight to three hours and it was found that this reduction

did not in any way affect the results obtained. The total nitrogen content of each sample was estimated by taking the mean of the results from three replicate samples. In the past the micro-Kjeldahl method of estimating nitrogen has been shown to give accurate and reproducible results when used with finely divided soils, a material similar to the finely ground compost (Bremner, 1960).

The pH of the central and outer regions of the windrow were determined every two days. For each region approximately 10 gms of fresh compost, taken from two or three separate sites, were homogenised with 100 ml of de-ionised water in a 'Waring-Blendor', the resulting suspension being cleared by centrifugation and the pH of the extract being taken with a Cambridge Model 78 pH meter.

The temperature of the windrow was checked daily for the first few weeks and from then on approximately every 2 days. The temperatures of the windrow were recorded at depths of 1½, 6 and 18 inches with a long-reach mercury thermometer, the average of six readings from different sites at each depth being recorded to the nearest degree. At one stage, the readings from the thermometer were checked by thermocouple and found to be accurate.

Results

The temperature changes which took place within

the windrow during the fifty days of composting are presented in Figure 3.3. Immediately after the construction of the windrow the refuse had a temperature of 28°C and during the following 48 hours there was an extremely rapid and large rise in temperature throughout the windrow. After 48 hours the temperature of the central region was 58°C, at a depth of 6 inches it was 64°C and at a depth of 1½ inches it was 48°C but in the next few days only slight, further increases were recorded. The maximum temperatures recorded were, for the central region 63°C on day 8, at a depth of 6 inches 66°C by day 4 and at a depth of 1½ inches 50°C also by day 4. After the maximum temperatures had been recorded, a drop in temperature was observed in all regions until after the windrow had been turned on day 11.

Immediately after 'turning' the windrow, the temperatures recorded on day 12 were, in the central region 46°C, at a depth of 6 inches 48°C and at a depth of 1½ inches 27°C. Following this fall, there was, once more, a rapid rise in temperature in all regions of the windrow. In the central region a peak temperature of 60°C was reached on day 22 and in this region the temperature remained above 50°C for 18 days until day 34, after which it decreased towards ambient, a temperature of 31°C being recorded on day 50 at the completion of composting. At a depth of 6 inches a maximum temperature, after turning, of 56°C was reached by day 14 and in this region the temperature on day 50 was 18°C only 4°C above the maximum air temperature.

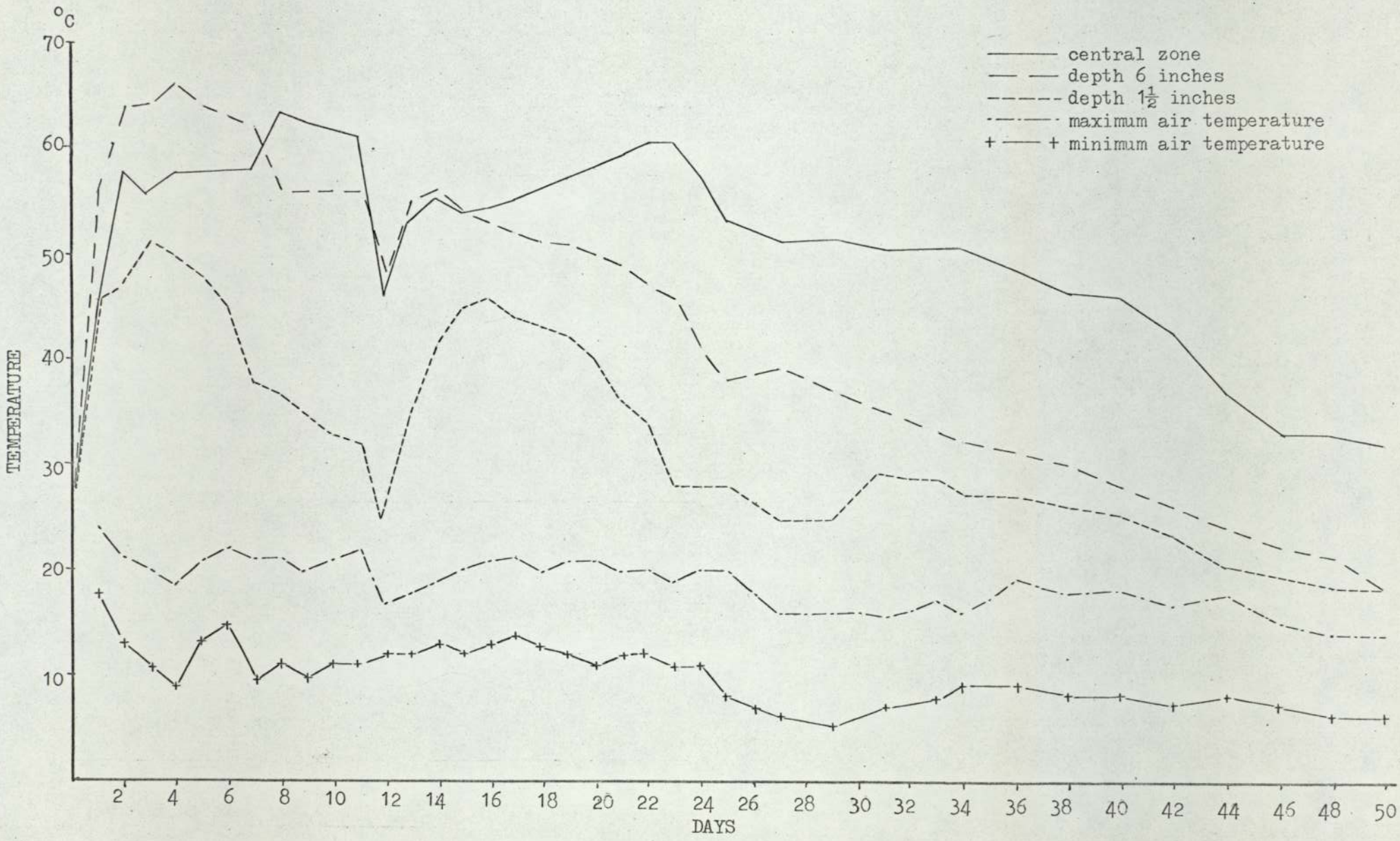


FIGURE 3.3 TEMPERATURE OF INNER AND OUTER REGIONS OF THE WINDROW AND MAXIMUM AND MINIMUM AIR TEMPERATURES

Throughout the 50 day composting period the air temperatures remained fairly constant, showing only a slight decline from start to finish. The maximum temperatures recorded fluctuated between the limits of 14 and 24°C and the minimum temperatures between 5 and 18°C. The lowest air temperatures were recorded between days 25 and 36 and this was reflected in the temperatures recorded in the windrow, which dropped more rapidly than at any other stage, especially in the outer regions.

The pH changes which occurred within the two regions of the windrow during the composting period are shown in Figure 3.4. The pH in the outer region rose very quickly from pH 6.02 to 7.96 in 48 hours and by day 4 it had risen still further to a peak of 8.13. After day 4 the pH dropped until by day 10 it was 7.6 after which it again rose until the windrow was turned on day 11. In the central region the pH initially rose much more slowly, reaching a first peak of 6.62 on day 4 after which it fell to pH 6.02 by day 8, only to increase, despite the turning of the windrow, until it reached a maximum of 7.95 on day 31, after which it remained almost constant until the completion of composting. The pH of the outer region, after turning, rose rapidly to a peak of 8.14 by day 19 and apart from minor fluctuations remained at this level until the completion of composting.

The changes in the carbon, nitrogen and moisture contents of the windrow are shown in Table 3.1. When a

FIGURE 3.4 pH OF THE CENTRAL AND OUTER REGIONS OF THE WINDROW

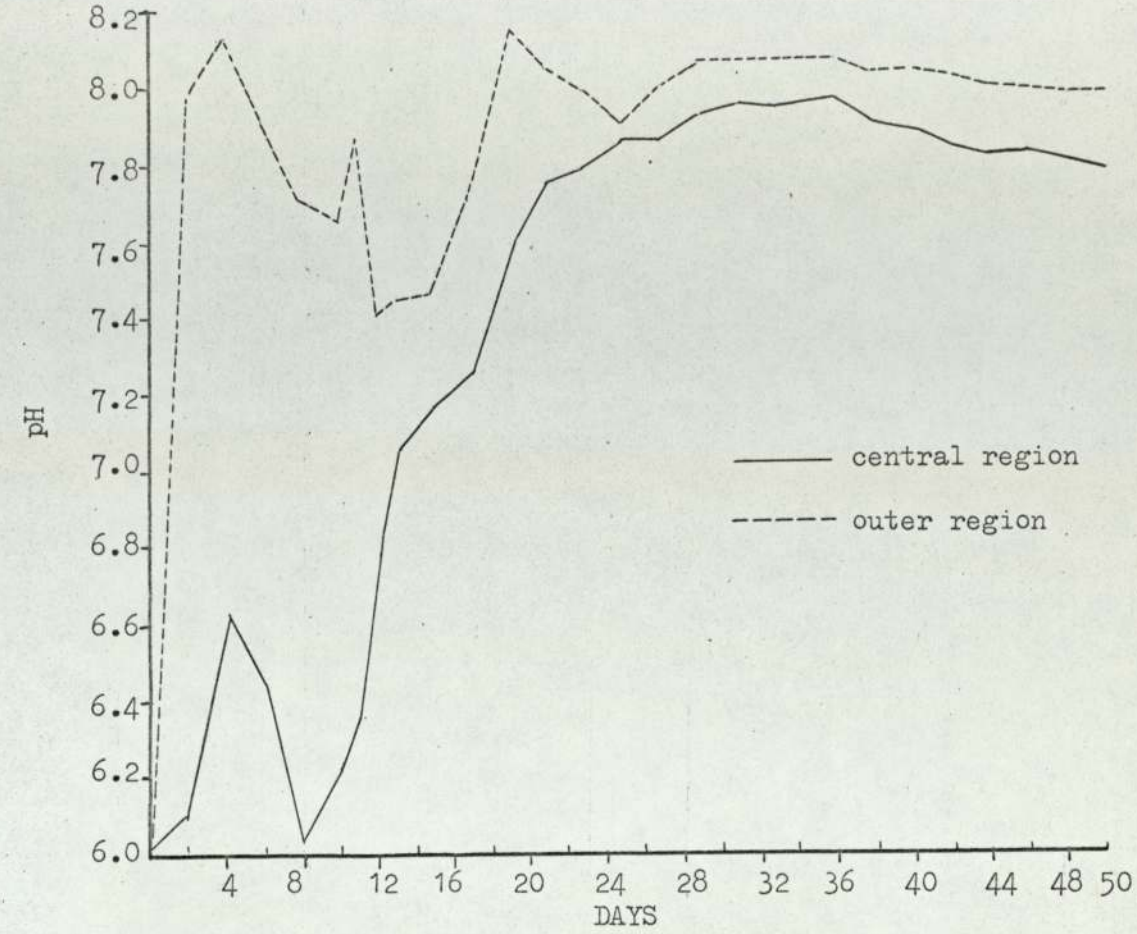


FIGURE 3.5 CHANGES IN THE C:N RATIO DURING COMPOSTING

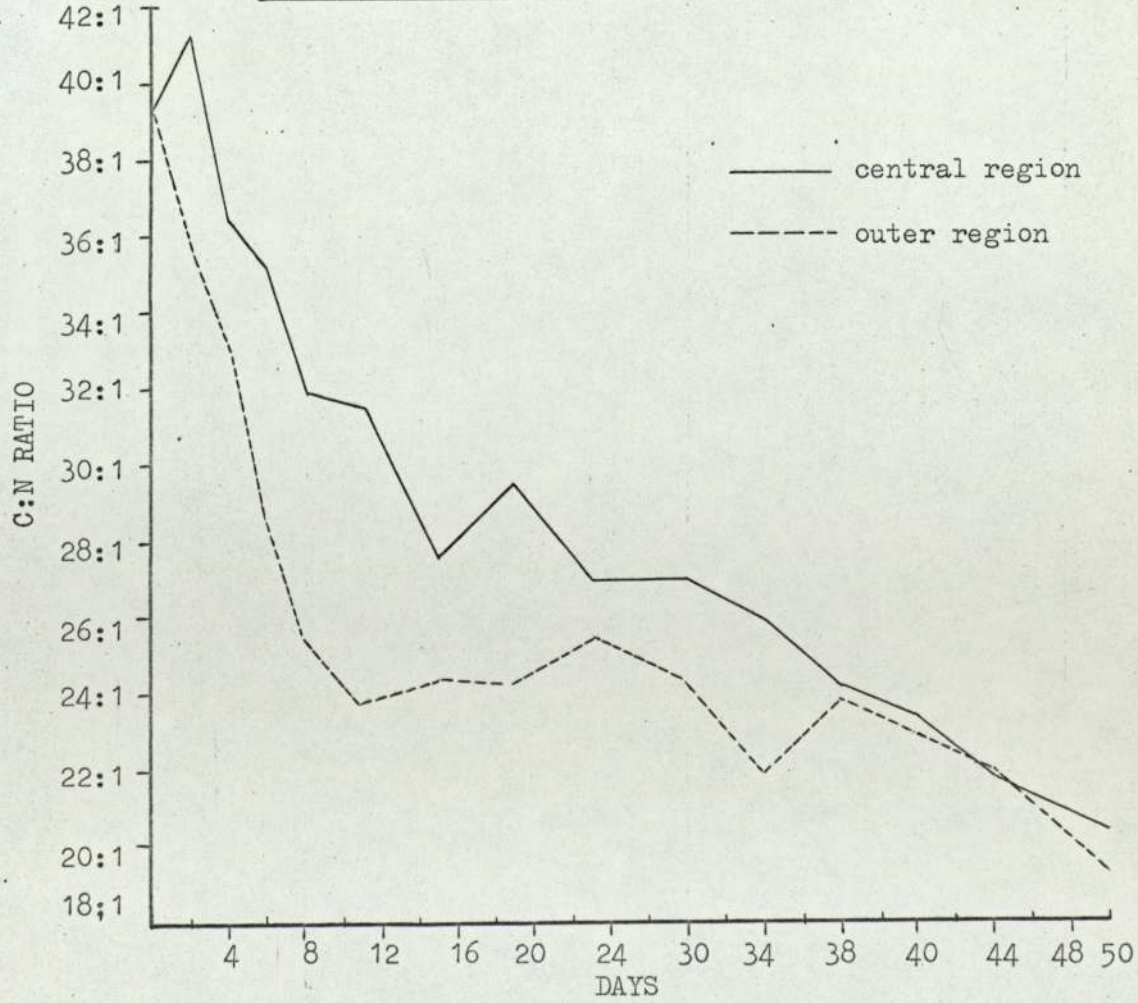


Table 3.1 Carbon, nitrogen and moisture contents of the two regions of the windrow

DAYS	CARBON CONTENT (%)		NITROGEN CONTENT (%)		C:N		MOISTURE CONTENT	
	CENTRAL	OUTER	CENTRAL	OUTER	CENTRAL	OUTER	CENTRAL	OUTER
0	28.5	28.5	0.737	0.737	38.7	38.7	43.3	43.3
2	29.3	28.8	0.710	0.812	41.2	35.5	39.1	40.4
4	28.4	28.3	0.782	0.855	36.3	33.1	38.9	45.0
6	27.9	27.1	0.824	0.987	35.1	28.5	36.3	42.5
8	27.5	26.4	0.861	1.035	31.9	25.5	33.5	39.6
11	26.6	23.1	0.852	0.974	31.4	23.7	33.8	43.5
15	23.1	23.1	0.841	0.951	27.5	24.3	39.7	44.7
19	24.9	22.1	0.846	0.900	29.4	24.1	39.2	40.8
23	25.0	22.8	0.929	0.901	26.9	25.3	33.6	43.8
28	24.2	22.0	0.897	0.910	27.0	24.2	31.5	43.8
32	22.6	21.2	0.875	0.963	25.8	21.8	29.0	41.5
36	21.3	20.4	0.887	0.856	24.1	23.7	28.9	40.0
40	20.7	18.8	0.892	0.828	23.3	22.9	28.5	40.5
44	20.2	19.2	0.936	0.884	21.7	21.8	26.1	39.2
50	18.0	16.7	0.895	0.873	20.2	19.2	25.4	39.0

plot of the C:N ratios was made as shown in Figure 3.5 it became apparent that degradation had proceeded much more rapidly in the outer region and that in both regions the maximum rate of degradation had occurred during the first 12 - 15 days of composting.

From Table 3.1 it can be seen that the moisture content of the central region fell much more rapidly than that of the outer region which at all times remained approximately constant at 40 percent. In the first 11 days of composting the moisture content of the central region fell from 43.3 percent to 33.8 percent; after turning and watering it rose to 39.7 percent but by the completion of composting on day 50 it had fallen to 25.4 percent.

The quantitative changes in the thermophilic and mesophilic microbial populations that occurred within the central region are shown in Figures 3.6 and 3.7 respectively. In this region the populations of both the thermophilic and mesophilic fungi decreased during the first two days as the temperature rose to 58^{o}C. The population of the mesophilic fungi, was reduced to a very low level but persisted throughout the period of peak heating and between days 23 and 28 there was a marked increase in their numbers which continued until day 50.

The fungi isolated at 25^{o}C from the central region of the windrow by Warcup's technique are listed in Table 3.2 which shows that the refuse initially contained

FIGURE 3.6 CHANGES IN THE THERMOPHILIC MICROBIAL POPULATIONS OF THE CENTRAL REGION OF THE WINDROW DURING COMPOSTING

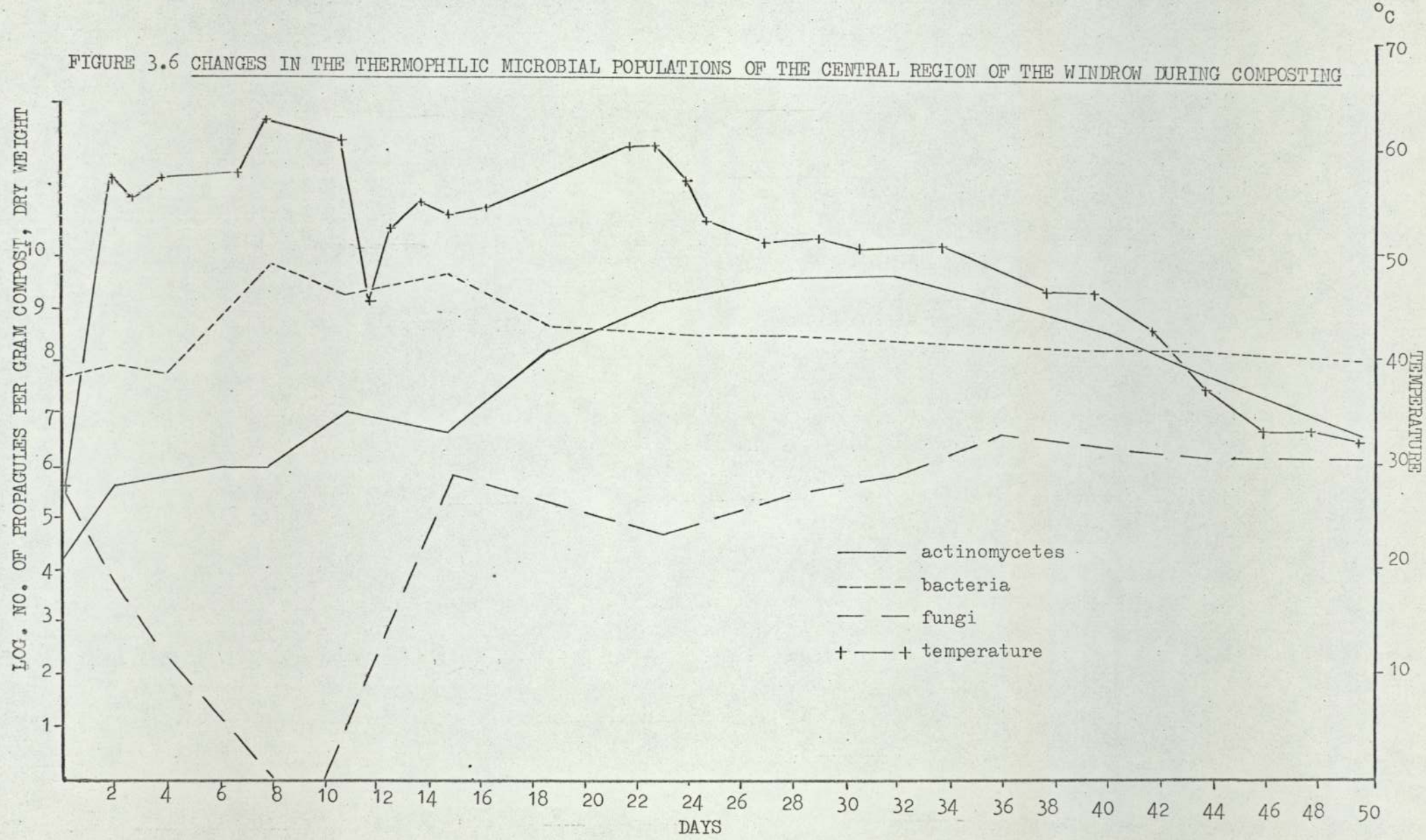


FIGURE 3.7 CHANGES IN THE MESOPHILIC MICROBIAL POPULATIONS OF THE CENTRAL REGION OF THE WINDROW DURING COMPOSTING.

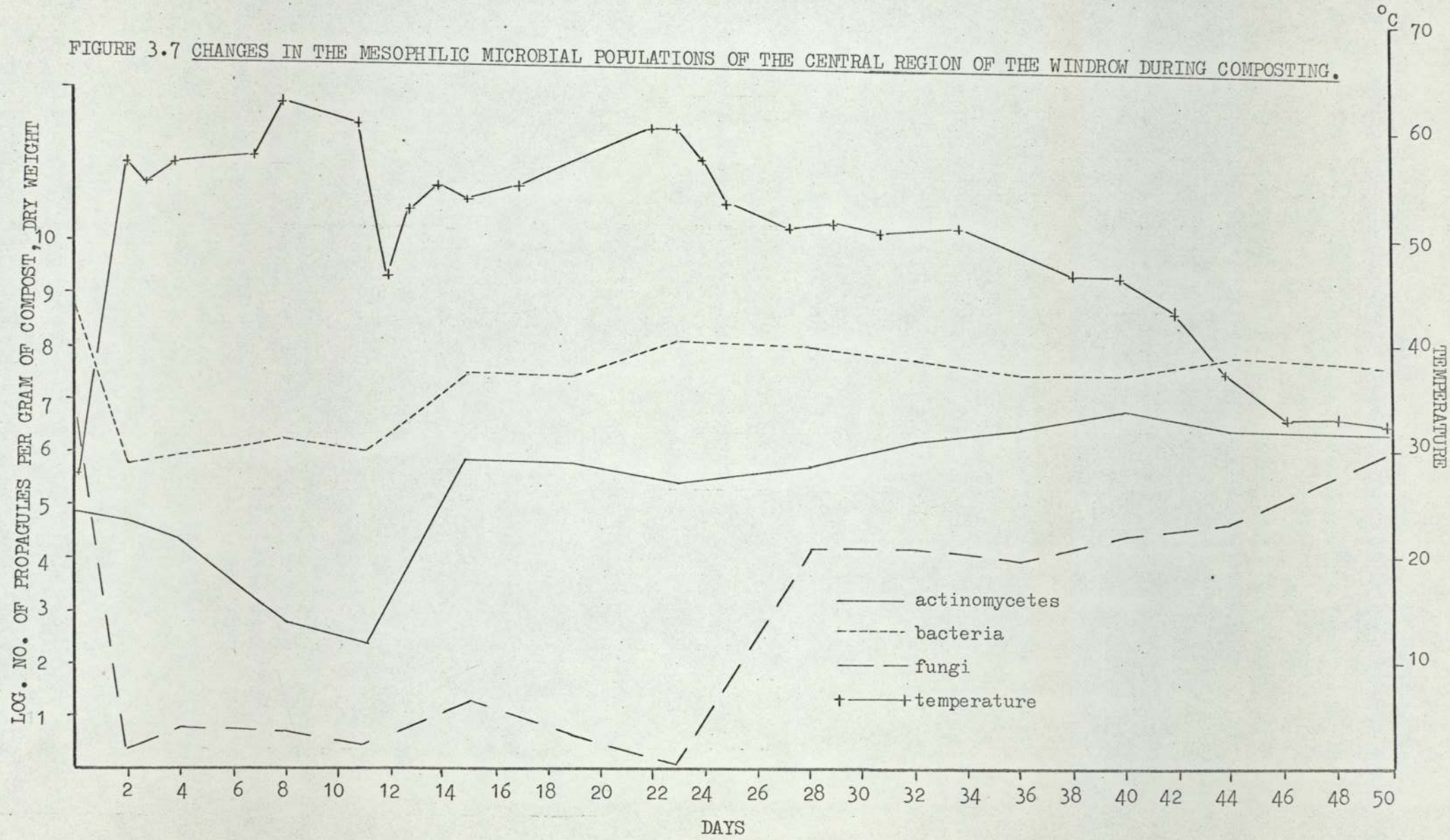


Table 3.2 (A) Percentage frequency of occurrence of fungi and actinomycetes isolated, from the central region of the windrow, on cellulose agar at 25°C by Warcup's technique

Species isolated \ Days	0	2	4	6	8	11	15	19	23	27	32	36	40	44	50
Arthrobotrys sp.								80	100						
Aspergillus sp.															
Aspergillus fumigatus	90	100	100	100			100	80	60	100	100	90	100	90	90
Aspergillus fumigatus (orange variant)				40			60	40	40	30	40		40	30	50
Cephalosporium sp.	20														
Cerastomella sp.															
Chaetomium aterrimum	50						20								
Eurotium sp.	20														
Fusarium sp.	90	70	50	40	60	40	90		50						
Gliocladium roseum															
Graphium sp.															
Humicola insolens*	10														
Humicola grisea	30						10								
Monilia brunea	20														
Mucor pusillus*	100	100	70	60			100	80	100	90	90	80	70	100	100
Penicillium funiculosum			20	30	40	30	30								
Penicillium sp.			100	90											
Paecilomyces varioti	20														
Piptocephalis sp.															
Sordaria fimicola	30														
Sporotrichum sp.	30														
Stachybotrys atra	20						10								
Stysanus sp.	80						10								
Trichoderma viride	10			10	10	10	20								
Ulocladium sp.	20														
Verticillium lateritium	40														
Actinomycetes	70	30	30		10	40	60	50	70	90	100	100	90	90	

* denotes thermopnile

Table 3.2 (B) Percentage frequency of occurrence of fungi and actinomycetes isolated, from the central region of the windrow, on glucose-starch agar at 25°C by Warcup's technique

Species isolated \ Days	0	2	4	6	8	11	15	19	23	27	32	36	40	44	50
Arthrobotrys sp.									20						
Aspergillus sp.	20														
Aspergillus fumigatus	90	100	100	100			100	70	50	90	100	100	100	100	90
Aspergillus fumigatus (orange variant)	30		50				20	90		50	70	50		30	70
Cephalosporium sp.	50														
Cerastomella sp.	30														
Chaetomium aterrimum	20														
Eurotium sp.	20	80													
Fusarium sp.	60		30	40		20									
Gliocladium roseum	20														
Graphium sp.	20														
Humicola insolens*															
Humicola grisea	10														
Monilia brunea															
Mucor pusillus*	90	90	70	50			100	100	90	100	70	80	80	90	90
Penicillium funiculosum	90		10	30	50										
Penicillium sp.	20														
Paecilomyces varioti	60														
Piptocephalis sp.	10														
Sordaria fimicola															
Sporotrichum sp.															
Stachybotrys atra															
Stysanus sp.	30														
Trichoderma viride	60		20												
Ulocladium sp.															
Verticillium lateritium															
Actinomycetes	80	40	30				30	30	40	60	80	60	100	70	70

* denotes thermophile

a varied selection of fungi with 26 different species being isolated from the initial sample of refuse. By day 4 the number of different species isolated, had been reduced to 8 including: two variants of Aspergillus fumigatus, Fusarium sp.,Mucor pusillus, Penicillium funiculosum, Penicillium sp., Trichoderma viride and several species of Actinomycetes. Of these fungi the two varients of the thermotolerant Aspergillus fumigatus and the thermophilic Mucor pusillus persisted, apart from the period between days 8 and 11, with high percentage frequencies of occurrence throughout the 50 days composting, these were also the three fungi most frequently isolated by the dilution technique. After the 'turning' of the windrow there was an increase in the number of species isolated on day 15, Chaetomium aterrimum, Humicola grisea, Stachybotrys atra and Stysanus sp. all being isolated for the first time since the construction of the windrow. These fungi did not persist, however, and were not isolated again on day 19. The mesophilic Actinomycetes in the central region persisted throughout the composting period their frequency of occurrence increasing in the later stages.

The mesophilic fungi isolated at 25°C, by the screened substrate tube, from the central region are listed in Table 3.3 which shows again that the three most active fungi in this region were Mucor pusillus and the variants of Aspergillus fumigatus. Trichoderma viride and the two species of Penicillium were isolated in the initial stages of composting but were not isolated by the screened substrate

Table 3.3 Mesophilic fungi and actinomycetes isolated by the screened substrate tube, from the central region of the windrow, on cellulose (C) and glucose-starch (GS) agar incubated at 25°C for 7 days

Species isolated \ Days	2		4		7		11		15		19		23		27		31		35		39		50	
	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus	+	+	+	+	+				+	+	+	+	+		+	+	+	+		+	+	+	+	+
Aspergillus fumigatus (orange variant)	+				+												+	+					+	
Aspergillus niger					+																			
Chaetomium thermophile																	+	+		+	+		+	+
Cladosporium sp.	+																							
Humicola insolens																					+		+	+
Mucor pusillus	+	+		+	+					+		+	+	+	+	+					+		-	+
Penicillium funiculosum					+	+																		
Penicillium sp.			+																					
Trichoderma viride				+		+																		
Actinomycetes																	+				+		+	
	First set of tubes								Second set of tubes								Third set of tubes							

+ denotes presence on the screened substrate tube

tube after day 7. Chaetomium thermophile and Humicola insolens, both thermophiles, were isolated, at 25°C, on day 31 from the third set of tubes, when the temperature of the windrow was 50°C.

On day 8 when the temperature was 63°C, the maximum attained, in the central region, the numbers of the thermophilic fungi present in this region were reduced to zero but they reappeared between days 10 and 15, most probably sometime after the windrow had been turned on day 11. However, the population of the thermophilic fungi decreased again after day 15, as the temperature rose to a second peak of 60°C on day 22. By day 25 the temperature had fallen to 53°C and from this stage the population of the thermophilic fungi increased, reaching a maximum on day 36 when the temperature was 48°C. Table 3.4 lists the thermophilic fungi and actinomycetes isolated from the central region by Warcup's technique at 50°C. Humicola lanuginosa was the most frequently isolated fungus both before and after the turning of the windrow. Chaetomium thermophile was isolated from the original refuse but did not appear in the central region of the windrow until day 15 after the windrow had been turned but it then had a frequency of occurrence of 100 percent which was maintained until the completion of composting on day 50. The fungi most frequently isolated from this region, by the dilution plate technique were the thermophiles, Chaetomium thermophile, Humicola lanuginosa, Mucor pusillus and the thermotolerant Aspergillus fumigatus. The fungi and actinomycetes isolated

Table 3.4 (A) Percentage frequency of occurrence of the thermophilic fungi and actinomycetes isolated, from the central region of the windrow, on cellulose agar at 50°C by Warcup's technique

Species isolated \ Days	0	2	4	6	8	11	15	19	23	28	32	36	40	44	50
Aspergillus fumigatus	50														
Chaetomium thermophile	30						100	100	100	100	100	100	100	100	100
Humicola insolens									30			20		40	
Humicola lanuginosa	100	60	100	100			100	100	100	100	100	100	100	100	100
Malbranchea pulchella	70								10	20	40	30	30	20	30
Mucor pusillus	50		40												
Streptomyces thermovulgaris	10														
Thermoactinomyces glaucus	80	100	100	30	40	50	60	40	100	90	50	50	60	30	20
Thermomonospora curvata	60	70	100	40	90	100	50	60	90	80	100	80	70	60	50
Thermomonospora fusca								20							

Table 3.4 (B) Percentage frequency of occurrence of the thermophilic fungi and actinomycetes isolated, from the central region of the windrow, on glucose-starch agar at 50°C by Warcup's technique

Species isolated \ Days	0	2	4	6	8	11	15	19	23	28	32	36	40	44	50
Aspergillus fumigatus	60		20												
Chaetomium thermophile	10						100	100	100	100	100	80	100	100	100
Humicola insolens															
Humicola lanuginosa	100	80	100	100			100	100	100	100	100	100	100	100	100
Malbranchea pulchella															
Mucor pusillus	100	60	100				60	40	80	40	60	50	30	70	50
Streptomyces thermovulgaris															
Thermoactinomyces glaucus	40	80	20	10	30	40	40		50	50	30	40	60	10	20
Thermomonospora curvata		80	90	50	80	60	60	50	70	70	80	90	80	66	50
Thermomonospora fusca															

at 50°C, by the screened substrate tube, from the central region of the windrow are listed in Table 3.5. Humicola lanuginosa and Chaetomium thermophile were again the most common of the thermophilic fungi and Humicola lanuginosa was the only thermophilic fungus to be isolated by this method before the windrow was turned on day 11. After the windrow had been turned and the temperature in this region had fallen from its peak of 63°C several other fungi were isolated by the screened substrate tube and in order of appearance they were, Mucor pusillus, Thermoascus aurantiacus, Aspergillus fumigatus, Malbranchea pulchella and the orange variant of Aspergillus fumigatus.

Unlike the thermophilic fungi the population of thermophilic actinomycetes in the central region increased rapidly during the first two days of composting and then increased steadily reaching a peak on day 28, following the second temperature peak, of 60°C in this region. The increase in the population of thermophilic actinomycetes in this region was closely watched by the increase in pH. After day 28 the population of the thermophilic actinomycetes remained at a constant level until the temperature dropped below 50°C on day 36 after which there was a sharp decline in their numbers. On the dilution plates, the dominant thermophilic actinomycetes in the central region were found to be Thermomonospora curvata and Streptomyces thermovulgaris. Four species of thermophilic actinomycetes were isolated by Warcup's technique and these were, Thermomonospora curvata, Thermoactinomyces glaucus,

Table 3.5 Thermophilic fungi and actinomycetes isolated at 50°C from the screened substrate tubes from the central region of the windrow on cellulose and glucose-starch agar (C and GS respectively)

Species isolated \ Days	2		4		7		11		15		19		23		27		31		35		39		50	
	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus																+							+	
Aspergillus fumigatus (orange variant)																							+	+
Chaetomiun thermophile									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Humicola lanuginosa	+	+	+	+					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malbranchea pulchella																			+		+		+	
Thermoascus aurantiacus															+					+		+		
Streptomyces thermo-vulgaris																							+	
Thermoactinomyces glaucus																							+	
Thermomonospora curvata			+		+	+	+		+	+	+	+	+		+	+	+	+	+	+	+	+		
Thermomonospora fusca				+																				
	First set of tubes								Second set of tubes								Third set of tubes							

+ denotes presence on the screened substrate tube

Thermomonospora fusca and Streptomyces thermovulgaris. Of these only Thermomonospora curvata and Thermoactinomyces glaucus were isolated regularly both having an average frequency of occurrence of over 50 percent. The thermophilic actinomycete most frequently isolated by the screened substrate tube was Thermomonospora curvata. In this investigation the thermophilic actinomycetes were identified according to the system of classification given by Waksman (1961).

During the first 8 days of composting the population of the thermophilic bacteria in the central region increased rapidly and then very gradually declined until the completion of composting. In the first two days of composting the numbers of mesophilic bacteria declined sharply and then remained at a constant level until after the windrow was turned on day 11 after which their numbers gradually increased reaching a peak on day 23 at which stage their numbers remained constant until the completion of composting. The population of the mesophilic actinomycetes in the central region also declined during the initial stages of composting but between days 11 and 15, after 'turning' there was a marked rise in their population which was halted by the increase in temperature to 60^{o}C by day 22. However after day 23 there was a rapid fall in the temperature and the population of the mesophilic actinomycetes increased reaching a peak on day 40.

The quantitative changes in the thermophilic and

mesophilic microbial populations of the outer region of the windrow are shown in Figures 3.8 and 3.9 respectively.

The rapid rise in the temperature of the outer region of the windrow during the first two days of composting coincided with a drop in the population of the thermophilic fungi. Between days 2 and 4 the population of the thermophilic fungi increased rapidly despite the high temperatures of 64 - 66°C at a depth of 6 inches. The increase in population probably occurred because the maximum temperature recorded at a depth of 1½ inches during this period was only 50°C. The increase in the population of the thermophilic fungi continued until the windrow was turned, after which a slight decline was observed until day 15 and after this time the population remained constant until the completion of composting. The fungi isolated on the Warcup plates at 50°C from the outer region of the windrow are listed in Table 3.6. The most commonly occurring fungi were Chaetomium thermophile, Humicola lanuginosa and Mucor pusillus the same three fungi that were most prevalent in the central region. The fungi appearing most frequently on the dilution plates incubated at 50°C were Chaetomium thermophile, Humicola lanuginosa, Mucor pusillus and Aspergillus fumigatus. The fungi most frequently isolated by the screened substrate tube at 50°C from the outer region were again, Chaetomium thermophile, Humicola lanuginosa and Mucor pusillus. In the later stages of composting Malbranchea pulchella was also frequently isolated by this technique, showing it to be active within the windrow, see Table 3.7.

FIGURE 3.8 CHANGES IN THE THERMOPHILIC MICROBIAL POPULATIONS OF THE OUTER REGION OF THE WINDROW DURING COMPOSTING.

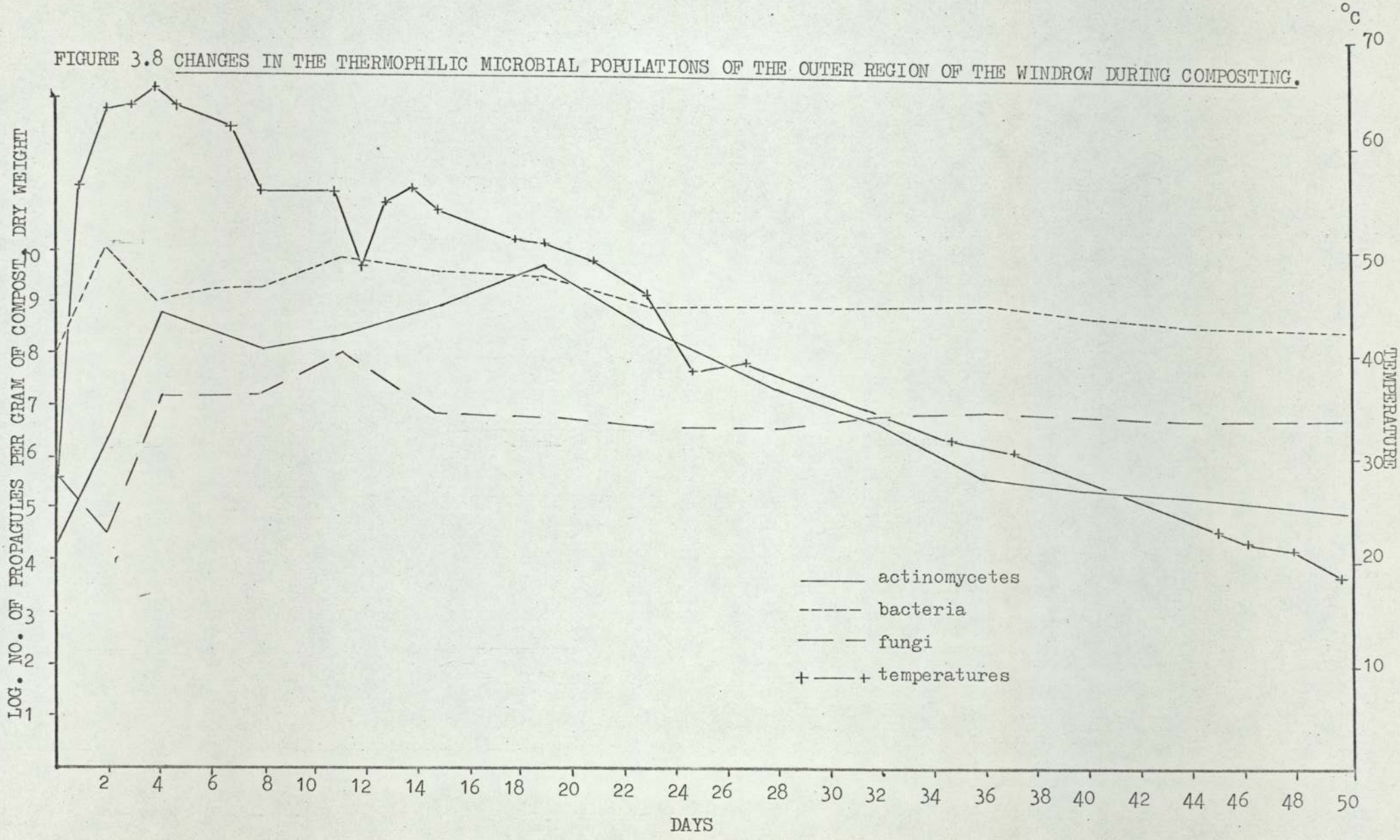


FIGURE 3.9 CHANGES IN THE MESOPHILIC MICROBIAL POPULATIONS OF THE OUTER REGION OF THE WINDROW DURING COMPOSTING

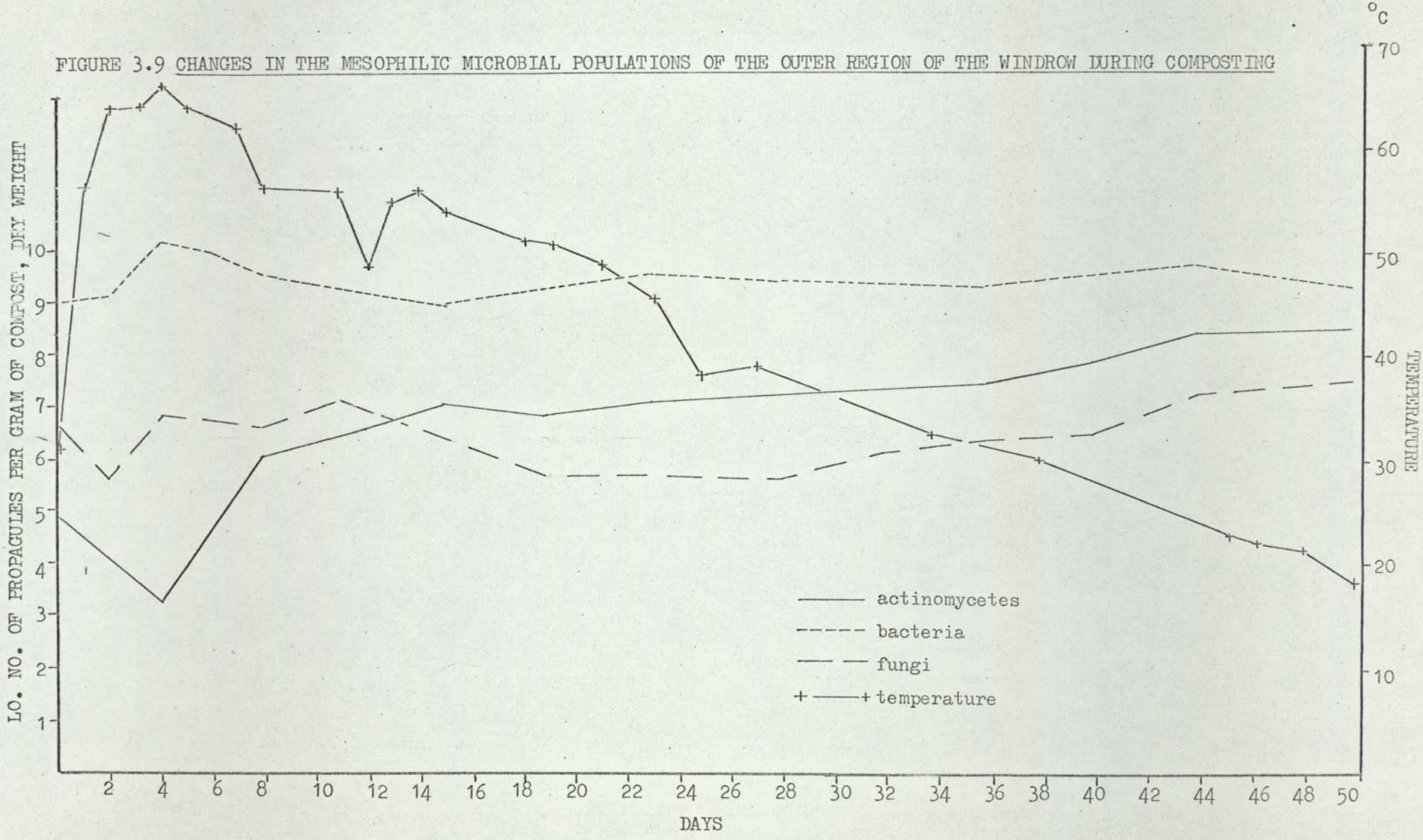


Table 3.6 (A) Percentage frequency of occurrence of thermophilic fungi and actinomycetes isolated, from the outer region of the windrow, on cellulose agar at 50°C by Warcup's technique

Species isolated \ Days	0	2	4	6	8	11	15	19	23	28	32	36	40	44	50
Aspergillus fumigatus	50										20		10		10
Chaetomium thermophile	30	80	100	100	100	100	100	100	100	100	100	100	100	100	100
Humicola insolens								60	90						
Humicola lanuginosa	100	100	100	100	100	100	100	100	70	80	100	100	100	100	100
Malbranchea pulchella	70									50	70	60	80	80	80
Mucor pusillus	50					80	10	20	70	90	100	90	100	88	100
Streptomyces thermovulgaris	10														
Thermoactinomyces glaucus	80	80			40	80			70		70			10	
Thermomonospora curvata	60	40	20	70	40	80	50	100	80	100	90	70	50	40	40
Thermomonospora fusca	30					30	70	100	50	20	30	10	10	10	20

Table 3.6 (B) Percentage frequency of occurrence of the thermophilic fungi and actinomycetes isolated, from the outer region of the windrow, on glucose-starch agar by Warcup's technique at 50°C

Species isolated \ Days	0	2	4	6	8	11	15	19	23	28	32	36	40	44	50
Aspergillus fumigatus	60	60	30				40						10		30
Chaetomium thermophile	10	50	60	70	80	100	100	100	100	100	100	100	100	100	100
Humicola insolens															
Humicola lanuginosa	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Malbranchea pulchella			20												
Mucor pusillus	100	70	100	100	100	70	100	100	100	90	100	100	70	100	100
Streptomyces thermovulgaris															
Thermoactinomyces glaucus	40	40	70		30	60			40		20				
Thermomonospora curvata		40	20	60	30	60	60	40	50	100	80	90	40	40	20
Thermomonospora fusca						40					10				

Table 3.7 Thermophilic fungi and actinomycetes isolated at 50°C from the screened substrate tubes from the outer region of the windrow, on cellulose (C) and glucose-starch (GS) agar

Species isolated \ Days	2		4		7		11		15		19		23		27		31		35		39		50	
	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS
Aspergillus fumigatus																								
Chaetomium thermophile				+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
Humicola insolens									+		+													
Humicola lanuginosa		+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malbranchea pulchella													+			+			+	+	+	+	+	
Mucor pusillus				+				+	+	+	+	+		+		+		+	+	+		+	+	+
Streptomyces thermo-vulgaris													+											
Thermoactinomyces glaucus					+	+	+						+	+			+		+	+				
Thermomonospora curvata			+		+		+		+		+	+	+	+	+	+	+	+	+		+	+	+	
	First set of tubes								Second set of tubes								Third set of tubes							

+ denotes presence on the screened substrate tube

In the outer region the population of mesophilic fungi remained constant until the windrow was turned after which the population declined until day 19. Between days 19 and 28 the population again remained constant but it increased from day 28 until the completion of composting on day 50. 26 species of fungi were isolated at 25°C on the Warcup isolation plates from the initial refuse, see Table 3.8, including two thermophiles Mucor pusillus and Humicola insolens and two variants of the thermotolerant Aspergillus fumigatus. During composting, the fungi most frequently isolated from the outer region at 25°C by the Warcup technique were Aspergillus fumigatus, Fusarium sp., Mucor pusillus, Penicillium funiculosum and Penicillium sp. After day 11 the nematode trapping fungus Arthrobotrys sp. was regularly isolated, its appearance coinciding with the presence of swarms of nematodes within this region. During the first 6 days the thermophilic Humicola insolens was regularly isolated at 25°C and it could also be observed growing on the surface of the windrow producing wefts of mycelium.

The fungi isolated at 25°C from the screened substrate tubes from the outer region of the windrow are listed in Table 3.9. The most frequently isolated fungi were Aspergillus fumigatus, Mucor pusillus and to a lesser extent Fusarium sp., Chaetomium aterrimum, Aspergillus fumigatus (O.V.) and Trichoderma viride.

During the first 4 days of composting an

Table 3.8 (A) Percentage frequency of occurrence of fungi and actinomycetes isolated, from the outer region of the windrow, on cellulose agar at 25°C by Warcup's technique

Species isolated \ Days	0	2	4	6	8	11	15	19	23	28	32	36	40	44	50
Arthrobotrys sp.						40	100	100	100	100	100	90	100	100	100
Aspergillus sp.											10				
Aspergillus fumigatus	90	100	100	100	100	90	100	90	70	100	100	100	100	90	100
Aspergillus fumigatus (orange variant)		40	20			20		10	30	40	30	40		40	30
Aspergillus niger															
Cephalosporium sp.	20														
Cerastomella sp.															
Chaetomium aterrimum	50		40	60	80	10	60	10	20	20	40		30	20	10
Eurotium sp.	20														
Fusarium sp.	90	60	60	60	70	60	40	100	80	70	50	60	70	80	60
Gliocladium roseum															
Graphium sp.		20					30	20				30			
Humicola grisea		30	20												
Humicola insolens*	10	50	60	10											
Monilia brunea	20														
Mucor pusillus*	100	80	60	50	70	90	70	40	100	90	80	70	70	90	80
Penicillium funiculosum			60	40	60	50		60		60	50	80	50	50	60
Penicillium sp.		10	70	40	40			70		40	50			60	60
Paecilomyces variotti	20														
Piptocephalis sp.															
Sordaria fimicola	30		10				40			10					
Sporotrichum sp.	30														
Stachybotrys atra	20														
Stysanus sp.	80														
Trichoderma viride	10		70				20		10	20		30	50	70	60
Ulocladium sp.	20														
Verticillium lateritium	40														
Actinomycetes	70	100	100	80	60	100	100	70	90	100	100	100	100	100	100

* denotes thermophile

Table 3.8 (B) Percentage frequency of occurrence of fungi and actinomycetes isolated, from the outer region of the windrow, on glucose-starch agar at 25°C by Warcup's technique

Species isolated \ Days	0	2	4	6	8	11	15	19	23	28	32	36	40	44	50
Arthrobotrys sp.						20	70	90	60	70	60	80	70	50	60
Aspergillus sp.	20	90	50			20	30	30			30	20	40	40	
Aspergillus fumigatus	90	100	100	90	100	100	100	100	100	90	100	100	90	90	100
Aspergillus fumigatus (orange variant)	30	20	10	20	20		10	20	20	10	20	10		10	
Aspergillus niger	50							30							
Cephalosporium sp.	50														
Cerastomella sp.	30														
Chaetomium aterrimum	20		30	20											
Eurotium sp.	20														
Fusarium sp.	60	70	50			30		50	60	30		30	40	10	30
Gliocladium roseum	20														
Graphium sp.	20							10							
Humicola grisea															
Humicola insolens*		10	20												
Monilia brunea															
Mucor pusillus*	90	100	30	50	100	80	100	100	70	100	100	100	80	100	100
Penicillium funiculosum	90		50	60	50	50	50	70		60	50	60	40	60	50
Penicillium sp.	20	10	50	40	30						50	50		40	30
Paecilomyces varioti	60														
Piptocephalis sp.	10														
Sordaria fimicola															
Sporotrichum sp.															
Stachybotrys atra															
Stysanus sp.	30														
Trichoderma viride	60		30				20			20				40	30
Ulocladium sp.															
Verticillium lateritium															
Actinomycetes	80	20	20	30	60	80	80	60	60	70	90	100	80	90	80

* denotes thermophile

Table 3.9 Fungi and actinomycetes isolated by the screened substrate tube from the outer regions of the windrow on cellulose (C) and glucose-starch (GS) agars incubated at 25°C

Species isolated \ Days	2		4		7		11		15		19		23		27		31		35		39		50	
	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS	C	GS
Arthrobotrys sp.													+	+			+		+	+				
Aspergillus fumigatus		+	+	+	+	+	+	+	+	+	+	+		+	+	+		+	+	+		+	+	+
Aspergillus fumigatus (orange variant)			+		+	+																		
Aspergillus sp.			+																					
Chaetomium aterrimum					+				+	+	+													
Coprinus sp.											+		+											
Fusarium sp.							+		+	+					+	+			+		+	+	+	+
Graphium sp.											+													
Humicola insolens																			+					
Mucor pusillus*		+		+	+		+	+	+	+		+	+	+	+	+		+	+	+		+	+	+
Penicillium funiculosum													+											
Penicillium sp.			+	+										+										
Stysanus sp.											+													
Trichoderma viride	+	+				+															+		+	
Actinomycetes																								

* denotes thermophile

extremely rapid and marked rise in the population of the thermophilic actinomycetes occurred in the outer region of the windrow. This coincided with a rise in temperature, at a depth of 6 inches, from 28°C to 66°C and a rise in pH from 6.02 to 8.13. After day 4 both the temperature and the pH fell and this was followed by a decline in the population of the thermophilic actinomycetes until day 8 after which time the population remained approximately constant until the windrow was turned. Between days 11 and 19 the population of thermophilic actinomycetes in the outer region gradually increased reaching a second peak on day 19 and once again the rise in population was accompanied by rises in both the temperature and the pH. After day 19 the population declined rapidly, until the completion of composting, following a sharp fall in the temperature in this region. Four species of thermophilic actinomycetes were isolated by Warcup's technique, (see Table 3.6) at 50°C, from the outer region and these were the same species which appeared in the central region. The most frequently occurring of these thermophilic actinomycetes was again Thermomonospora curvata although its frequency of occurrence dropped in the later stages of composting as the temperature fell below 40°C. On the dilution plates incubated at 50°C Thermomonospora curvata and Streptomyces thermovulgaris were the most frequently occurring actinomycetes. Thermomonospora curvata and Thermoactinomyces glaucus were the only two species to be regularly isolated by the screened substrate tubes from the outer region of the windrow.

The population of the mesophilic actinomycetes, in the outer region, declined rapidly during the first four days of composting as the temperature rose to 66°C at a depth of 6 inches. As the temperature fell from this peak, between days 4 and 11, the population of mesophilic actinomycetes increased rapidly. This rate of increase slowed down once the windrow had been turned and the population gradually increased until the completion of composting.

During the first two days of composting the population of the thermophilic bacteria in the outer region increased rapidly after which, apart from minor fluctuations, it remained constant until the completion of composting. The population of the mesophilic bacteria in this region remained almost constant throughout the composting period.

Discussion

The final analysis of the compost, on day 50, revealed that it had an average carbon content of 17.35 percent and a nitrogen content of 0.88 percent, giving a final C:N ratio of 19.7:1. These values are typical of a stable compost (Berkeley Project, 1953; H.M.S.O., 1971) and compare favourably with the values given by Chesterfield Rural District Council (see Appendix III) for their composts.

The temperature readings obtained from the small experimental windrow are similar to those reported for large windrows, consisting of many tons of refuse

(Stutzenberger, Kaufman and Lossin, 1970) and are, therefore, typical of the normal process of aerobic thermophilic decomposition associated with the windrowing of refuse.

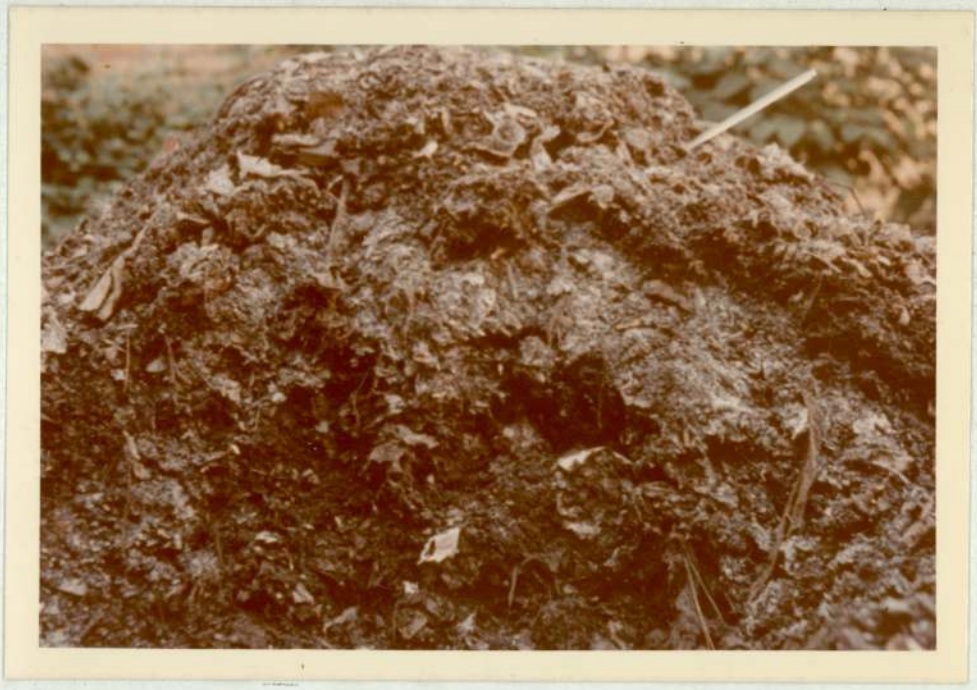
The results obtained from the three isolation techniques were generally consistent, the fungi most frequently isolated by Warcup's technique also being isolated by the dilution technique and to a lesser extent by the more selective screened substrate tube. This finding indicates the validity of the dilution technique when it is used for comparative purposes as in the present investigation.

With the small experimental windrow the rise in temperature in both regions was very rapid being in the region of 60°C throughout the windrow after 48 hours. The maximum temperature for growth of most mesophilic micro-organisms is generally considered to be around 40°C (Cochrane, 1958; Snell, 1960 and Chang and Hudson, 1967). Thus the extremely rapid rise in the temperature of the windrow meant that the mesophilic phase must have been extremely rapid, that is less than 24 hours, or non-existent. Within 48 hours after construction of the windrow the populations of mesophilic actinomycetes, bacteria and fungi in both regions of the windrow had either decreased or remained constant, indicating that they were not active, to any extent, and therefore unlikely to be the cause of thermogenesis. The thermophilic populations of actinomycetes and bacteria increased during the first 48 hours of

composting, the rise in the actinomycete population being particularly noticeable, especially in the outer region where in the first 4 days there was, approximately, a 10,000 fold increase in the number of propagules detected. During this period the population of the thermophilic fungi declined in both regions.

On the consideration of rising populations it would appear that the thermophilic bacteria and actinomycetes were responsible for thermogenesis within the windrow and not the mesophilic populations as reported elsewhere (A.P.W.A., 1961; Burman, 1961; White, 1965; Arditti, 1967). This hypothesis is supported by the fact that some thermophilic bacteria are known to be active from temperatures of 25°C upwards and that thermophilic actinomycetes have also been reported to be active at temperatures as low as 28°C (Snell, 1960), which was the initial temperature of the windrow.

During the thermophilic phase of the windrow, which in the central region lasted until day 40, the thermophilic actinomycetes were the dominant group of micro-organisms throughout the windrow. The increase in the population of the thermophilic actinomycetes was much more rapid in the outer region and the white mycelium of these organisms could, after the first two days, be observed completely covering the compost, below the surface, to a depth of 12 inches and this is illustrated in Photograph 3.1. The development of this population in the central region was



PHOTOGRAPH 3.1 SECTION THROUGH THE EXPERIMENTAL WINDROW SHOWING THE DEVELOPMENT OF THERMOPHILIC ACTINOMYCETES IN THE OUTER LAYERS.

greatly speeded up by turning the windrow on day 11. It was after the turning of the windrow that the pH in this region rose rapidly followed a day or two later by a corresponding increase in the numbers of the thermophilic actinomycetes. Jann, Howard and Salle (1959) demonstrated that an alkaline pH is necessary before rapid aerobic composting can occur; alkaline pH's are of course favourable to the development of actinomycetes and the research of Jann, Howard and Salle (1959) could indicate the importance of the thermophilic actinomycetes to the aerobic composting of refuse.

The most frequently occurring species of thermophilic actinomycetes, isolated by all three techniques, was Thermomonospora curvata. Fergus (1964) found that this thermophilic actinomycete was the most abundant species in composts of horse manure and straw and, as previously stated, Stutzenberger (1971) has implicated Thermomonospora curvata as a major cellulose decomposer in refuse composts. There now seems little doubt that some of the thermophilic actinomycetes are major cellulose decomposers in composts and evidence which strongly supports this hypothesis has been provided by Hayes (1969).

In the early stages of composting the populations of the thermophilic fungi declined in both regions of the windrow because of the rapid rise in temperature. The thermophilic fungi in the central region were 'killed off' by day 8 when the temperature had reached 63^{o}C and were

next isolated in this region on day 15 after the windrow had been turned and material from the cooler extremes of the windrow containing viable propagules was introduced to the region. The sensitivity of the thermophilic fungi to temperatures over 60°C was demonstrated by Fergus and Amelung (1971) who found in an examination of the heat resistance of 15 species of thermophilic fungi, that only Humicola lanuginosa remained viable for longer than 12 hours at a temperature of 62°C. In the present investigation the maximum development of the thermophilic fungi, in the central region, occurred between days 24 and 40 when the temperature was maintained between 45 and 55°C, a period which was similar to the 'plateau' temperatures described by Chang and Hudson (1967) for their wheat straw composts. The population of the thermophilic fungi in the outer region was not so severely affected by the extremes of temperature because of the gradient which existed between the compost at a depth of 6 inches and the outermost layers, for example a peak temperature of 66°C was recorded at a depth of 6 inches on day 4 but the corresponding temperature at a depth of 1½ inches was 50°C, very nearly optimum for the growth of the majority of the thermophilic fungi. The development of the thermophilic fungi was much greater in the outer region, the peak population being more than ten times the size of the corresponding peak in the central region. Burman (1961) and Fergus (1964) reported that the development of the thermophilic fungi in composts is mainly restricted to the outermost layers, a fact which agrees with the findings of the present investigation.

In both regions the most commonly occurring thermophilic fungi, with all the isolation techniques used, were in order of frequency, Humicola lanuginosa, Chaetomium thermophile and Mucor pusillus. The thermotolerant Aspergillus fumigatus was consistently isolated on the dilution and Warcup isolation plates but it was isolated infrequently from the screened substrate tubes. Aspergillus fumigatus is known as an almost ubiquitous inhabitant of composts (Raper and Fennell, 1965; Tansey, 1971) and the thermotolerant strain is highly cellulolytic (Loginova and Tashpulatov, 1964; Rogers, Coleman, Spino and Purcell, 1972; Malik, 1970) and may therefore play a significant role in the decomposition of cellulose in composts during the post-thermophilic phase. Chaetomium thermophile was the most consistently isolated cellulolytic thermophilic fungus from both regions of the windrow, in the central region it was not isolated until after the windrow had been turned but from this stage it was isolated with a high frequency of occurrence and appeared on all the plates prepared from the screened substrate tube showing it to be active in the composting process. In the outer region the frequency of occurrence of Chaetomium thermophile steadily increased from its relatively low level in the initial refuse until by day 8 it had a frequency of occurrence of 100 percent. The highly cellulolytic nature of Chaetomium thermophile has been well documented (Chang, 1967; Fergus, 1969; Malik, 1970 and Tansey, 1971), its presence in composts has been frequently reported (Fergus, 1964; Chang and Hudson, 1967; Mills, 1973) and the combination of these two factors

indicate that Chaetomium thermophile could be one of the major cellulose decomposers in composting.

The optimum pH for the growth of fungi is usually stated to be between pH 5.5 and 6.5 whereas the pH throughout the windrow when Chaetomium thermophile became prevalent was about pH 8 thus raising the question of whether it could be cellulolytically active under such conditions. Malik (1970) compared the cellulolytic abilities of the thermophilic fungi at different pH values and found that, although the optimum pH for Chaetomium thermophile was between pH 6 and pH 7, its cellulolytic ability was only slightly impaired at pH 8. A study of the effect of pH on the ecology of cellulolytic thermophilic fungi (Malik and Eggins, 1972) has shown that Chaetomiun thermophile can maintain a high frequency of occurrence in the colonisation of cellulosic substrates even at high pH values. Thus the ability of Chaetomium thermophile to colonise and degrade cellulosic substrates at pH 8, coupled with its frequent occurrence in the experimental windrow, support the hypothesis that it could be one of the major cellulose decomposers in composting.

Humicola lanuginosa was nearly always isolated in association with Chaetomium thermophile and on the isolation plates the mycelium of Humicola lanuginosa could be seen growing over and amongst the perithercia and mycelium of Chaetomium thermophile and also over the mycelium of the actinomycetes. Chang (1969) reported that Humicola lanuginosa

appeared to grow more vigorously in mixed cultures than alone and its frequent association with cellulolytic organisms led Chang (1967) to suggest that it is a secondary sugar fungus as defined by Garrett (1951). Mucor pusillus was consistently isolated throughout the composting period by the dilution plate and Warcup's technique but it was only occasionally isolated from the screened substrate tube. Chang and Hudson (1967) reported that Mucor pusillus appeared only in the initial stages of their wheat straw compost and accordingly classified it as a saprophytic sugar fungus. However, in the present investigation Mucor pusillus persisted throughout the composting period and fulfilled the role of a secondary fungus like Humicola lanuginosa.

Some interesting points arise from the behaviour of the mesophilic fungi during the composting process. In the central region the population of the mesophilic fungi declined to a very low level during the first 48 hours of composting but unlike the thermophilic fungi they were not killed off and persisted in low numbers until day 28, from which time their numbers increased until the completion of composting. Although some mesophilic fungi persisted through the period of peak heating none were isolated by the screened substrate tube between days 7 and 15 showing them to be inactive. Whilst studying the role of pure cultures of mesophilic fungi in thermogenesis Norman (1930) discovered that some fungi could raise the temperature of sterile straw in Dewar flasks to temperatures well above their normal growth range, even above their known thermal death points

and viable cultures could still be isolated during the period of peak heating. The results of Norman (1930) and those of the present investigation, concerning the survival of mesophilic fungi, indicate that in laboratory conditions on synthetic media that an organism may behave differently than it does in its natural environment. Recent research has shown that some pathogens namely Salmonella paratyphi and Escherichia coli can also survive the period of peak heating produced in the windrowing of refuse (Parrakora, Müller and Strauch, 1970) when theoretically they should be killed off after an hour's exposure to a temperature of 55°C (Berkeley Report, 1953).

In the central region three species of mesophilic fungi Fusarium sp., Penicillium funiculosum and Trichoderma viride survived the period of peak heating before the windrow was turned on day 11. The number of species of fungi isolated at 25°C from the central region was reduced from the 26 present in the original refuse to only 8 by day 4. In the outer region the mesophilic fungi were present in greater variety and abundance throughout the period of composting presumably because of the cooler outer layers. In both regions of the windrow the numbers of fungi isolated at 25°C increased in the later stages of composting but in the central region this was almost exclusively due to an increase in the frequency of the two variants of Aspergillus fumigatus and the thermophilic Mucor pusillus. It would therefore seem unlikely that the mesophilic fungi could play a very important role in the type of composting process studied in the

present investigation.

Mesophilic actinomycetes consistently appeared on the Warcup isolation plates prepared at 25°C throughout the 50 days of composting although they were only rarely isolated by the screened substrate tube. During the later stages of composting as the windrow cooled down their numbers increased and in the past they have been linked with the decomposition, in the cooling phase, of the more durable of the components of the compost, such as lignin (Burman, 1961).

The plot of the C:N ratios, Figure 3.5, revealed that decomposition proceeded much more rapidly in the outer region of the windrow. This almost certainly occurred because the better aeration of the windrow in this region probably promoted the much more rapid rises in the populations of the thermophilic actinomycetes and bacteria, than those observed in the central region. In the outer region and to a lesser extent the central region the maximum rate of decomposition coincided with the increases in the populations of the thermophilic actinomycetes. Although the C:N ratio is a useful guide to the state of decomposition that exists in composts of organic wastes it does not reveal which constituents of the wastes have been degraded and whether in this case the thermophilic actinomycetes had degraded the cellulose content or utilised the more readily available carbohydrates.

With composts of wheat straw, Chang (1967) found that the rate of decomposition of cellulose varied with the age of the compost, the greatest losses occurring during the initial heating stages as the temperature rose to its peak and also during the 'plateau period' after the temperature had fallen from its peak to approximately 50°C. Chang (1967) concluded, from these observations, that cellulose might be important in thermogenesis. From the earlier study on the ecology of these same composts, Chang and Hudson (1967) had found that the thermophilic actinomycetes were abundant in both the periods described by Chang (1967) whereas the thermophilic fungi were abundant only in the plateau period. This clearly links both these groups of organisms with the decomposition of cellulose in composts.

In the present investigation the thermophilic actinomycetes were most numerous in the outer region of the windrow during the first 3 weeks of composting and their numbers only started to decline to any marked extent after the first 5 weeks of composting when the temperature fell below 50°C. The period of peak development of the thermophilic actinomycetes in the experimental windrow coincides closely with the periods of maximum cellulose decomposition recorded by Stutzenberger, Kaufman and Lossin (1970) and Mills (1973), both of whom found that at least 45 percent of the cellulose was degraded during the first 6 weeks of the windrowing of refuse. This evidence strongly supports the hypothesis that thermophilic actinomycetes such as Thermomonospora curvata

are the most important organisms involved in the composting of refuse.

When the increases in the populations of the thermophilic fungi are compared with those of the populations of the thermophilic actinomycetes it appears that the thermophilic fungi can only play a minor role in the decomposition of cellulose. In both regions there was, eventually, a 10,000 fold increase in the numbers of the thermophilic actinomycetes compared with a 10 fold increase in the central region and a 100 fold increase in the outer region of the populations of thermophilic fungi. Such evidence alone, is insufficient to decide the importance of the relative activities of these two groups of micro-organisms in composts of refuse. Chaetomium thermophile a highly cellulolytic thermophilic fungus was consistently isolated from both regions of the experimental windrow by the screened substrate tube showing it to be active and this could well mean that species of the thermophilic fungi though not so numerous as the thermophilic actinomycetes may play a significant role in the decomposition of cellulose in composts of refuse. This hypothesis can only be proved by further research on characterising the cellulases present in composts of refuse and also those of the thermophilic fungi.

At the present time it can be stated that there is very definite evidence that thermophilic actinomycetes are major cellulose decomposers in composts of refuse and

that as cellulose decomposition is now the rate limiting step in this process it could possibly be improved by encouraging the development of thermophilic actinomycetes.

CHAPTER 4 Pages

Chapter 4

THE ANALYSIS OF THE NEWSPRINT USED AND THE SELECTION OF FUNGI SUITABLE FOR USE IN THE DEVELOPMENT OF PROCESSES FOR THE UPGRADING OF NEWSPRINT

Introduction

The recent estimates that world food production would have to be trebled or even quadrupled by the year 2,000 (De Maeyer, 1968) stimulated research into the possible use of micro-organisms as a potential source of food for man and fodder for animals. The use of fungi as food is not new, higher fungi in the form of mushrooms, boletes, morels and truffles have been consumed as delicacies by man for centuries. The cultivation of fungal mycelium as a possible food source, particularly protein, has been a relatively recent development. It has been reported (Litchfield, 1968) that the idea of the microbiological conversion of inorganic nitrogen and carbohydrate materials such as straw, sawdust or plant residues to protein was first utilised by Pringsheim and Lichtenstein in 1920 who reported the feeding of animals with Aspergillus fumigatus grown on straw supplemented with inorganic nitrogen fertiliser.

The literature concerned with the production of foods and feeds from fungi has been reviewed by Thatcher (1954) and the contribution that fungi can make to the worlds protein supply has been discussed by Rose (1961), Bunker (1963, 1968), Gray (1966) and Litchfield (1968). Thatcher (1954) points

out that the basis of commercial food synthesis by fungi is the high degree of efficiency of these organisms in using energy from cheap carbohydrates to convert elementary nutrients into proteins, vitamins and fat. Gray (1962) assessed the world's food situation and found that proteins were in short supply whereas there was an adequate supply of fats and an excess of carbohydrates. Consequently he suggested that the excess of carbohydrates be converted by micro-organisms to protein to meet the deficit in the world's supply. Gray, Och and Abou-El-Seoud (1965) proposed that the Fungi Imperfecti could be used to convert the excess of carbohydrates to a potential source of edible protein. The Fungi Imperfecti were thought (Gray, 1962; Gray, Och and Abou-El-Seoud, 1965) to be suitable agents of biosynthesis because of their ubiquity, the large numbers of different forms and their fast growing mould-like habit. In subsequent screening programmes Gray and associates (1966-67) presented evidence that cheap carbohydrates such as sweet potatoes, manioc, sugar beet, rice and sugar cane juice could be used as substrates by the Fungi Imperfecti for the production of protein.

The advantages of the micro-fungi, such as the Fungi Imperfecti, over the yeasts and bacteria as agents of biosynthesis for protein have been discussed by Spicer (1971). Fungi, generally, possess a better protein profile than many species of bacteria or yeast, essential amino acids such as methionine and cystine can be presented in greater concentrations, up to 4% of the total protein which is nearly twice the quantity in yeast or bacteria. Micro-fungi are

already accepted in many parts of the world, notably Japan, Indonesia and other Asian countries, as a staple ingredient of the daily diet. Miso, tempeh and ontjom are examples of this type of diet eaten in various parts of Asia; it has been calculated that the average Indonesian man eats 154gm per day of Rhizopus sp. mycelium (Spicer, 1971). In the western hemisphere fungi are consumed in mould fermented foods such as Stilton, Camembert and other cheeses. Yeasts have a high nucleic acid content which can result in the production of a feedstuff with an extremely bitter taste and the production in the body of high levels of uric acid which can cause damage to the kidneys (Bunker, 1963). The net protein utilisation of yeasts has been reported as being around 35 - 40% requiring supplementation with 0.3 - 0.5% methionine to raise it to 70 - 75%. Species of micro-fungi have been found which do not suffer from the limitations described for yeast and with the supplementation of 0.2% methionine products equalling the standard of egg protein have been obtained. Unlike yeasts some fungi can be used to completely replace conventional proteins in feedstuffs. It has been found that the addition of yeasts to feedstuffs in concentrations of greater than 10% of the total weight depresses the growth rates of animals.

Burt (1972), stated that if the rate in increase of the human population is restricted to 1.7% compound per annum and if the average patterns of human food consumption per capita remain as they were in 1959-61 then the world supply of concentrate feeds for animal production will be in substantial deficit by the end of this century. If the

available supply of such feeds were used to support pig and poultry production, equivalent to the estimated requirements of the expected human population, production of ruminant meat would in theory become zero and milk production would be less than half of the estimated requirement. Assuming these estimates by Burt (1972) to be valid the deficit in feeds will have to be made up by improving the utilisation of raw materials used in concentrate feeds and by upgrading others presently considered to be unsuitable. The upgrading of waste cellulose to a palatable, easily digestible and nutritious feedstuff would reduce this deficit. Chahal, Munshi and Cheema (1969) pointed out that the only carbohydrate waste available in abundance in many of the developing countries, such as India where protein malnutrition is especially acute, is cellulose. It has already been reported in this thesis that in industrial nations such as the U.K. and the U.S.A. the disposal of cellulose in the form of waste paper presents a serious problem. Cellulose would therefore appear to be the cheapest carbohydrate which is readily available, in many areas of the world, for use in the production of a protein and vitamin enriched feedstuff.

The use of cellulose, hemicelluloses or lignin as substrates for the production of feedstuffs by fermentation has not been widely considered because it has been believed that fermentations designed to utilise these substrates could be specialised and of long duration and therefore may well not be economically feasible. The development of an economically feasible process of cellulose fermentation (upgrading) requires the selection of a micro-organism

capable of causing the rapid breakdown of the components present in a cellulosic substrate such as newsprint. The micro-fungi have been shown to possess many advantages over the bacteria and yeasts (Spicer, 1971) for the production of feedstuffs and many species are also known to be cellulolytic. In the present investigation an analysis of the newsprint used is undertaken and the use of this newsprint in a selective isolation technique and a linear growth test for the selection of cellulolytic fungi suitable for use in the development of processes for upgrading newsprint are described.

Materials and Methods

(i) Analysis of the newsprint

In the initial stages of the project it became apparent that a standard form of waste newsprint would be needed for the consistency required by all experimental purposes. The newsprint used for such purposes throughout the present investigation was all obtained, in an ink-free condition, from the tail-end of one roll supplied by a local newspaper. Obtaining the newsprint from one roll minimised any possible variation in its composition as it would almost certainly have been made from the same batch of wood pulp. It has previously been mentioned that newsprint consists of ground and pulped softwoods having essentially the same composition as woods of this type i.e. 40-50% cellulose, 15-35% lignin and 25-35% hemi-celluloses (Wenzl, 1970). These figures include a wide variation and it was thought necessary to accurately ascertain at least the hemi-celluloses, cellulose and lignin contents of the

newsprint used and accordingly analyses were made of these fractions.

The isolation of cellulose from wood or other plant materials requires its separation from extractives (readily soluble materials), lignin and other non-cellulose components. In the methods most frequently used for the isolation and determination of cellulose the other constituents must be removed as completely as possible by procedures of extraction or solubilisation leaving behind a residue which is largely cellulose. The major step in all such procedures is the removal of lignin and therefore the isolation of cellulose becomes primarily a delignification procedure (Browning, 1967). Some delignification procedures remove a portion of the hemi-celluloses along with the lignin leaving with the cellulose variable amounts of the hemi-celluloses depending on the nature of the delignification process. It is, however, desirable to isolate the cellulose by the removal of the lignin without loss of any of the polysaccharides, that is the hemi-cellulose fraction. Isolation methods which can successfully achieve this aim have been developed and they yield a product known as holocellulose which includes both the cellulose and hemicelluloses originally present in wood.

Delignification of wood was achieved as early as 1868 by treating it with chlorine water (Browning, 1967). In 1880 the application of chlorine gas by Cross and Bevan first demonstrated the utility of halogenation and their procedure has remained for many years one of the classical

methods of wood chemistry and is now the basis of the method used for the determination of cellulose in wood materials given by the T.A.P.P.I. standard 17-m55. This process involves the use of gaseous chlorine which reacts with the lignin to form substitution and oxidation products which are soluble in alcohol, aqueous alkaline solutions and boiling sodium sulphite solution. The original method of Cross and Bevan involved a pre-treatment of the wood samples by boiling in a solution of dilute alkali usually 1% sodium hydroxide before chlorination took place. This procedure was found to give variable results because although the alkali pre-treatment is advantageous in expediting the removal of lignin it is undesirable in that the hemicelluloses may be partly attacked and suffer loss, lowering the yield obtained. Dean and Tower (1907) modified the original method by missing out the alkali pre-treatment and obtained consistent results. Chlorine gas is an objectionable reagent to have in most laboratories and methods of delignification were developed by Ross, Davidson and Houghton (1929); and Norman and Jenkins (1933) which although using the principle of chlorination did not require the presence of chlorine gas in large quantities. Both these methods involve the use of hypochlorite solutions with the addition of a dilute acid for the generation of chlorine in solution, the sample for analysis is present in the solution and so the method basically becomes one of chlorination. The substitution and oxidation products produced from the lignin were removed by boiling the sample in sodium sulphite solution. In the present investigation the method of Norman and Jenkins (1933) was used with reference to the amended and

concise account of it, given by Browning (1967).

The newsprint was prepared for analysis by grinding small pieces of it in a laboratory scale Culatti 75watt hammermill to produce a finely divided flour. The paper which after grinding would pass through a sieve of 80 mesh but which was retained by a sieve of 60 mesh was used for analysis. The material for analysis was dried to constant weight at 80°C and approximately 2gms were accurately weighed out and extracted with a mixture of 33 volumes of 95% ethanol and 67 volumes of benzene in a Soxhlet appartus for 6 hours. After extraction the material was dried, re-weighed, placed in a beaker and treated with 100ml. of 3% sodium sulphite solution. The mixture was brought to the boil after which the material was separated from the sodium sulphite solution by centrifugation and then carefully washed with distilled water The material was then transferred back to the beaker made up to 100ml. with distilled water, to this mixture was added 5ml. of sodium hypochlorite solution containing 15% available chlorine (available chlorine refers to the chlorine liberated by the action of dilute acids and is expressed as a percentage by weight of the solution or bleaching powder), the mixture was allowed to stand at room temperature for 10 minutes after which time the solid material was separated out by centrifugation and washed with distilled water. The material was transferred to a beaker with 50ml. of distilled water and 50ml. of 6% sodium sulphite solution. The mixture was boiled for 20 minutes, the solid material then being separated out by centrifugation and washed again. The treatments with the

15% sodium hypochlorite solution and the 6% sodium sulphite solution were repeated once more after which the material was suspended in 50ml. of distilled water, 5ml. of sodium hypochlorite solution, containing 3% available chlorine and 2ml of sulphuric acid at a concentration of 20%; the mixture was allowed to stand for 10 minutes after which time the residual material was separated out, washed and suspended in 50ml of distilled water and 50ml of 6% sodium sulphite solution. On addition of the sodium sulphite solution an intense purple colouration developed which gradually disappeared on heating, the material becoming orange to brown in colour. This colouration is known as the Mäule reaction which is a reaction between the sodium sulphite and the chlorinated lignin and it serves to indicate the presence of lignin in the sample being analysed. The alternate treatments with the acid hypochlorite and the sodium sulphite were repeated until the purple colouration no longer developed. Finally the solid material was separated out, washed thoroughly with hot water, dried at 100°C and reweighed. The final product consisted of a cream to white coloured powder.

Throughout the whole experimental procedure great care was taken to avoid any loss of the solid material during transfers between various receptacles and also to prevent bumping in the beaker when the material was being heated with solutions of sodium sulphite. Determinations were made with five replicate samples.

The stock solution of sodium hypochlorite was obtained from the supplier with an available chlorine content

of between 15-17%. The available chlorine contents of the solutions used were determined by the following procedure of volumetric analysis described by Vogel (1951). A volume of 5ml of the stock solution of sodium hypochlorite was pippetted into a 250ml conical flask, 25ml of distilled waster was added followed by 2gm of iodate free potassium iodide and 10ml of glacial acetic acid. The liberated iodine was titrated against standard 0·1N sodium thiosulphate,* 2ml of starch solution (1gm of starch in 100ml of water) was added when the solution had turned pale yellow, the titration was then continued until the blue colour formed on addition of the starch solution had just disappeared. The availability of the chlorine in the hypochlorite solution was then calculated on the basis of:-

$$1ml. - Na_2S_2O_3 = 0.03546g. Cl.$$

Once the available chlorine content of the hypochlorite solution had been determined it was possible to make up the solutions of the required strength for the estimation of the cellulose in the newsprint.

* Sodium thiosulphate is usually obtained as $Na_2S_2O_3 \cdot 5H_2O$ which exists in a state of high purity but because of uncertainty as to the exact water content due to its efflorescent nature the substance is unsuitable for use as a primary standard and therefore must be standardised before use. In the present investigation an approximately 0.1N solution was prepared by dissolving 25gm of the A.R. crystallised sodium thiosulphate in water and accurately standardising it by an iodometric procedure with potassium iodate outlined by Vogel (1951). The normality of sodium thiosulphate could be calculated from the fact that:-

$$1ml. N\text{-}Na_2S_2O_3 = 0.03657g. KIO_3$$

Norman and Jenkins (1933) stated that the hemicelluloses (they described them as cellulosans or polysaccharides) which are very intimately associated with pure cellulose in nearly all natural celluloses should remain intact in any method of cellulose estimation and were of the opinion that their method, as described above did not extensively remove the hemicelluloses. The final product of this extraction process therefore, consists of cellulose and the hemicellulose and is similar to the holocellulose described by Browning (1967) but is usually known as 'Cross and Bevan' cellulose. An analysis of the product, obtained by this extraction process from the treatment of softwoods by Norman and Jenkins (1933), revealed that the residual lignin content was as low as 0.1-0.5% on the basis of the cellulose.

The hemicellulose fraction of the newsprint was determined using newsprint as the starting point for the analysis. Approximately 2gm of oven dried 60-80 mesh powdered newsprint was accurately weighed and then extracted with 80% ethanol in a Soxhlet apparatus until it was free of sugars when tested with fresh anthrone reagent (0.2% anthrone in concentrated sulphuric acid). The material was then dried, weighed and the ethanol soluble fraction determined. The starch dextrins and glycogen were removed by the method of Weinmann (1947) as follows, approximately 2gm of the dried ethanol extracted material were weighed out accurately into a conical flask and 10ml water added. The flask was covered with a glass funnel and the mixture heated for 30 minutes on a boiling water bath to gelatinise the

starch content. The mixture was cooled to room temperature and 10ml. of an acetate buffer (3 vol. 0.2N acetic acid plus 2 vols. 0.2N sodium acetate) pH 4.45 were added with 10ml. of 0.5% diastase solution. The flask was tightly stoppered and incubated at 37°C for 2 days after which time the residue was filtered, washed, oven dried at 80°C and re-weighed. A known weight of this residue was taken and the hemicelluloses extracted from it using a one step extraction (Chang, 1967) to remove both the hemicelluloses designated A and B (Browning, 1967) with 24% potassium hydroxide solution. The material was allowed to stand in the potassium hydroxide solution for 4 hours at a temperature of 25°C after which time the residue was filtered, washed with dilute hydrochloric acid and distilled water and then dried, reweighed and the hemicellulose fraction determined. The residue remaining consisted of cellulose and lignin, and analytical methods do exist for determining the composition of such materials. The most frequently used technique is to hydrolyse the polysaccharides with cold 72% sulphuric acid and then diluting the acid and boiling the mixture. The process yields a product known as 'Klason Lignin' and in the past it has been widely used. This method suffers from one serious drawback because it was found that lignin like material could be produced by prolonged exposure of the cellulose, hemicelluloses and other polysaccharides to 72% sulphuric acid (Norman and Jenkins, 1933; Schubert, 1956; Browning, 1967). This obviously gives a false impression of the lignin content and in the present investigation it was thought necessary to use a separate method such as the hypochlorite process for the estimation of the cellulose and lignin contents.

(ii) Selective isolation technique

The selective isolation technique used was essentially the same as that used in Chapter 2 for the isolation of the thermophilic and mesophilic fungi from the amended samples of refuse and compost. In this case thermophilic and mesophilic fungi were isolated from a soil amended with powdered newsprint. The soil used was obtained from a pastureland in Clent, near Stourbridge, Worcestershire, which has not been treated with any fertiliser or herbicide for the past forty years. The top layers of the soil down to a depth of 6-7 inches consist of a dark reddish-brown clay loam and only this portion of the soil was collected for use in this investigation. This soil had previously been used for various aspects of mycological research and was found to contain a large number of fungal species including many of the confirmed thermophilic fungi (Eggins and Malik, 1969; Malik, 1970).

The newsprint used was reduced to a very fine powder by first passing lengths of it through a document shredder and secondly by ball-milling this shredded paper, in a dry state, for 72 hours. The resulting powdered newsprint was used for the amendment of the pastureland soil and was added so that it amounted to 4% of the wet weight of the soil. The moisture content of the soil was determined and adjusted to 25% of its dry weight, the final pH of the soil was between pH66 and pH68. The amended soil was divided into 11 equal portions each of which was placed in a set of 10 sterile Petri dishes. All the sets of the Petri dishes were incubated at 50°C in a high humidity incubator. One

set of 10 Petri dishes were sacrificed at a time and standard Warcup (1951) soil plates were prepared from the amended soil after Ohr., 12hrs., 1 day and 2, 3, 4, 6, 8, 10, 12 and 14 days of incubation. The soil from each Petri dish in each soil was used to incubate a total of 9 plates which consisted of 3 cellulose agar plates, 3 glucose-starch agar plates, as previously described in Chapter2, and 3 plates of newsprint agar which consisted of the mineral salts agar used by Eggins and Pugh, (1962) plus a 1% suspension of newsprint ball-milled for 72 hours to produce a very finely divided suspension. The plates were incubated at 50°C and after 7 days the fungal species were recorded and where possible isolated into pure culture. After the procedure had been completed at 50°C it was repeated with an incubation temperature of 25°C for the isolation of mesophilic fungi with the ability to degrade newsprint. In both cases the frequency of occurrence of a species was determined by recording its presence or absence in each sample, a fungus being given a positive record if it appeared on any of the three replicate plates.

(iii) Linear growth tests

The fungi selected on the basis of the elective isolation technique described above and also on the basis of reports in the literature, concerning their cellulolytic ability were subjected to a linear growth test. The fungi selected for this growth test are listed in the results section of this chapter.

A widely used method of measuring the linear growth of the fungi is the measurement of the colony diameter. The

diameter of the fungal colony has been proposed by Brancato and Golding (1953) as a reliable measure of growth. Brancato and Golding (1953) found that the diameter of the colony lent itself satisfactorily to the measurement of the growth rate because in such a situation they reported that there was no acceleration of the growth rate with time. Within a short time of germination many fungi attain a characteristic radial growth rate and Brancato and Golding (1953) found that this rate of growth was maintained over a period of time varying with the organism and the medium; they also found that the difference in the growth rates of a fungus on two different media was significant.

In these experiments two different media were used to assess the growth rate of the test species, these were the cellulose and newsprint agars used in the elective isolation technique. Three replicate plates were used for each species with each medium, inoculation of the plates and the measurement of the colony diameter was carried out exactly as described in Chapter 2. Prior to inoculation the fungi had been cultured on cellulose and newsprint agars and the newsprint agar plates were inoculated with mycelium grown on newsprint agar and the cellulose plates with mycelium grown on cellulose agar. The cultures of the thermophilic fungi were incubated at 48°C and those of the mesophilic fungi at 25°C. The colony diameters were measured every 24 hours.

Results

(i) Analysis of the newsprint

The results of the analysis, by the method of Norman and Jenkins (1933), to determine the cellulose and lignin contents of the newsprint are given in table 4.1 below.

Table 4.1 Results of the hypochlorite analysis for the cellulose and lignin contents of the newsprint.

Replicate Sample	Cross and Bevan Cellulose Content (Percentage Weight Remaining)	Lignin Content (Percentage Weight Loss)
1	67.81%	32.19%
2	69.95%	30.05%
3	74.39%	25.61%
4	67.60%	32.40%
5	67.65%	32.35%
Averages	69.47%	30.53%

Thus the lignin content of the newsprint was taken to be 30.53% of the dry weight and the Cross and Bevan cellulose content as 69.47% of the dry weight. The close agreement of the results obtained for the five replicate samples, a maximum variation of 7% for all the samples and just over 2% for four of the samples indicated the reliability of this technique.

The hemicelluloses and ethanol soluble and diastase soluble fractions of the newsprint are given in table 4.2. The ethanol soluble fraction consisted of simple sugars,

glucosides, essential oils, colouring matter and resinous materials and the diastase soluble fraction consisted of starch, dextrins and glycogen.

Table 4.2 The hemicellulose, ethanol soluble and diastase soluble fraction of the newsprint.

Replicate Sample	* Ethanol Soluble Fraction	* Diastase Soluble Fraction	* Hemicelluloses
1	3.10%	7.46%	14.52%
2	3.44%	7.41%	14.62%
3	3.25%	7.42%	14.60%
4	3.38%	7.44%	14.57%
5	3.16%	7.41%	14.59%
Averages	3.27%	7.43%	14.58%

* Figures given have been corrected for the percentage dry weight of the total composition of the newsprint.

The total dry weight of the newsprint removed by the successive extraction of the ethanol solubles, diastase solubles and hemicelluloses amounted to 3.27 + 7.43 + 14.58 = 25.28%. The removal of these three fractions left a residue of cellulose and lignin consisting of 74.72% of the dry weight of the newsprint. The lignin content of the newsprint as determined by the hypochlorite extraction amounted to 30.53% of the dry weight. The cellulose content of the newsprint was therefore calculated as 74.72 - 30.53 = 44.19% of the total dry weight. The composition of the newsprint obtained for the experimental purposes of the present investigation can be stated as having the composition given in table 4.3.

Table 4.3 Composition of the newsprint used in all experimental purposes.

	Percentage of Dry Weight
Ethanol Solubles (sugars, glucosides, oils, colouring and resinous materials)	3.27%
Diastase Solubles (Starches, Dextrins and Glycogens)	7.43%
Hemicelluloses	14.58%
Cellulose	44.19%
Lignin	30.53%
Total	100

(ii) Selective isolation technique

The frequencies of the fungi isolated at 50°C from the amended and incubated soil samples on the newsprint (NPA), cellulose (CA) and glucose-starch (GS) agars are presented in tables 4.4, 4.5 and 4.6 respectively. All of the thermophilic fungi listed by Cooney and Emerson (1964) were isolated plus Talaromyces emersonii Stolk, a second variety of Sporotrichum thermophile and the thermotolerant Aspergillus fumigatus.

The most frequently isolated fungi from the initial (Ohrs of incubation) soil samples, at 50°C, on the newsprint agar were Chaetomium thermophile, Aspergillus fumigatus, Humicola lanuginosa and the thermophilic actinomycetes. After 12 hours incubation of the newsprint enriched soil the frequencies of occurrence of these fungi and the actinomycetes

Table 4.4 Percentage frequency of occurrence of cultures recorded on newsprint agar plates incubated at 50°C for 7 days after inoculation with newsprint enriched and incubated soil.

Fungi	Period of incubation of enriched soil										
	0Hr	12Hr	1Day	2Days	3Days	4Days	6Days	8Days	10Days	12Days	14Days
Aspergillus fumigatus	40	50	60	90	60	30	10	30	20	60	90
Cephalosporium sp.	10	20					10		30		
Chaetomium thermophile	60	80	100	100	100	80	100	100	100	100	100
Humicola grisea	20	10		10	20	20	40	50	40	30	30
Humicola insolens		10									
Humicola lanuginosa	70	90	80	100	100	100	100	100	100	100	30
Malbranchea pulchella	20	50									
Mucor pusillus		10			10	20	10				
Sporotrichum thermophile var 1	10	10	20	10	10	10					
Sporotrichum thermophile var 2	10	10	20	40	40	40	30	40	20	10	
Talaromyces duponti		30									
Talaromyces emersonii								10			
Thermoascus aurantiacus	10	20									
Torula thermophila											
Actinomycetes	80	90	80	90	100	100	100	90	100	100	70

all increased on the newsprint agar. In the following days of incubation, Chaetomium thermophile, Humicola lanuginosa and species of the thermophilic actinomycetes became dominant on the newsprint agar all averaging percentage frequencies of occurrence of very nearly 100. The percentage frequency of occurrence of Aspergillus fumigatus increased from 40 to 90% in 2 days then decreased to 20% after 10 days of incubation and finally increased to 90% at the end of the 14 day incubation period for the newsprint enriched soil. The frequencies of occurrence of Humicola grisea and Sporotrichum thermophile var 2 were initially very low but after 6-8 days of incubation their frequencies of occurrence substantially increased only to decline again in the later stages of incubation. Sporotrichum thermophile var 1 maintained its initial low frequency of occurrence until the fourth day of incubation after which time it was not isolated again. Humicola insolens, Malbranchea pulchella var sulfurea, Talaromyces duponti and Thermoascus aurantiacus were only isolated during the first 24 hours of incubation. Mucor pusillus and Talaromyces emersonii were only occasionally isolated during the fourteen days incubation of the newsprint enriched soil.

Inoculations of the cellulose agar, Table 4.5, with the newsprint enriched soil produced similar results to those obtained with the newsprint agar. The fungi most frequently isolated from the soil samples which were not incubated were again, Aspergillus fumigatus, Chaetomium thermophile, Humicola lanuginosa and the thermophilic

Table 4.5 Percentage frequency of occurrence of cultures recorded on cellulose agar plates incubated at 50°C for 7 days after inoculation with newsprint enriched and incubated soil.

Fungi	Period of incubation of enriched soil										
	0Hr	12Hr	1Day	2Days	3Days	4Days	6Days	8Days	10Days	12Days	14Days
Aspergillus fumigatus	40	50	40	100	60	20	10	10	10	90	90
Cephalosporium sp.	20	60	10	70	50	20	60	80	60	100	50
Chaetomium thermophile	50	100	90	100	100	100	100	100	100	100	100
Humicola grisea	20	10	10	10	30	40	20	10			
Humicola insolens	30	50	10	10	10						
Humicola lanuginosa	100	90	90	100	100	100	70	100	90	80	70
Malbranchea pulchella											
Mucor pusillus							10	10			
Sporotrichum thermophile var 1		10	10	30	20	20					
Sporotrichum thermophile var 2	10	10	20	40	60	50	70	80	70	70	50
Talaromyces duponti											
Talaromyces emersonii				10		10	20		30		
Thermoascus aurantiacus		10	30	10		10					
Torula thermophila	10										
Actinomycetes	40	100	20	30	70	60	70	90	100	60	70

actinomycetes. Throughout the 14 day period of incubation Chaetomium thermophile, Humicola lanuginosa, were the dominant fungi being consistently isolated with frequencies of occurrence averaging nearly 100%. The isolation of Aspergillus fumigatus followed a similar pattern to that shown on the newsprint agar with an initial increase after 2-3 days of incubation of the soil followed by a decline until day 12 after which time its frequency of occurrence increased sharply. Cephalosporium sp. and Sporotrichum thermophile var 2 were consistently isolated throughout the period of incubation with percentage frequencies of occurrence averaging over 50. In both cases this represented a substantial increase over the frequencies of occurrence obtained on the newsprint agar. The pattern of isolation of Sporotrichum thermophile var 1 on cellulose agar was also similar to that displayed on the newsprint agar being consistently isolated with a low frequency of occurrence during the first 4 days of incubation of the soil. Humicola insolens was also isolated with a low frequency of occurrence during the first 3 days of incubation. Thermoascus aurantiacus and Talaromyces emersonii were only isolated occasionally, and Torula thermophila was isolated only from the initial sample of the soil, it was the only time that this fungus was isolated from the newsprint enriched soil.

Inoculation of the glucose-starch agar with the newsprint enriched soil gave a completely different picture of isolations. Initially, the most frequently isolated organisms were Cephalosporium sp. and Thermoascus aurantiacus. Both of these fungi persisted throughout the 14 day incubation

Table 4.6 Percentage frequency of occurrence of cultures recorded on glucose-starch agar plates incubated at 50°C for 7 days after inoculation with newsprint enriched and incubated soil.

Fungi	Period of incubation of enriched soil										
	0Hr	12Hr	1Day	2Days	3Days	4Days	6Days	8Days	10Days	12Days	14Days
Aspergillus fumigatus	30	70	80	100	90	60	50	50	50	100	90
Cephalosporium sp.	40	40	60	80	30	10	70	100	80	100	100
Chaetomium thermophile			40	80	100	20	10	10	30		
Humicola grisea	10										
Humicola insolens		10			10						10
Humicola lanuginosa	20	70	50	70	100	60	80	60	70	30	30
Malbranchea pulchella	10	70									
Mucor pusillus		20				10	30				
Sporotrichum thermophile var 1					30	10		10		10	
Sporotrichum thermophile var 2											
Talaromyces duponti		10	40								
Talaromyces emersonii				30	20	40	60	40	10	20	40
Thermoascus aurantiacus	40	40	50	30	50	30	40		10	10	10
Torula thermophila											
Actinomycetes	20	20	10	10	50	50	60	80	90	10	10

period with the frequency of occurrence of Cephalosporium sp. reaching a maximum of 100% after 3 days declining to 50% on days 8 and 10 but increasing to a maximum of 100 once more after 12 days of incubation. After 3 days of incubation the frequency of occurrence of Thermoascus aurantiacus gradually decreased. Aspergillus fumigatus and Humicola lanuginosa initially had low frequencies of occurrence but these steadily increased and both fungi were isolated throughout the period of incubation. Chaetomium thermophile was not isolated until after 24 hours of incubation of the soil and by day 3 it had obtained a frequency of occurrence of 100%. However, its frequency of occurrence after this time dropped very quickly and it was not isolated after the tenth day of incubation of the soil. Humicola grisea, Humicola insolens, Malbranchea pulchella, Mucor pusillus, Sporotrichum thermophile var 1 and Talaromyces duponti were infrequently isolated on the glucose-starch agar. Between days 2 and 14 Talaromyces emersonii was frequently isolated. The thermophilic actinomycetes were isolated throughout the incubation period reaching a maximum percentage frequency of occurrence of 90% on day 10. Usually the frequency of occurrence of the thermophilic actinomycetes was much lower on the glucose-starch agar than on the newsprint and cellulose agars.

The results of the isolations made on the newsprint and cellulose agars showed a close similarity, with cellulolytic fungi such as Aspergillus fumigatus, Chaetomium thermophile, Humicola grisea, Sporotrichum thermophile var 2 and the thermophilic actinomycetes all being isolated with

increasing frequencies of occurrence during the 14 day incubation period of the enriched soil. Humicola lanuginosa which is known not to be cellulolytic (Pugh, Blakeman and Morgan-Jones, 1964; Chang, 1967) was frequently isolated on both the newsprint and cellulose agars and was always found in close association with Chaetomium thermophile and the thermophilic cellulolytic actinomycetes acting in the role of a secondary sugar fungus as defined by Garrett (1963). The main differences between the results obtained on the newsprint and cellulose agar were the frequent isolations of Cephalosporium sp. and to a lesser extent Humicola insolens on the cellulose agar but not on the newsprint agar and the more frequent isolation of the thermophilic actinomycetes on the newsprint agar. On the glucose-starch agar there was generally a greater frequency of occurrence of the non-cellulolytic or weakly cellulolytic fungi such as Cephalosporium sp., Talaromyces emersonii and Thermoascus aurantiacus and a lesser frequency of occurrence of the strongly cellulolytic and secondary sugar fungi.

The two varieties of Sporotrichum thermophile isolated from this soil on the cellulose and newsprint agars were quite distinct. The first variety isolated was cream to white in colour at all temperatures of incubation, it grew well at temperatures as low as 25°C but would not grow above 55°C and its optimum temperature for growth was between 45 and 50°C. It grew well on YPSS agar but on the cellulose and newsprint agars the aerial mycelium produced was very sparse and sporulation rare. On YPSS agar the conidia produced were usually within the range of 3-6μ x

2-4μ and were oval to pyriform in shape. This variety was characterised by the production of a purplish green pigment at incubation temperatures greater than 50°C. The second variety of Sporotrichum thermophile also had cream coloured mycelium and spores at temperatures of incubation above 48°C. Below 48°C the mycelium always became cinammon to dark orange in colour. On both YPSS and the cellulose and newsprint agars Sporotrichum thermophile var 2 always sporulated heavily. The conidia were again oval to pyriform in shape and were usually within the range 3-6μ x 2-4μ. At temperatures above 48°C the second variety was characterised by the production of a very bright yellow pigment and a 'woolly' mycelium the colour and texture of sheepskin. This isolate grew well at temperatures as low as 25°C covering a cellulose agar plate within 7 days and also producing zones of clearing. It would not however, grow at temperatures above 55°C, its optimum temperature for growth was between 45 and 48°C. This second variety closely resembles the asexual stages of Thielavia thermophila as described by Hedger and Hudson (1970) but at no time during the course of the present investigation were any cleistothecia produced.

The frequencies of occurrence of the fungi isolated at 25°C from the amended and incubated soil samples on the newsprint, cellulose and glucose-starch agars are presented in tables 4.7, 4.8 and 4.9 respectively.

Initially on the newsprint agar the most frequently occurring fungi were Fusarium solani, Mucor globosus, Sordaria fimicola, Zygorhyncus moelleri and Trichoderma

Table 4.7 Percentage frequency of occurrence of cultures recorded on newsprint agar incubated at 25°C for 7 days after inoculation with newsprint enriched and incubated soil.

Fungi	Period of incubation of enriched soil									
	0Hr	1Day	2Days	3Days	4Days	6Days	8Days	10Days	12Days	14Days
Aspergillus fumigatus	30	50	10			30			10	
Aspergillus niger							10		20	10
Aspergillus sp.										
Aureobasidium pullulans						20				
Eurotium sp.				10						
Fusarium solani	100	100	100	100	90	100	100	100	100	90
Gliocladium roseum	20	60	30	20	10	30	20	30	30	30
Geotrichum candidum	20									
Humicola grisea	20				80	70	50	10	40	40
Mucor globosus	50	30	10	70	50	40	60	10	60	30
Paecilomyces varioti		50	40	40	90	90	90	60	80	70
Penicillium sp.1	20	30	60	80	100	70	100	80	100	80
Penicillium sp.2	10				10			50	60	80
Penicillium sp.3		10								
Rhizoctonia sp.	10		20							
Sordaria fimicola	100	100	60	40	40				30	30
Stachybotrys atra						20				
Stysanus sp.						10			10	
Trichoderma viride	100	100	100	100	100	100	100	100	100	100
Trichocladium asperum										
Verticillium sp.										
Zygorhyncus moelleri	80	90	70	30	40	20				10
Actinomycetes	20		30			30			20	10

viride. Of these fungi both Fusarium solani and Trichoderma viride were consistently isolated with near maximum frequencies of occurrence throughout the 14 days of incubation. Mucor globosus was also consistently isolated during this period but with a much lower frequency of occurrence and Zygorhyncus moelleri was only infrequently isolated after the sixth day of incubation of the soil. Gliocladium roseum, Paecilomyces varioti and Penicillium sp.1 were frequently isolated throughout the incubation period. Humicola grisea and Penicillium sp.2 were rarely isolated in the initial stages but were both consistently isolated in the later stages of incubation. The remaining species isolated appeared sporadically and a pattern of isolation could not be discerned.

On the cellulose agar plates, Fusarium solani, Gliocladium roseum, Humicola grisea, Sordaria fimicola, Trichoderma viride and Zygorhyncus moelleri were the fungi most frequently isolated from the initial soil samples. Fusarium solani, Giocladium roseum and Trichoderma viride were isolated with near maximum frequencies of occurrence throughout the incubation period while Humicola grisea was consistently isolated from each soil sample with lower frequencies of occurrence. Once again the frequencies of occurrence of Sordaria fimicola and Zygorhyncus moelleri decreased during the later stages of the period of incubation. The frequencies of occurrence of Penicillium sp.1 and Paecilomyces varioti although initially low increased after 24 hours of incubation and remained high above 50% for the duration of the incubation period. Penicillium sp.2

Table 4.8 Percentage frequency of occurrence of cultures recorded on cellulose agar plates incubated at 25°C for 7 days after inoculation with newsprint enriched and incubated soil.

Fungi	Period of incubation of enriched soil									
	0Hr	1Day	2Days	3Days	4Days	6Days	8Days	10Days	12Days	14Days
Aspergillus fumigatus						20	10			
Aspergillus niger							10	10	30	
Aspergillus sp.										
Aureobasidium pullulans										
Eurotium sp.								10		
Fusarium solani	100	100	100	100	100	100	100	100	100	100
Gliocladium roseum	100	100	100	60	70	90	80	100	90	60
Geotrichum candidum										
Humicola grisea	60	70	40	30	80	70	90	80	90	80
Mucor globosus	10	20	20	40	20	50	50	10	30	10
Paecilomyces varioti	20	80	60	70	100	70	100	60	100	100
Penicillium sp.1	20	40	20	70	90	90	90	80	90	80
Penicillium sp.2		30			10	50			70	80
Penicillium sp.3										
Rhizoctonia sp.			10			10		10		10
Sordaria fimicola	70	100	40	20	30		30		30	60
Stachybotrys atra										
Stysanus sp.				10	10	10			40	
Trichoderma viride	100	100	100	100	100	100	100	100	100	100
Trichocladium asperum										
Verticillium sp.	20									
Zygorhyncus moelleri	70	80	60	30	40					
Actinomycetes	50	60	10			10	20			

was again frequently isolated in the later stage of incubation.

On the glucose-starch plates the fungi most frequently isolated from the initial samples were Fusarium solani, Mucor globosus and Trichoderma viride. These three fungi remained prevalent during the 14 day incubation period of the soil. After the first 12 hours of incubation Penicillium sp.1 and 2 were also isolated with high frequencies of occurrence throughout the incubation period. Eurotium sp., Gliocladium roseum and Paecilomyces varioti were isolated sporadically during the incubation period. Zygorhyncus moelleri and Sordaria fimicola once again only appeared in the initial stages of incubation.

The mesophilic fungi had a similar pattern of isolation on the newsprint and cellulose agars with cellulolytic species such as Fusarium solani, Trichoderma viride, Paecilomyces varioti, Penicillium sp.1 and Sordaria fimicola (in the early stages) being consistently isolated throughout the period of incubation with high frequencies of occurrence. Gliocladium roseum and Humicola grisea both cellulolytic fungi were consistently isolated on the newsprint and cellulose agars but appeared on the cellulose agar with much higher frequencies of occurrence. Of the sugar fungi Zygorhyncus moelleri was frequently isolated during the earlier stages of incubation but not in the later stages showing it to fit into the category of saprophytic fungi (Garrett, 1951) which utilise and deplete the more readily degradable simple carbon substrates. The pattern of iso-

Table 4.9 Percentage frequency of occurrence of cultures recorded on glucose-starch agar incubated at 25°C for 7 days after inoculation with amended and incubated soil.

Fungi	Period of incubation of enriched soil									
	0Hr	1Day	2Days	3Days	4Days	6Days	8Days	10Days	12Days	14Days
Aspergillus fumigatus			10							
Aspergillus niger										
Aspergillus sp.										
Aureobasidium pullulans										
Eurotium sp.	10	10	20					20		
Fusarium solani	100	90	100	100	90	100	90	100	100	100
Gliocladium roseum		10	50		40	60			10	
Geotrichum candidum		10								
Humicola grisea										
Mucor globosus	80	40	20	60	50	60	80	30	70	40
Paecilomyces varioti					20	20				20
Penicillium sp.1	40	70	90	90	90	100	90	80	70	40
Penicillium sp.2		90	100	30	80	70	100	90	100	100
Penicillium sp.3										
Rhizoctonia sp.										
Sordaria fimicola	10		10	10						
Stachybotrys atra										
Stysanus sp.										
Trichoderma viride	100	100	100	100	100	100	100	100	100	100
Trichocladium asperum			10		10					
Verticillium sp.										
Zygorhyncus moelleri	30	20	30	30	10			20		
Actinomycetes					10					

lation on the glucose-starch plates was again much different to that obtained on the cellulose and newsprint agars. Fewer cellulolytic fungi, with the exceptions of Fusarium solani, Penicillium sp. 1 and 2 and Trichoderma viride, being isolated with high frequencies of occurrence throughout the period of incubation. Of the sugar fungi only Mucor globosus was isolated with a high frequency of occurrence on the glucose-starch agar.

From the results of the selective isolation technique carried out at 50°C the following thermophilic fungi were isolated for the linear growth test:-

Cephalosporium sp.
Chaetomium thermophile var coprophile
Chaetomium thermophile var disstum
Humicola grisea var thermoidea
Humicola insolens
Malbranchea pulchella
Sporotrichum thermophile var 1
Sporotrichum thermophile var 2
Torula thermophila

These are all thermophilic cellulolytic fungi which had been isolated from the enriched and incubated soil samples and also from samples of refuse and compost. The ability to clear cellulose agar had been observed with all of the thermophilic isolated above. In tables 4.4, 4.5 and 4.6 the varieties of Chaetomium thermophile were not identified separately as they are difficult to distinguish on isolation

plates. The main difference between the two varieties is the manner of the distribution of perithecia. The perithecia of Chaetomium thermophile var coprophile are usually gregarious and those of Chaetomium thermophile var dissitum are rarely gregarious (Cooney and Emerson, 1964). An additional species of the thermophilic fungi, Thielavia thermophila, was added to the above list of those fungi isolated from the soil. Thielavia thermophila had been reported by Mills (1973) to have cellulolytic ability.

From the results of the selective isolation technique carried out at 25°C the following fungi were selected for the linear growth test:-

Fusarium solani	Penicillium sp.1
Gliocladium roseum	Penicillium sp.2
Humicola grisea	Sordaria fimicola
Paecilomyces varioti	Trichoderma viride

Many species of mesophilic cellulolytic fungi were isolated from the samples of refuse and compost and from the experimental windrow and the following fungi were selected from these isolates on the basis of their ability to clear cellulose agar in pure cultures.

Alternaria sp.	Ulocladium sp.
Chaetomium globosum	Verticillium lateritium
Gliomastix sp.	Verticillium sp.
Stysanus sp.	

Detailed accounts of the microbiological degradation of cellulose have been presented by Siu (1951) and Gascoigne and Gascoigne (1960) and from their classification of the cellulolytic abilities of individual species and genera of the fungi the following species were selected for the linear growth test:-

Chaetomium affine
Chaetomium indicum
Myrothecium verrucaria
Cladosporium cladosporiodes

Chahal and Gray (1971) reported that two species of the Mycelia Sterilia, Rhizoctonia solani and Sclerotium rolfsii produced moderate to good growth on wood pulp and cultures of these organisms were acquired for the screening programme. Myrothecium verrucaria reported by Siu (1951) to be strongly cellulolytic has been reported by Updegraff (1971) to give very good growth on suspensions of ball-milled newsprint. Two species of Basidiomycetes, Coprinus sp. and Lentinus tigrinus, and a blue staining fungus Scytalidium lignicola were also used in the screening programme. The strong cellulolytic ability of species of Coprinus isolated from straw composts has been noted by Eastwood (1952) and Chang (1967). Lentinus tigrinus a white rot was listed by Siu (1951) as having lignolytic ability and Scytalidium lignicola was reported by King (1972) to have strong cellulolytic ability.

The results of the linear growth tests for these fungi are presented in Fig. 4.1 for the thermophiles and Fig.

FIGURE 4.1 LINEAR GROWTH OF SOME OF THE THERMOPHILIC FUNGI ON CELLULOSE AND NEWSPRINT AGARS.

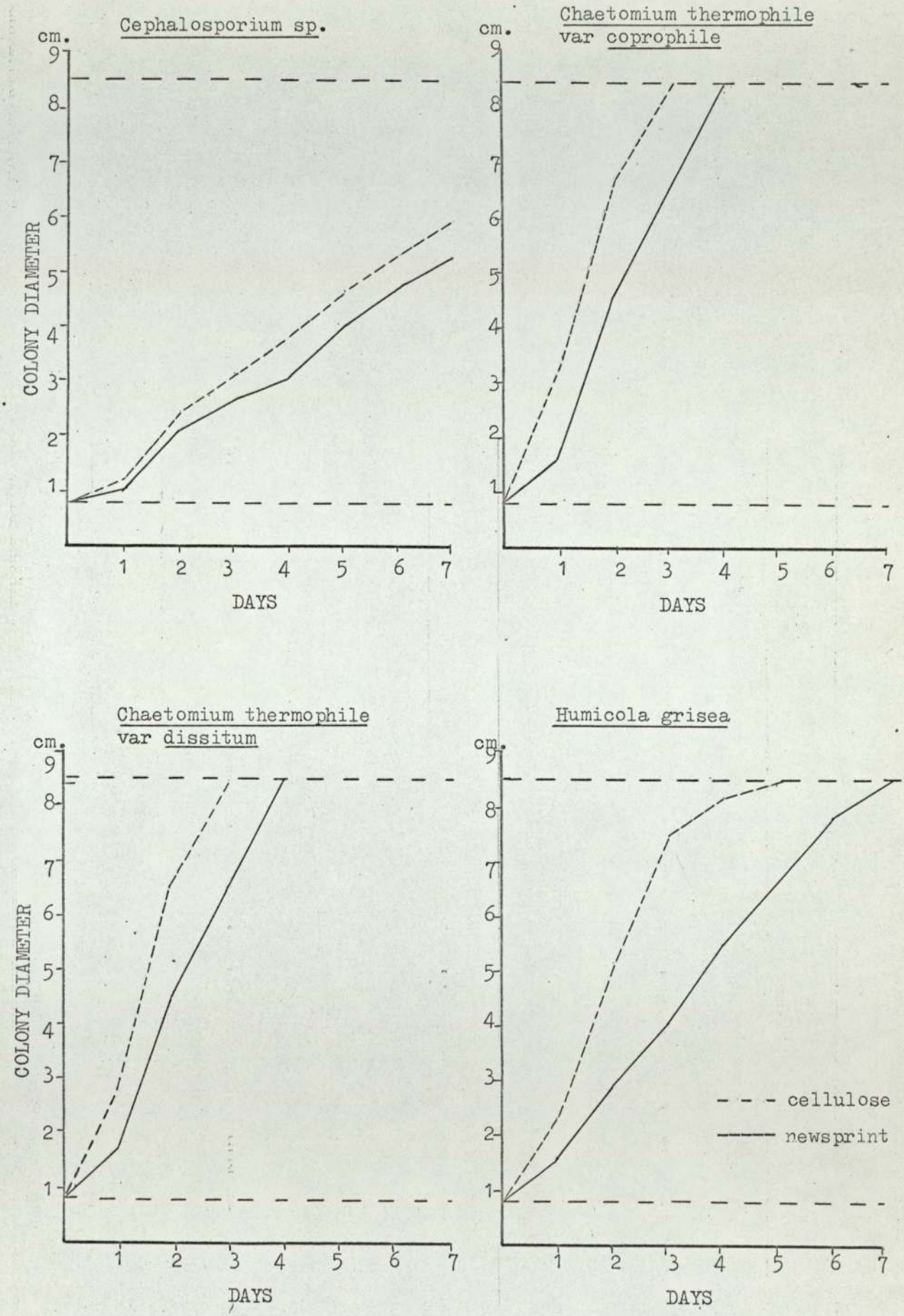


FIGURE 4.1 continued.

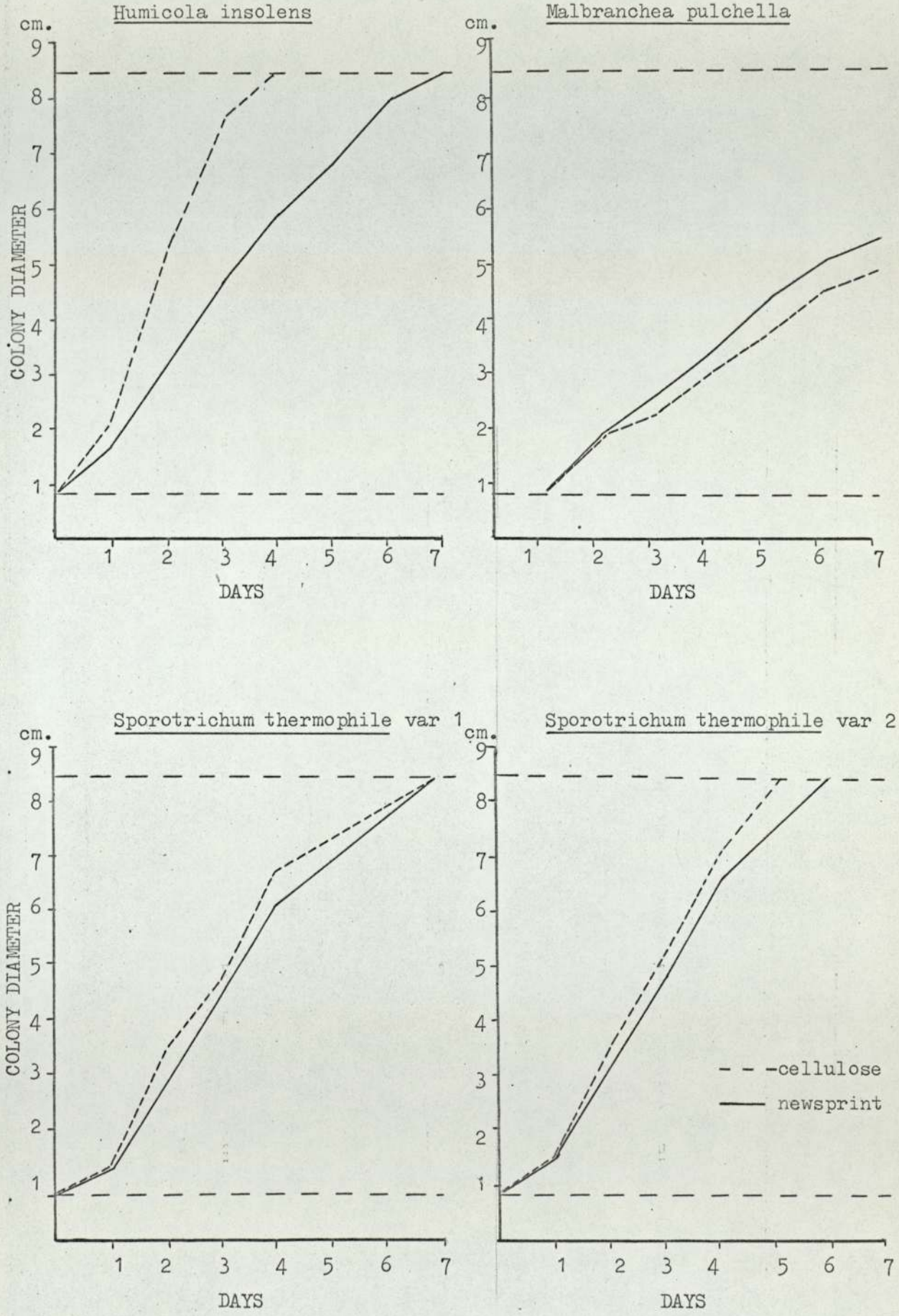
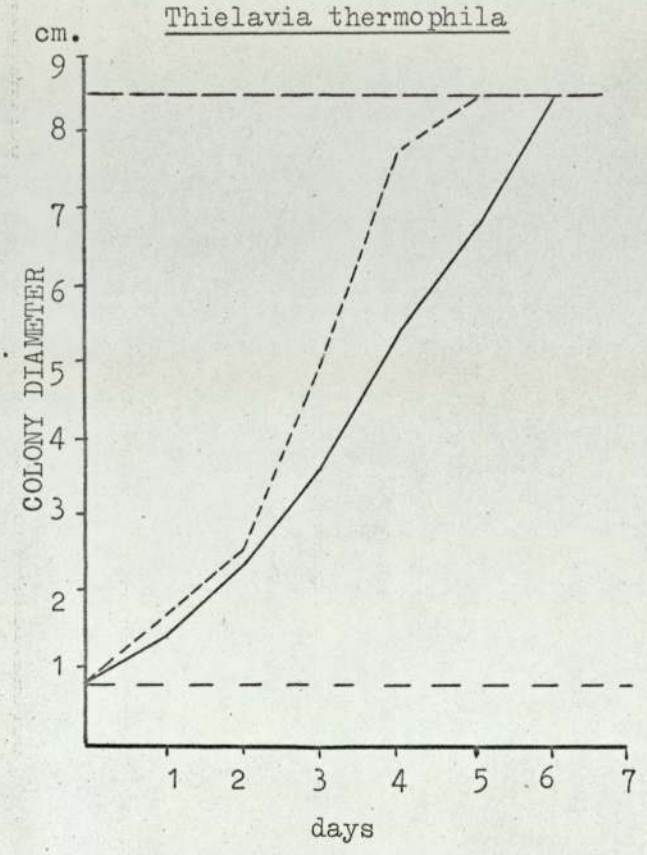
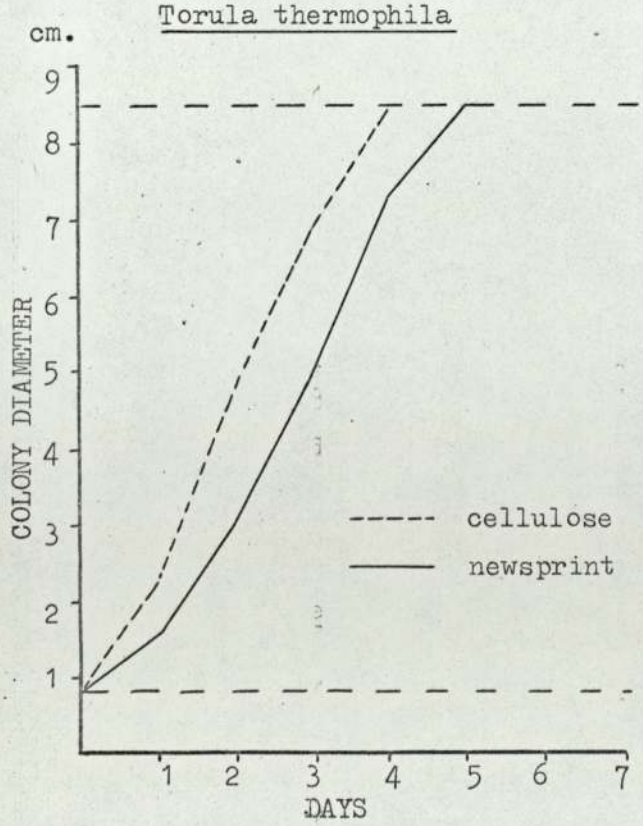


FIGURE 4.1 continued.





4.2 for the mesophiles. From the graphs presented in these figures it can be seen that the fastest growing thermophilic fungi were the two varieties of Chaetomium thermophile, coprophile and dissitum. Both these fungi attained the maximum possible colony diameter (8.5cm) in 3 days on the cellulose agar and 4 days on the newsprint agar. Humicola insolens, Sporotrichum thermophile var 2 and Torula thermophila, were the next fastest growing fungi covering the cellulose plates within 4 days and the newsprint plates in 7, 6 and 5 days respectively. Only two of the thermophilic fungi tested failed to cover the entire area of the cellulose and newsprint plates within 7 days, these were Cephalosporium sp. and Malbranchea pulchella. Malbranchea pulchella was also the only thermophilic fungus to have a faster growth rate on the newsprint agar than on the cellulose agar, the remaining thermophilic fungi, with the exception of Sporotrichum thermophile var 1 which had identical growth rates on both media, grew substantially quicker on the cellulose agar. Not one of the thermophilic fungi tested could produce a complete clearing of the newsprint agar. However, when the plates were examined carefully a partial clearing of the newsprint agar could be observed in all cases.

The fastest growing mesophilic fungus was Trichoderma viride which covered the cellulose plates in 3 days and the newsprint plates in 4 days equalling the growth rate attained by the fastest growing thermophilic fungi. The only other mesophilic fungus, from the 22 tested, to equal the growth rates of even the slower growing thermophilic fungi (with

FIGURE 4.2 LINEAR GROWTH OF SOME MESOPHILIC FUNGI ON CELLULOSE AND NEWSPRINT AGARS AT 25° C.

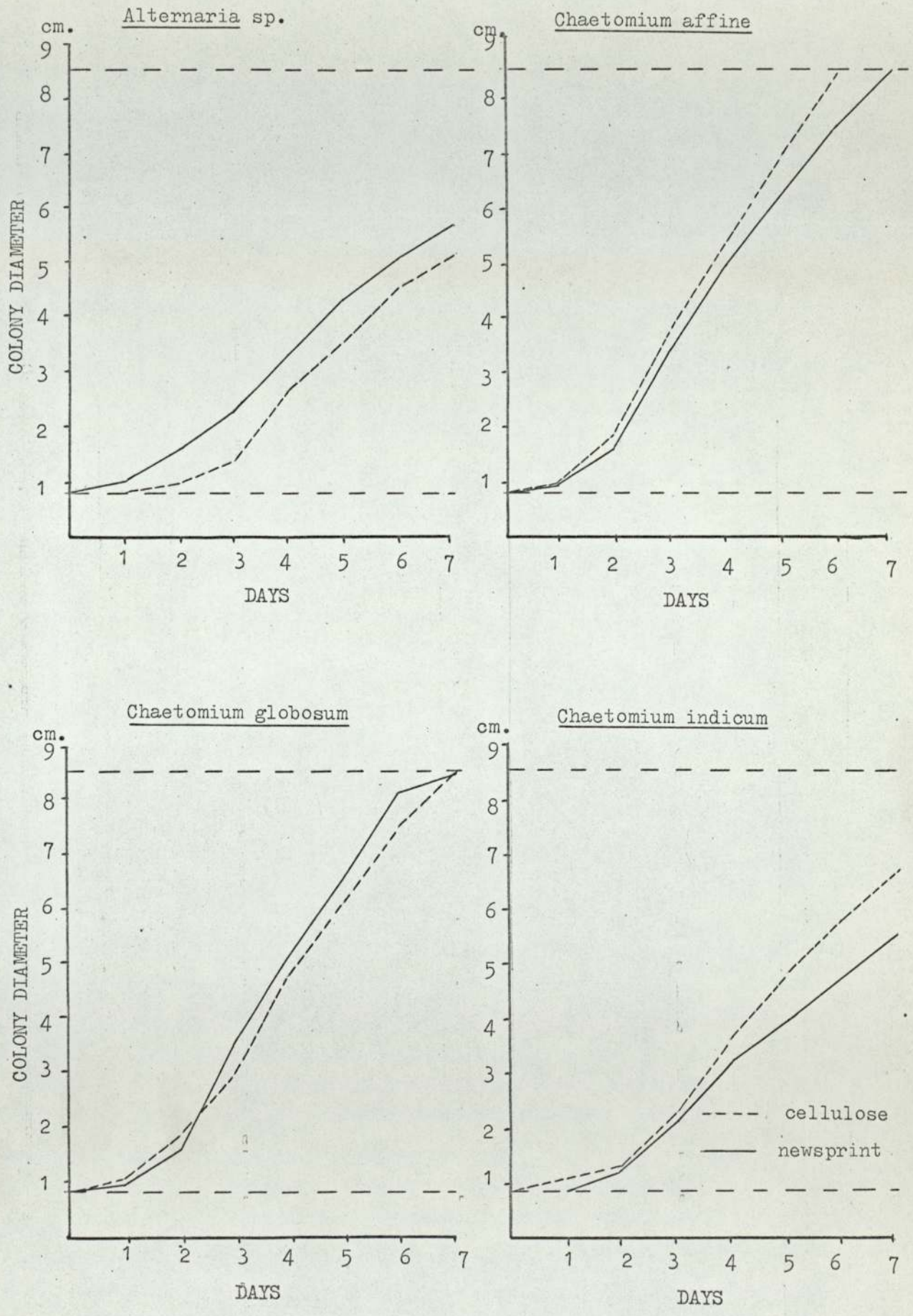


FIGURE 4.2 continued.

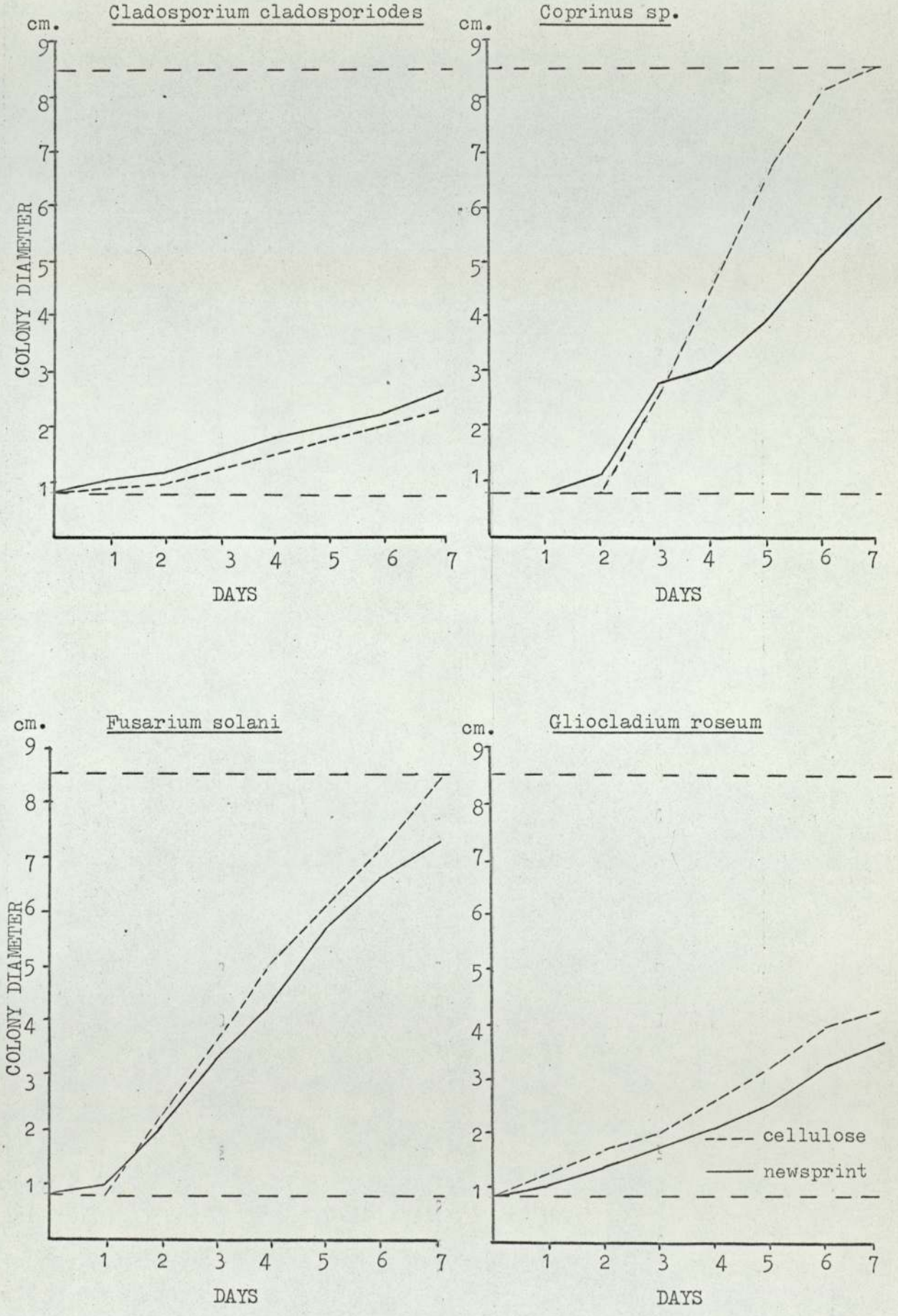


FIGURE 4.2 continued.

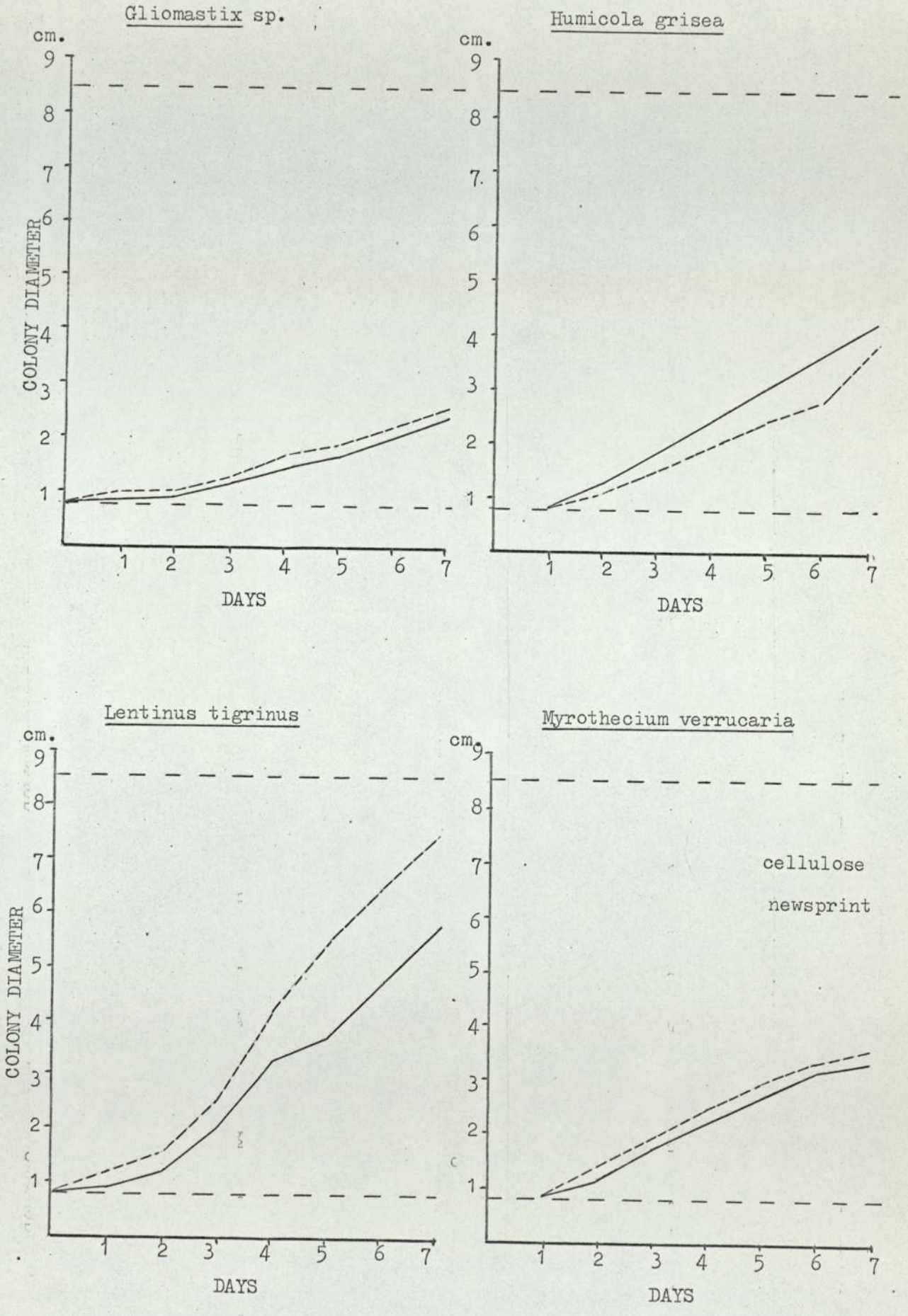


FIGURE 4.2 continued.

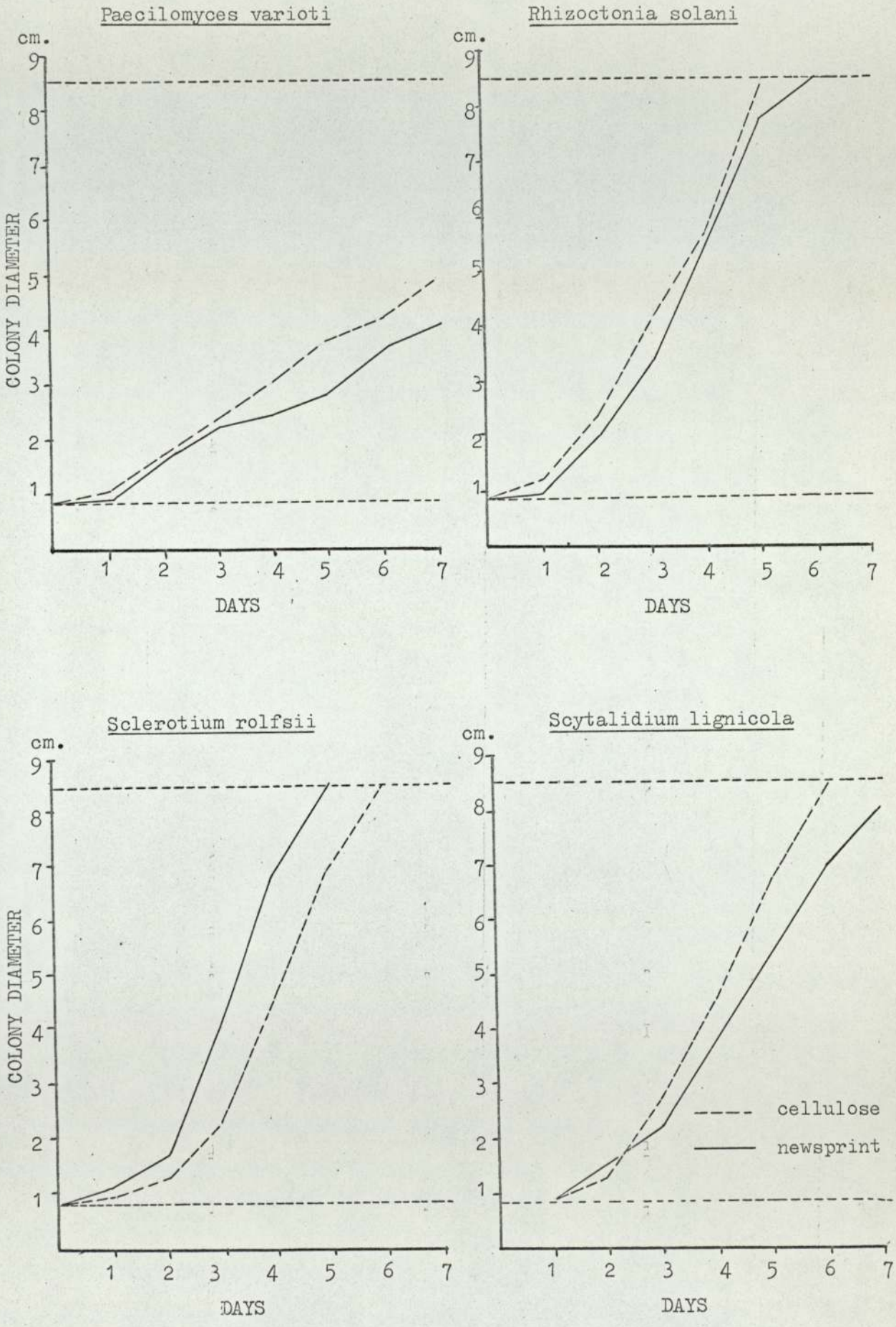


FIGURE 4.2 continued.

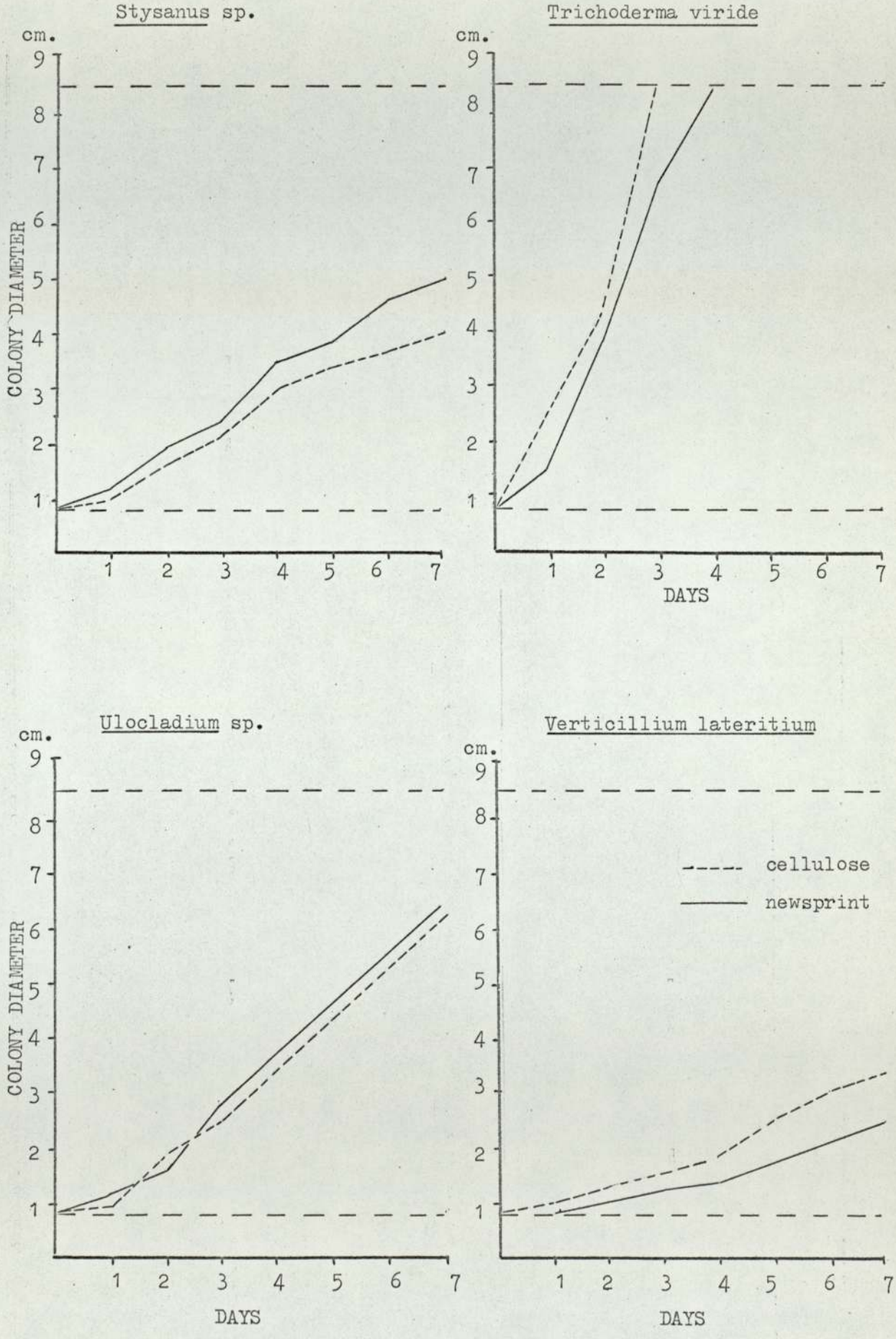
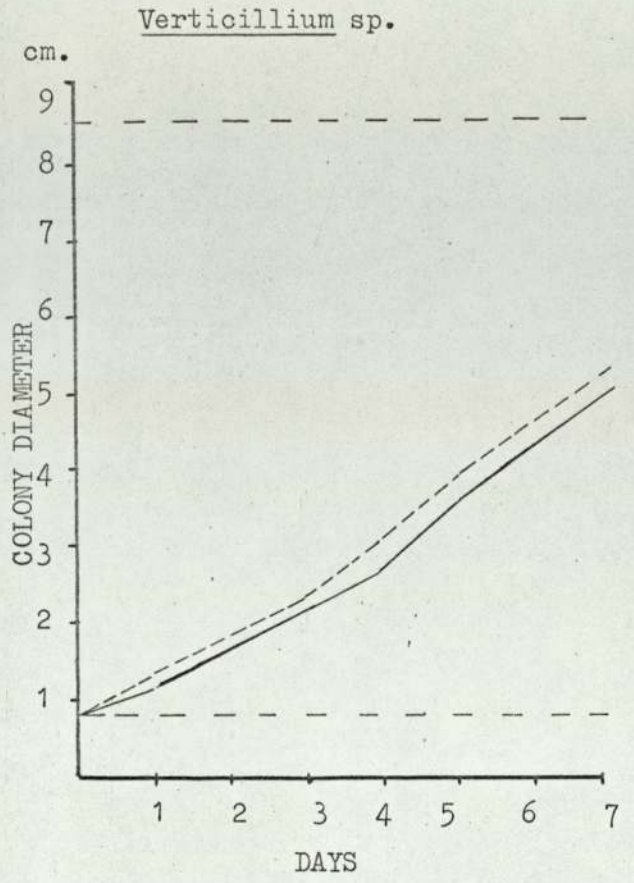
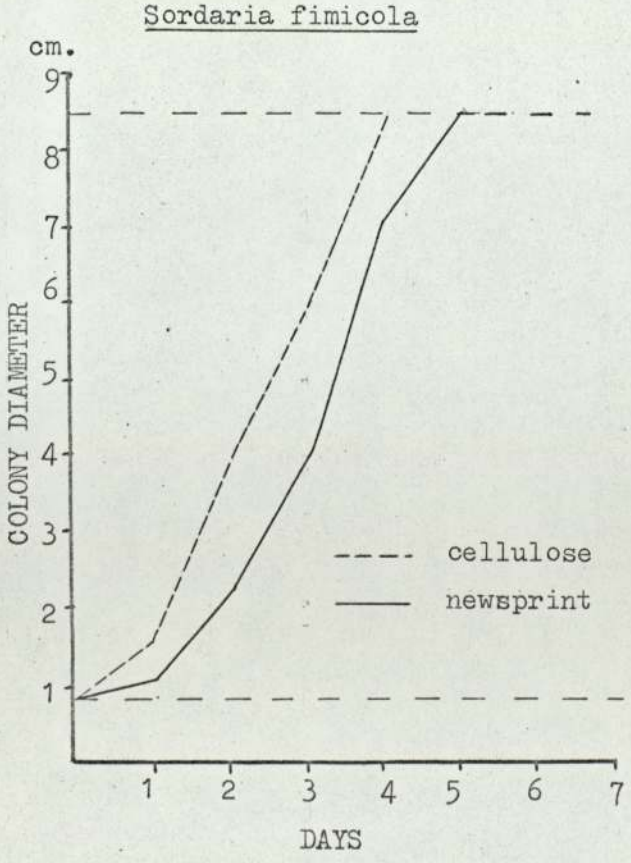


FIGURE 4.2 continued.





the exception of Cephalosporium sp. and Malbranchea pulchella) were in order of growth rates, Sordaria fimicola, Chaetomium affine, Rhizoctonia solani, Sclerotium rolfsii, Chaetomium globosum, Scytalidium lignicola, Fusarium solani and Coprinus sp. The remaining mesophilic fungi tested failed to cover the total area of the cellulose and newsprint agar plates after 7 days of incubation. Alternaria sp., Chaetomium globosum, Cladosporium cladosporiodes, Humicola grisea, Sclerotium rolfsii, Stysanus sp. and Ulocladium sp. were the only mesophilic fungi to exhibit faster growth rates on the newsprint agar. The difference between the growth rates of the mesophilic fungi on the cellulose and newsprint agars was usually marginal unlike that displayed by the thermophilic fungi. All of the mesophilic fungi, with the exception of Lentinus tigrinus produced clearing of the cellulose agar. The mycelium produced by Lentinus tigrinus was very sparse and appeared unable to utilise the cellulose or newsprint carbon sources. Not one of the mesophilic fungi tested could completely clear the newsprint agar although partial clearings were produced by all of the fungi except Lentinus tigrinus. Particularly noticeable were the extent of the zones of partial clearings produced by Chaetomium affine, Coprinus sp., Rhizoctonia solani and Trichoderma viride. These clearings matched in extent the ones produced by the most vigorous of the thermophilic fungi.

Discussion

The composition of the newsprint revealed by the analyses employed is in close agreement with those given by

Wenzl (1970) and Updegraff (1971) thus showing these techniques to be accurate.

The enrichment technique used was successful in the selection of a number of highly cellulolytic fungi which were then screened on the basis of linear growth rates. The difference in the growth rates exhibited by the majority of the fungi on the cellulose and newsprint agars supports the opinion of Brancato and Golding (1953) that the difference in the growth rates of a fungus on different substrates could be assessed on the criterion of colony diameter produced on an agar medium. However, it must also be observed that the fungi which grew rapidly on one medium also grew rapdily on the other medium i.e. in no case did a fungus grow slowly on one medium and very rapidly on the other. This finding agrees with those of King (1972) who examined the growth rates of many of the blue-staining fungi on cellulose pectin and starch agars and found that although variations existed between the growth rates of a fungus grown on these three substrates, the fungi which grew fast on one substrate tended to grow fast, with some variation, on the other substrates. Cochrane (1958) has expressed the opinion that the criterion of linear growth must be used with caution in nutritional studies and suggests that the burden of proof that fungal spread on agar is a fair index of growth rests on the investigator who chooses to use the method. In the present investigation only one thermophilic fungus and seven mesophilic fungi, out of the thirty-two tested, grew faster on the newsprint agar than on the cellulose agar. Although the difference in growth rates on the two media was

only marginal with many of the mesophilic fungi, it is clear that the majority of the fungi grew faster on the cellulose agar indicating the existence of a factor retarding the growth of the fungi on the newsprint agar.

The failure of any of the thermophilic or mesophilic fungi to produce a complete clearing of the newsprint agar indicated the incomplete utilisation of all the available constituents of the newsprint. Razzell (1971) stated that the problem of degrading newsprint is mainly a problem of lignin decomposition and that lignin in quantities as small as 5% of the total weight of the cellulose fibres decreases the breakdown of that cellulose by as much as 25% when compared with the breakdown of fully delignified fibres. Olson, Peterson and Sherrard (1937) have shown that as the lignin content of sawdust is lowered then its fermentability increases and therefore to obtain a good fermentation (with the thermophilic bacteria) the lignin content should be below 2%. The lignin content of the newsprint used in the present investigation was estimated to be 30.53%, by weight, and this could account for the slower growth of the majority of the fungi on the newsprint agar and the incomplete utilisation of the newsprint, thus resulting in the partial clearing observed in that agar.

The faster growth rates of most of the thermophilic fungi on the newsprint and cellulose agars and the reports in the literature of the enhanced cellulolytic activity of these fungi were the decisive factors in reaching the conclusion to use only thermophilic fungi in the development of

a process for the upgrading of waste newsprint. The eight thermophilic fungiselected for additional screening were:- Chaetomium thermophile var coprophile, Chaetomium thermophile var dissitum, Humicola grisea, Humicola insolens, Sporotrichum thermophile var 1, Sporotrichum thermophile var 2, Thielavia thermophila and Torula thermophila. The additional screening was carried out with shake-flask cultures on suspensions of ball-milled newsprint; the effects of two different nitrogen sources were also evaluated.

CHAPTER 5 Pages

Chapter 5

THE GROWTH OF THERMOPHILIC CELLULOLYTIC FUNGI IN SHAKE-FLASK CULTURES AND A SYSTEM OF SUBMERGED FERMENTATION ON SUSPENSIONS OF BALL-MILLED NEWSPRINT

Introduction

Initially the biodegradation of cellulose was attributed to a single enzyme (Whitaker, 1953) or to a component known as C_1 which was thought to produce shorter chains for more conventional hydrolysis by a Cx cellulase (Reese, Siu and Levinson, 1950). Thus a controversy existed over the number of enzymes involved in the degradation of fibrous cellulose, not only over the existence of the C_1 and Cx factors but also over the number of Cx factors which existed. Working with the cellulase system of Myrothecium verrucaria, Selby, Maitland and Thompson (1963) reported the possible existence of two components, a carboxymethyl cellulase and the labile C_1 component essential for attack on fibrous cotton. The existence of these two components was later confirmed by Selby and Maitland (1965). The existence of a C_1 factor was reported independently by Mandels and Reese (1964) working with the cellulose systems of Trichoderma viride. In spite of the confirmation of the existence of the C_1 and Cx factors the exact role of the C_1 component remained, until very recently, something of a mystery. The C_1 component appeared to have all the characteristics of an enzyme except that its precise function could not be identified (Selby, 1968). With most cellulolytic fungi C_1 is

always absent from the culture medium; the very rapid adsorption of this component by hydrocellulose has been reported by Selby (1968). Proposals which have been put forward to explain the role of C_1 include, the theory that C_1 aids the adsorption of Cx on cellulose. It was thought that this could possibly occur because of the supra-molecular structural arrangement of cellulose which in the cotton fibre consists of completely crystalline elementary fibrils each containing about 100 cellulose chains. The regular array of molecular chains is thus disturbed at intervals by the occurrence of chain ends which results in an accompanying disturbance of hydrogen bonding between chains in the vicinity of the chain ends which although insufficient to enable Cx working alone to remove soluble sugars could possibly lead to a single bond rupture by Cx possibly aided by C_1 followed by the absorption of C_1. It has been postulated by Selby (1968) that such a system could produce a highly localised disturbance of bonding and consequent loosening of a short length of surface chain which would then be more susceptible to extensive attack by Cx. King and Vessal (1969) reported that the C_1 component was a hydrolase enzyme attacking crystalline cellulose and producing cellobiose as 97% of the product. Recent evidence presented by Halliwell, Griffin and Vincent (1972) has confirmed the hypothesis of King and Vessal (1969) by demonstrating that component C_1, acting as a cellobiohydrolase, hydrolysed native cellulose to cellobiose in a solubilising reaction promoted by the presence of the enzyme cellobiase.

Thus as currently understood the cellulase complex contains the following components (in order of attack):-

i) C_1 a cellobiohydrolase which in the presence of cellobiase attacks crystalline cellulose to yield cellobiose.

ii) The glucanase (Cx) components which are hydrolytic and have the capacity to attack amorphous or soluble cellulose derivatives such as carboxymethyl cellulose. There are two types of glucanase, the endo-β-1-4-glucanase which has the dominant role of initiating the attack on amorphous cellulose yielding mainly cellodextrins and a trace of glucose by a random attack on internal linkages and the exo-β-1-4-glucanase which successively removes singleglucose units from the non-reducing end of the cellulose chain completing the hydrolysis of the products from the endo β-1-4-glucanase.

iii) The β-glucosidases involved in the breakdown of cellulose are active on the β dimers of glucose including cellobiose. The β glucosidases and the exo-β-1-4 glucanases share common substrates, cellobiose to cellohexaose, the small oligomers being more rapidly hydrolysed by the β-glucosidases.

The multi-enzymic cellulase system as described above achieves a maximum rate of attack on native cellulose such

as cotton fibre and a maximum completeness of conversion to glucose. The cellulose in wood and woody materials such as newsprint has been shown by analysis to make up only about 40% of the total wood, (excluding hemicelluloses), being either chemically bound or physically intermixed with the more resistant lignin which in turn confer a similar resistance on the cellulose (Siu and Reese, 1953). The lignocellulosic substance of wood cells is reported to be more resistant to degradation by micro-organisms than most other types of plant tissue (Scheffer and Cowling, 1966). The effects of lignin on the decay of cellulose were briefly mentioned in the previous chapter when it was stated that the problem of upgrading newsprint was not primarily a problem of cellulose decomposition but of lignin decomposition because lignification of wood cell walls is by far the most important non-toxic factor that contributes to the natural resistance of sapwood and heartwood to microbial deterioration. Many virulently cellulolytic micro-organisms including fungi and rumen bacteria are prevented from degrading wood by the intimate nature of the association between lignin and the wood polysaccharides (Scheffer and Cowling, 1966). It is thought that the lignin acts as a physical barrier preventing the cellulases of many micro-organisms from reaching a sufficient number of glycosidic bonds in the polysaccharides to permit significant hydrolysis. This means that all micro-organisms that degrade wood cell walls (the structural elements of newsprint) possess the necessary enzymes not only to degrade wood polysaccharides but also to depolymerise lignin or at least disrupt its association with the polysaccharides.

Previous attempts to ferment the cellulose of wood have usually tried to disrupt its association with lignin either by grinding or hydrolysis before attempting the fermentation stage. Research with thermophilic cellulolytic bacteria grown on finely ground wood pulp has shown that an appreciable part of its cellulose can be fermented, the finer the grinding the higher the percentage of cellulose fermented, (Virtanen, Koistinen and Kiuru, 1938). These results were thought to indicate that cellulose is not chemically bound with lignin in wood. Olson, Peterson and Sherrard (1937) examined the effect of the presence of lignin in wood pulp and similar products on the fermentation of cellulose by thermophilic enrichment cultures and found that in order to obtain extensive fermentation the lignin content had to be less than 1%. The presence of 2-4% lignin in pulps reduced the amount of cellulose fermented to 50-60%, the addition of isolated lignin or lignin containing materials such as groundwood did not interfere with the fermentation of pure cellulose. Olson, Peterson and Sherrard (1937) concluded that the effect produced by the presence of lignin is not physical but due to a chemical union between lignin and cellulose. Fuller and Norman (1943) state that ball-mill grinding would not change the micro-structure of wood or alter the fact that lignin and cellulose are interpenetrating systems and they are of the opinion that the conclusions about a chemical union between cellulose and lignin are not justified because physical contact between the cellulose and the lignin even after ball-milling would prevent complete contact with the extracellular enzymes of a micro-organism. The explanation of the effect of lignin on cellulose availa-

bility given by Fuller and Norman (1943) was primarily a physical one because ball-milling breaks the ligno-cellulose matrix into such fine particles that a large amount, but not all, of the polysaccharide surface is exposed and freed of its protective association with lignin and rendered susceptible to enzymatic dissolution.

One of the first attempts to utilise cellulose wastes such as wood for the production of feedstuffs was the production of Candida utilis and Candida arborea grown on wood hydrolysates (Rose, 1961). Such methods of fermentation have so far failed to find any application in an open economy but the recent upsurge of interest in the disposal of large quantities of cellulose wastes and the worldwide shortage of protein rich feedstuffs has led to increased interest in these types of fermentation. Attempts have been made to ferment alkaline hydrolysed bagasse by Cellulomonas sp. and it has been claimed that the product obtained was competitive with more conventional proteins (Dunlap, 1969; Dunlap and Callihan, 1969;Han ,Dunlap & Callihan,1969).A process for the hydrolysis of the waste paper and vegetable wastes in refuse for the production of glucose for fermentation with Candida utilis has been described (Meller, 1969), but at the present time it is not thought to be economically viable. Other processes for the acid hydrolysis of cellulose wastes to fermentable sugars have been described by Padilla and Hoskins (1968) and Kobayashi (1971). More recently attempts have been made to achieve continuous enzymatic saccharification of cellulose with the cellulase enzymes extracted from the culture filtrates of Trichoderma viride (Anon, 1969;

Ghose, 1969). The enzymatic hydrolysis of bagasse with the cellulases of Chaetomium globosum has also been attempted and was found to be at least 60% as efficient as acid hydrolysis (Agarwal and Rastog, 1967). Other pretreatments of cellulose substrates to increase the rate of fermentation for the production of microbial protein have included electron irradiation and photochemical treatment. Rogers, Coleman, Spino and Purcell (1972) found that photochemical treatment of cellulose was a more effective form of pretreatment than either acid/alkaline hydrolysis or electron irradiation for producing fungal protein in the form of Aspergillus fumigatus.

Attempts have also been made to ferment cellulose substrates directly without the need for any form of pre-treatment or saccharification. Stranks (1957) has reported the fermentation of cellulose by rumen micro-organisms, 84% of a 4% cellulose suspension being fermented in 72 hours at 40°C. This method of fermentation was proposed as a means of utilisation for low forms of wood waste. Stranks (1968) also studied the growth of Aureobasidium pullulans on ball-milled Aspen and proposed that it could be used where low grade forage material is to be upgraded for consumption as fodder. Aureobasidium pullulans, however, could only utilise the hemicellulose fraction of the wood and only produced a limited amount of breakdown of the Aspen. Updegraff (1971) employed Myrothecium verrucaria to breakdown suspensions of ball-milled newsprint and synthesise fungal protein in a system of submerged fermentation. Updegraff (1971) concluded, because of the low yields and production rate of his isolate that feed material could not be produced at a com-

petitive price in this manner. Chahal and Gray (1969, 1970) and Chahal, Munshi and Cheema (1969) investigated the possibility of producing fungal protein from delignified wood pulp. They used only mesophilic fungi and found that four species, Myrothecium verrucaria, Chaetomium globosum, Rhizoctonia solani and Trichoderma sp. were able to grow well on this substrate. Of these fungi Rhizoctonia solani achieved the best growth, one variety was reported to produce a product after 4 days incubation in a shake-flask culture with 23.2% of protein containing 21 of the 22 amino acids. Chahal and Gray (1969) and Jones and Irvine (1972) found that for fungal growth and protein synthesis urea was the best of the nitrogen sources tested, these included nitrate and ammonium nitrogen.

Chahal and Gray (1969) have also reported that cellulolytic fungi such as Myrothecium verrucaria require different types of media for growth and synthesis of fungal protein than those which have been used to measure cellulolytic activity and loss in tensile strength of fabric described by Greathouse, Klemme and Barker (1942), and Darby and Mandels (1954). Consequently in the present investigation a secondary screening of the eight thermophilic fungi selected by the linear growth test was undertaken in shake-flask cultures with two cheap and readily available nitrogen sources, ammonium sulphate and urea, using suspensions of ball-milled newsprint as the substrate. The most vigourously growing fungi in the shake-flask cultures were selected for further investigations which were carried out in the more optimum conditions provided by a laboratory scale 1 litre tower fermenter.

Materials and Methods

(i) Shake-flask cultures

A further screening of the eight species of thermophilic fungi selected on the basis of the linear growth test was carried out using shake flask cultures. The substrate used in the shake-flask cultures was the ball-milled suspension of newsprint as used in the newsprint agar. The mineral salts solution used in the present investigation was basically the one developed by Chahal and Gray (1969) for growing cellulolytic fungi on wood pulp. The basal synthetic medium developed by Chahal and Gray (1969) was devised by comparing the growth of Myrothecium verrucaria and the weight loss in wood pulp caused by this fungus on different media used by other investigators. The composition of this basal synthetic medium is given below.

Basal synthetic medium developed by Chahal and Gray (1969):-

Nitrogen source	400mg N.
Potassium di-hydrogen phosphate	2.5gm
Crystalline magnesium sulphate	1.25gm
Dextrose	1gm
Vitamin solution Trace elements solution }	These solutions were replaced by using 0.5gm of Oxoid yeast extract
Zinc sulphate solution (44gm/litre)	1ml
Ferric chloride solution (1.92gm/litre)	1ml

Make up to 1 litre with a 1% suspension of ball-milled newsprint.

The basal synthetic medium has a pH of 5.6 to 5.8, approxi-

mately the same as the pH of the mineral salts of Eggins and Pugh's (1962) cellulose medium. This pH was considered suitable for the growth of the thermophilic fungi on a cellulosic substrate such as newsprint. Lilly and Barnett (1951) reported that an initial pH of 5 to 6 is considered satisfactory for the growth of the majority of the fungi and Malik (1970) found that for the majority of the thermophilic fungi the optimum pH for maximum cellulolytic activity was between 5 and 7.

Twenty-five ml. portions of the medium were dispensed into 100 ml. conical flasks which were sealed with cotton-wool plugs and autoclaved at 15 lb. p.s.i. for 20 minutes. The effect of two different nitrogen sources, ammonium sulphate and urea on the growth of the fungi was examined. The required amount of ammonium sulphate to give a nitrogen content of 400mg per litre was added to the medium which was then dispensed and autoclaved in the normal way. The medium with urea as a nitrogen source was autoclaved before the addition of the urea. A solution of urea, of known concentration, was prepared and sterilised by filtration through a Millipore bacterial filter. The required volume of sterile urea solution was then added aseptically to each flask to give a nitrogen content equivalent to 400mg N/litre. Two concentrations of ball-milled newsprint suspensions were used, 1% and 2%, with the 2% suspensions the quantities of the mineral salts per unit volume were doubled with the exceptions of the nitrogen sources, i.e. ammonium sulphate, urea and yeast extract. The medium containing a 1% suspension of ball-milled newsprint thus had a C:N ratio of ap-

proximately 13:1 and the medium with the 2% suspension a C:N ratio of 26:1, (the carbon content of the newsprint was determined as 52.92% by the 'ashing' method described in Chapter 3).

The shake-flasks were inoculated by the addition of one 8mm agar plug taken from a 7 day old culture of the desired fungus grown on newsprint agar. The inoculated flasks were incubated at 48°C in a Gallenkamp shaking reaction incubator type 1H 350 set to give a stroke of 1.75 inches at a speed of 100 strokes per minute. This was the maximum speed and hence aeration rate which could be safely used without the contents of the flask splashing against the cotton-wool plug. Two replicate shake-flasks were sacrificed every 12 hours, i.e. after 12, 24, 36 and 48 hours. The contents from each flask, consisting of the residual suspension and fungal mycelium, were harvested on dried and weighed filter papers in a Buchner funnel, washed thoroughly, oven-dried at 60°C overnight, placed in a dessicator for 24 hours and weighed.

The growth of the fungi was measured in terms of crude protein synthesised and by weight loss produced in the suspension by the fungus. The weight loss was determined by the difference in weights between the contents obtained from the experimental flasks and the control flasks, which had received the same treatment as the experimental flasks except for inoculation. Control flasks were set up for every new batch of medium prepared and the weight losses in the experimental flasks were expressed as percentages of the total

weight of the suspension obtained from the control flasks. The nitrogen contents of the products obtained from the shake flasks were determined by the method of micro-Kjeldahl (detailed in Chapter 3) and the protein contents were estimated by employing the conversion factor (6.25 x N) as the nitrogen content of most proteins has been estimated at 16.0% (Foster, 1949). Samples of the contents of the shake flasks were obtained for the micro-Kjeldahls by carefully teasing the dried residue away from the filter paper, in most cases no difficulty was experienced in cleanly separating the residue from the filter paper.

For each of the eight species of thermophilic fungi a series of shake-flask cultures were set up for the two concentrations of ball-milled newsprint with each of the two nitrogen sources used.

(ii) Submerged fermentation

Studies of the submerged fermentation of suspensions of ball-milled newsprint were undertaken, using the two most vigorously growing thermophilic fungi from the shake-flask cultures, in a laboratory scale tower fermenter.

The tower or tubular fermenter can be described as an elongated non-mechanically stirred fermenter with an aspect ratio (height to diameter) of at least 6:1 on the tubular section or 10:1 overall, through which there is a unidirectional flow of medium or gases. (Greenshields and Smith, 1971; Greenshields, Morris, Daunter, Alagaratnam and Imrie, 1971). The tower fermenter used in this study, shown in Fig 5.1, had an operating volume of 1 litre and was fitted

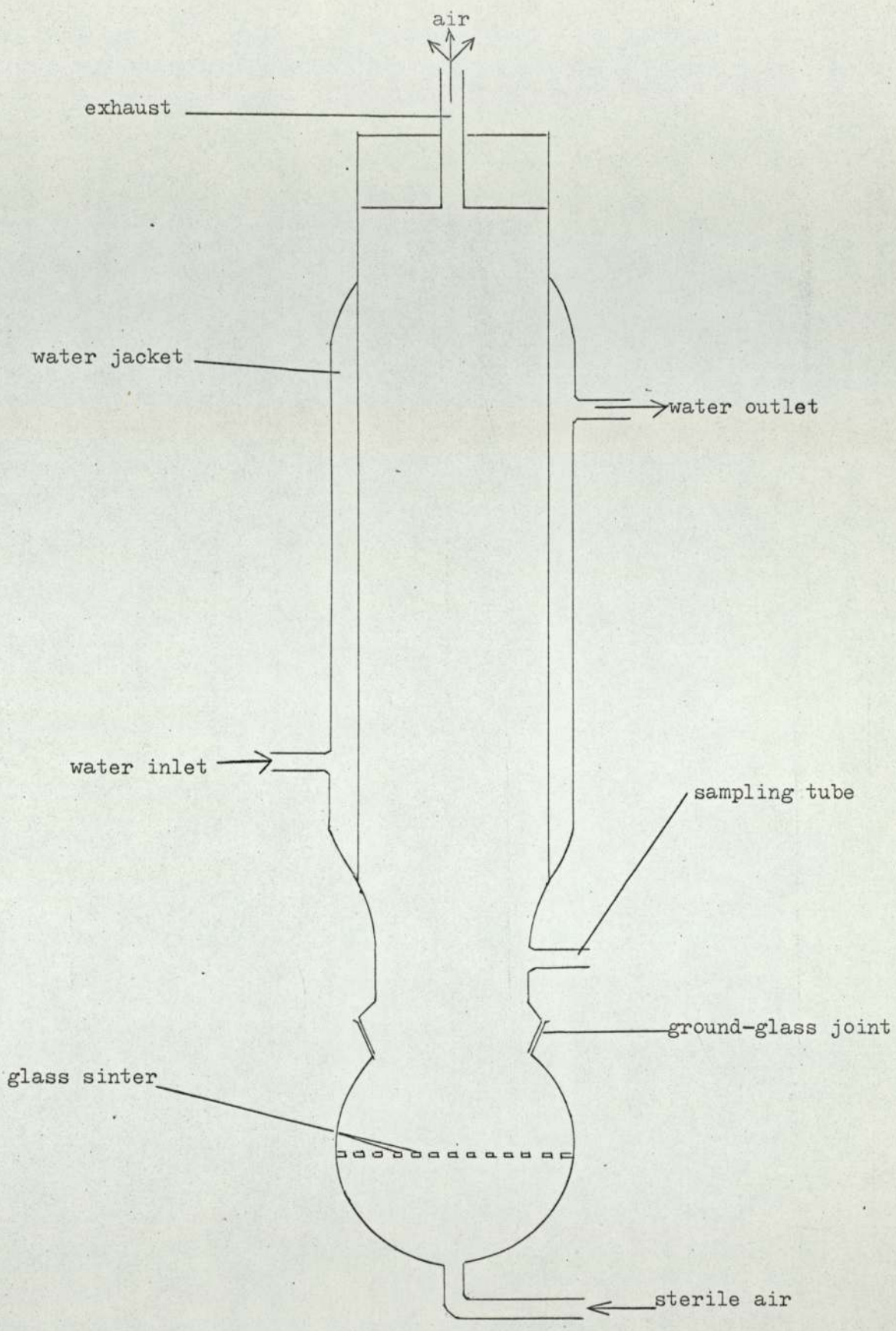


FIGURE 5.1 ONE-LITRE TOWER FERMENTER

with a water jacket for the purpose of temperature regulation. Aeration was provided by pumping air through the glass sinter and into the fermentation medium. It was the air pressure behind the sinter which supported the column of liquid above the sinter and prevented the liquid from passing through the pores of the sinter. As the air passed through the sinter into the fermentation liquid many air bubbles were formed and as these bubbles rose from the bottom to the top of the tubular section of the tower they served to provide an efficient form of agitation. Aeration was provided by a Gallenkamp A.F.410 air pump and the rate of aeration was monitored by a rotameter connected to the exhaust of the tower. The rate of aeration was maintained at 1 vol./vol./min. which was found by Greenshields et al., (1971) to be adequate for tower fermenters of this size.

In the present investigation the temperature of the fermentation medium was maintained at 48°C, the optimum temperature for growth of the two thermophilic fungi used. The air was pre-heated by bubbling it through a water bottle at 48°C and sterilised by filtration through a glass-wool filter. The pre-heating of the air reduced the rate of evaporation from the fermentation liquid to a minimum. The rate of evaporation was measured by placing a condenser between the exhaust of the tower and the rotameter.

The fermenter was sterilised by passing live steam through the sampling tube into the tubular section of the tower for at least two hours. After the completion of steaming the sampling tube was sealed with a clamp and a

steady stream of sterile air was passed through the fermenter to prevent contaminants being drawn into the fermenter through the exhaust as it cooled down. After the tower had cooled and the temperature of the water passing through the water jacket of the tower had stabilised at 48°C the inoculated fermentation medium was carefully poured into the top of the tower after the removal of the exhaust bung, aseptic precautions were taken during this procedure. During the early stages of the fermentation the rate of flow of air through the fermenter was found to fluctuate extensively and constant adjustments of the pump were required. However, after an hour or so the reading on the rotameter became steady and the rate of aeration could be accurately set for the remainder of the fermentation period.

The fermentation medium used was the one described for the shake-flask cultures developed by Chahal and Gray (1969), with a 1% suspension of ball-milled newsprint as the substrate and urea as the main nitrogen source. The medium with the exception of urea was sterilised by autoclaving, the sterile urea solution being aseptically added later. The fermentation medium was inoculated with the mycelium produced in 4 of the shake-flasks after 48 hours of incubation. Gundersen (1962) found that uniformity in amount and physiological age of the inocula of fungi was of major importance for obtaining reproducible results and so in this study the same method of preparing the inoculum was used for all the tower fermentations carried out. The mycelium from the 4 shake-flasks was homogenised, in a sterilised stainless steel attachment of a Waring blender with a

measured amount of the fermentation medium, and then added to the main body of the fermentation medium. It has been reported (Foster, 1949) that this system of inoculation starts the new culture with a greatly reduced lag phase since growth starts from each viable particle regardless of how small it is. One of the most common faults that has been reported in the submerged cultivation of fungi is the use of too small an inoculum which delays the development of the culture. The best results are usually obtained when the fermentation medium is inoculated with 5-10% of its volume with either a heavy pre-germinated culture or with the same amount of a fully grown vegetative submerged culture (Foster, 1949). In the present investigation the total volume of vegetative mycelium taken from the 4 shake-flasks was 100ml and this was added to 900ml of the fermentation medium representing an inoculation level of 10%.

Initially in the first few fermentations some problems were encountered with foaming of the medium but it was found that this could be almost completely prevented by the addition of small quantities of an inert silicone based anti-foam agent, 'Silcolapse'. The anti-foam agent was added during the fermentation whenever foaming occurred but care was taken to add only the minimum amount required in order not to markedly affect the dissolved oxygen concentration within the fermenter (Greenshields, Morris, Daunter and Alagaratman and Imrie, 1971).

Samples of the fermentation medium were taken at various stages during the fermentation, depending upon the

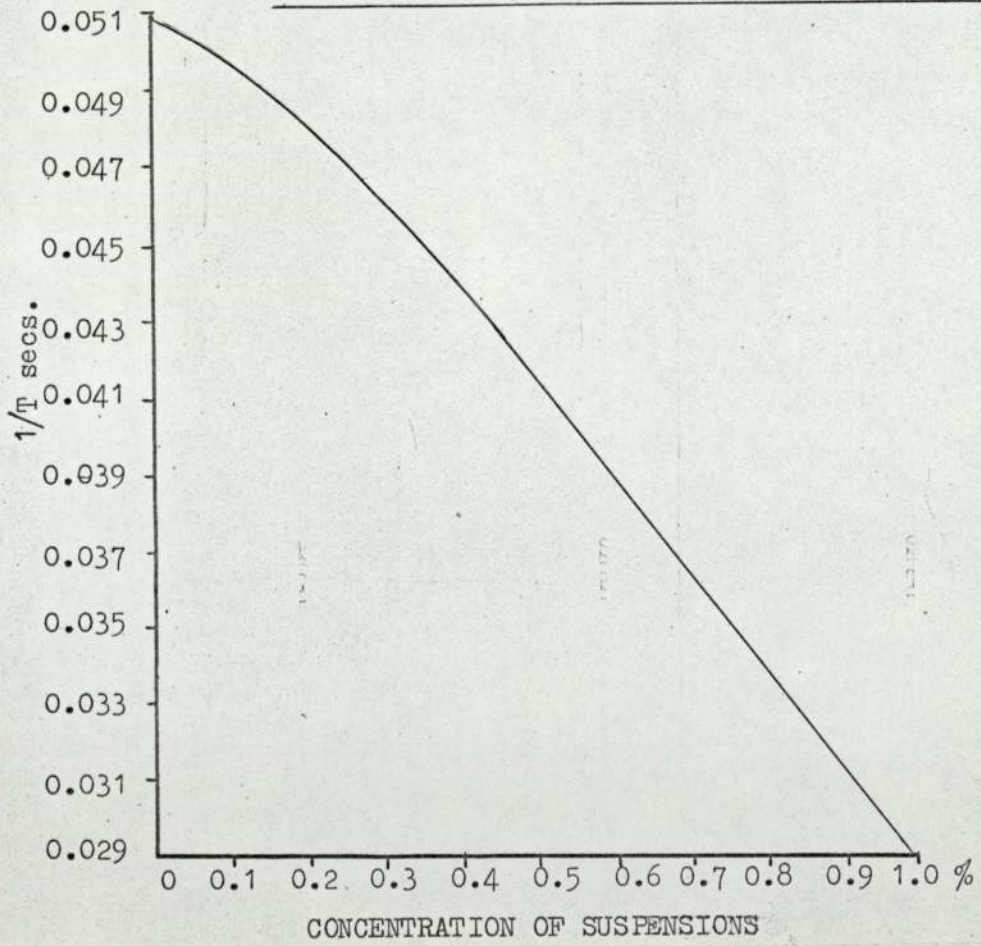
fungus used, from the sampling tube attached to the sidearm on the tubular section of the fermenter. Before each sample was taken the contents in the sidearm were drained off and discarded as these were not subject to the changes occurring in the main body of the fermenter. At each sampling time 60-70ml of the fermentation medium was collected and the pH of each sample recorded. Exactly 25mls of the sample were then measured out and the solid materials present (mycelium and residual suspension) were harvested on a dried and weighed filter paper in a Buchner funnel, washed, oven-dried at 60°C and weighed. The filtrate was also collected and kept for analysis. The nitrogen contents of the solid residue obtained on the filter paper and of the culture filtrates were determined by the method of micro-Kjeldahl and the protein content of the residue was determined by the factor (6.25 x N). The mycelium present in the samples of the fermentation medium could be easily and effectively separated from the suspension of ball-milled newsprint with an 80 mesh sieve, Brookes, Stanton and Wallbridge (1969) had found that a 100 mesh sieve was effective for separating fungal mycelium from cassava flour in a system of submerged fermentation. The suspension of newsprint from a 25ml sample was washed gently through the sieve, collected and harvested on a dried and weighed filter paper in a Buchner funnel, washed, oven-dried at 60°C, placed in a dessicator and weighed. The quantity of ball-milled newsprint remaining per unit volume of the fermentation medium could thus be determined at any stage of the fermentation. The mycelium collected on the sieve from a 25ml sample was all carefully washed off and harvested on dried and weighed filter paper.

Thus the quantity of mycelium produced per unit volume of the fermentation medium could also be determined at any stage of the fermentation. The protein content of the mycelium was determined by the method of micro-Kjeldahl.

The results of the analyses presented above were only available after the fermentation had finished and could not be used to monitor the fermentation while it was in progress. However, the rate of flow of the suspension of ball-milled newsprint through a reverse flow viscometer was found to be proportional to the concentration of the suspension and thus presented a method of monitoring the progress of the fermentation. Accordingly suspensions of varying concentrations between 0 and 1% were prepared and their rate of flow through the reverse flow viscometer (Gallenkamp No.VS340 Volac size C) was measured at 48°C, being timed with a stop watch. A plot was made of the reciprocals of the times (in seconds) that were taken for each concentration of the suspension to flow through the viscometer, this plot is presented in Fig. 5.2. From the plot, the quantity of the suspension remaining in the medium at any stage in the fermentation, could be estimated by removing a sample of the medium, separating the mycelium and measuring the rate of flow of the suspension. This method was found to give accurate estimation of the progress of any of the fermentations, this was verified by the results obtained in the subsequent analyses of the dried samples.

The effect of different concentrations of urea on the growth of one of the thermophilic fungi was examined,

FIGURE 5.2 RATE OF FLOW(1/T)OF STANDARD SUSPENSIONS OF BALL-MILLED NEWSPRINT THROUGHA REVERSE-FLOW VISCOMETER, AT 48°C.



urea was used to give the following concentrations of nitrogen per litre, 100, 200, 300, 400, 500 and 600mg. These concentrations gave the following C:N ratios, 53:1, 26:1, 18:1, 13:1, 11:1 and 9:1 respectively. The growth rates achieved by a second thermophilic fungus on ball-milled newsprint and a pure form of cellulose, ball-milled Whatman's Chromedia CF11, were compared. In this case both media had a nitrogen content of 400mg/litre with urea as the main source of nitrogen.

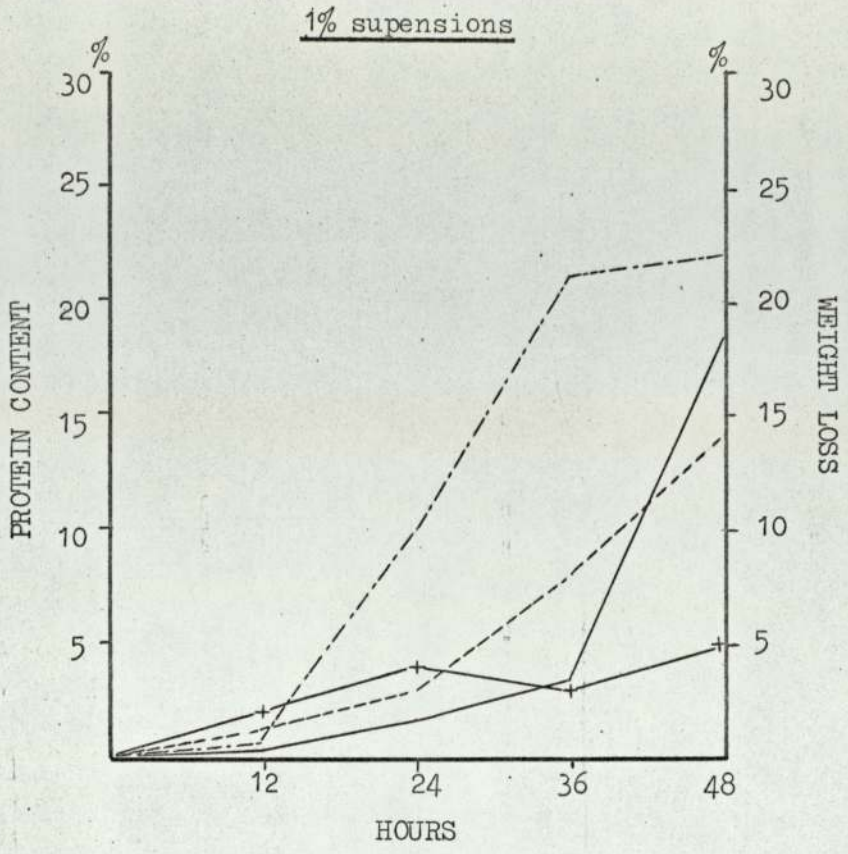
Results

(i) Shake-flask cultures

The results of the growth of the eight thermophilic fungi on the 1 and 2% suspensions of ball-milled newsprint in the shake-flask cultures with urea and ammonium sulphate as the two main nitrogen sources are presented in Figs. 5.3(a) to 5.3(h).

With the exception of Sporotrichum thermophile var 1 all of the fungi produced larger quantities of protein, hence better growth, on the 1% suspensions with C:N ratios of 13:1 than on the 2% suspensions with C:N ratios of 26:1 regardless of whether the nitrogen source was urea or ammonium sulphate. After 48 hours of incubation Sporotrichum thermophile var 1 on the 2% suspension with ammonium sulphate gave a product with a protein content of 5% whereas on the corresponding 1% suspension the product contained only 1% protein. However, with the suspensions containing urea, Sporotrichum thermophile var 1 grew better on the 1% suspension as did the remainder of the fungi.

FIGURE 5.3(a) GROWTH OF Chaetomium thermophile var coprophile ON SUSPENSIONS OF BALL-MILLED NEWSPRINT.



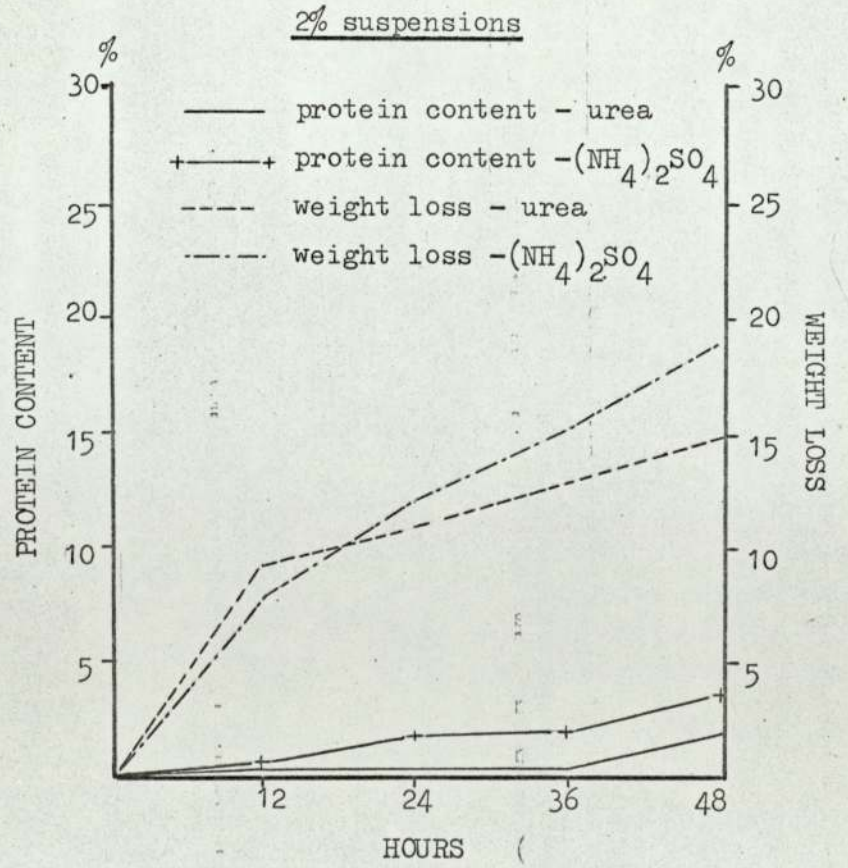
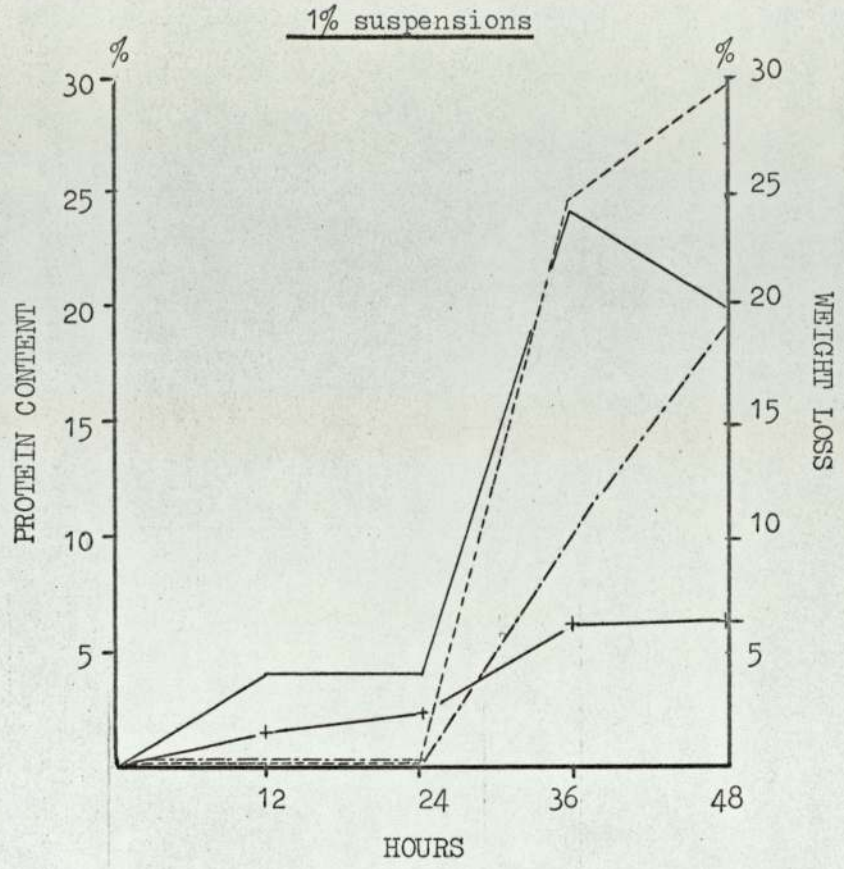


FIGURE 5.3(b) GROWTH OF Chaetomium thermophile var dissitum ON SUSPENSIONS OF BALL-MILLED NEWSPRINT.



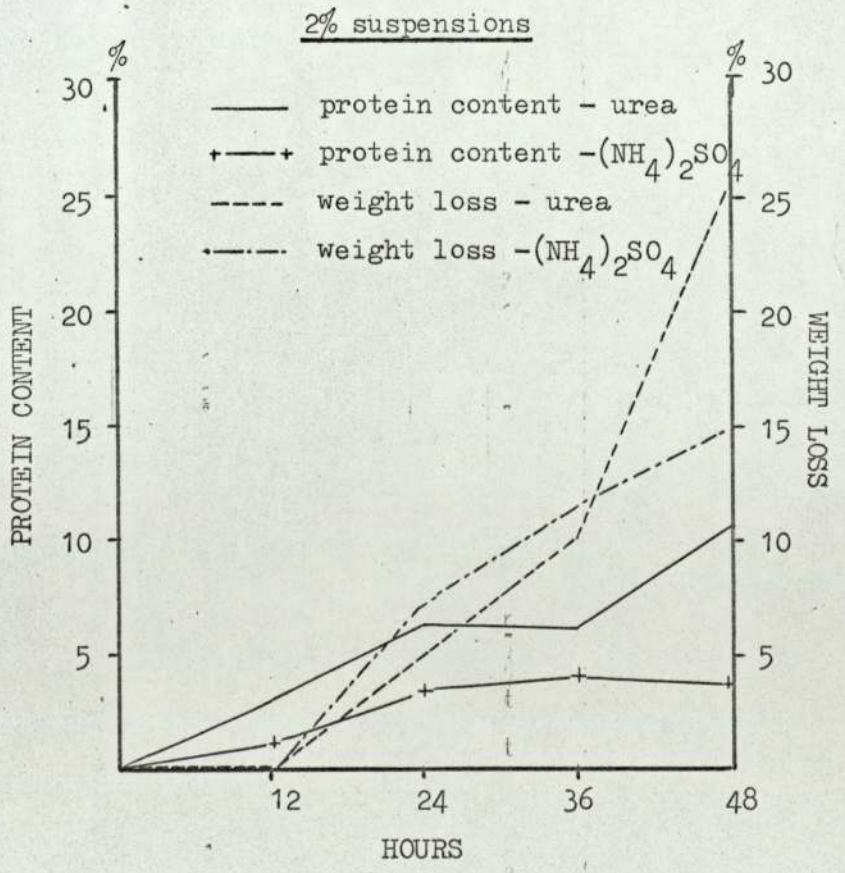
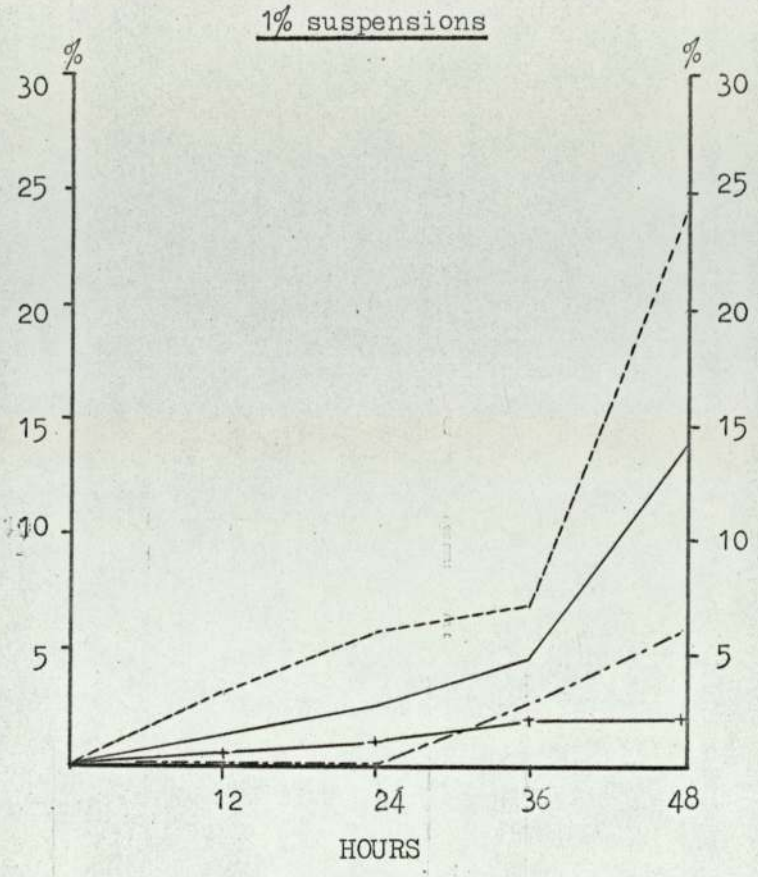


FIGURE 5.3(c) GROWTH OF *Humicola grisea* var *thermoidea* ON SUSPENSIONS OF BALL-MILLED NEWSPRINT.



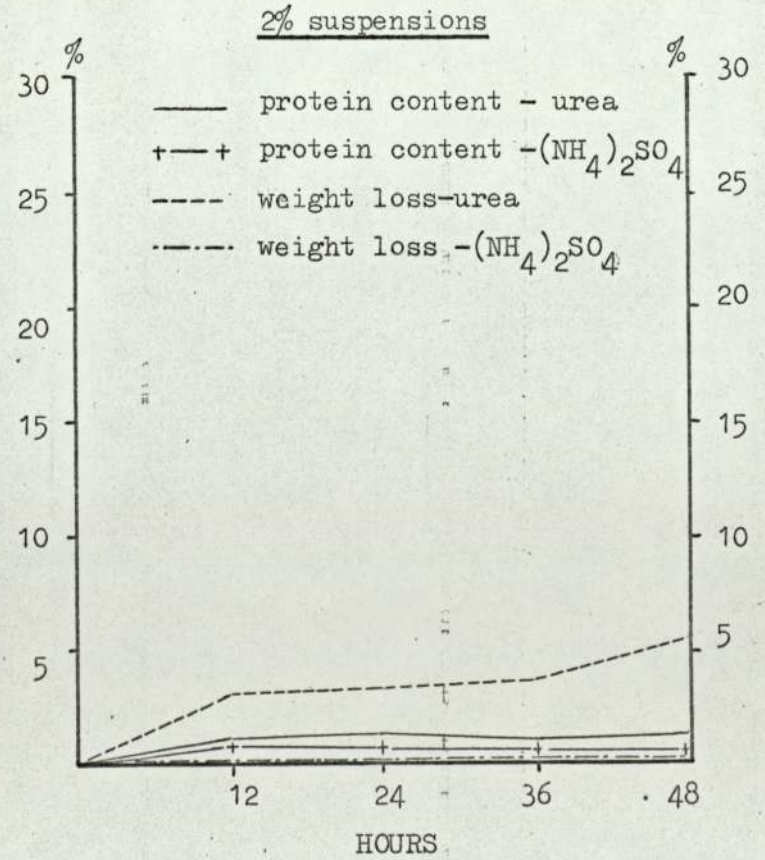
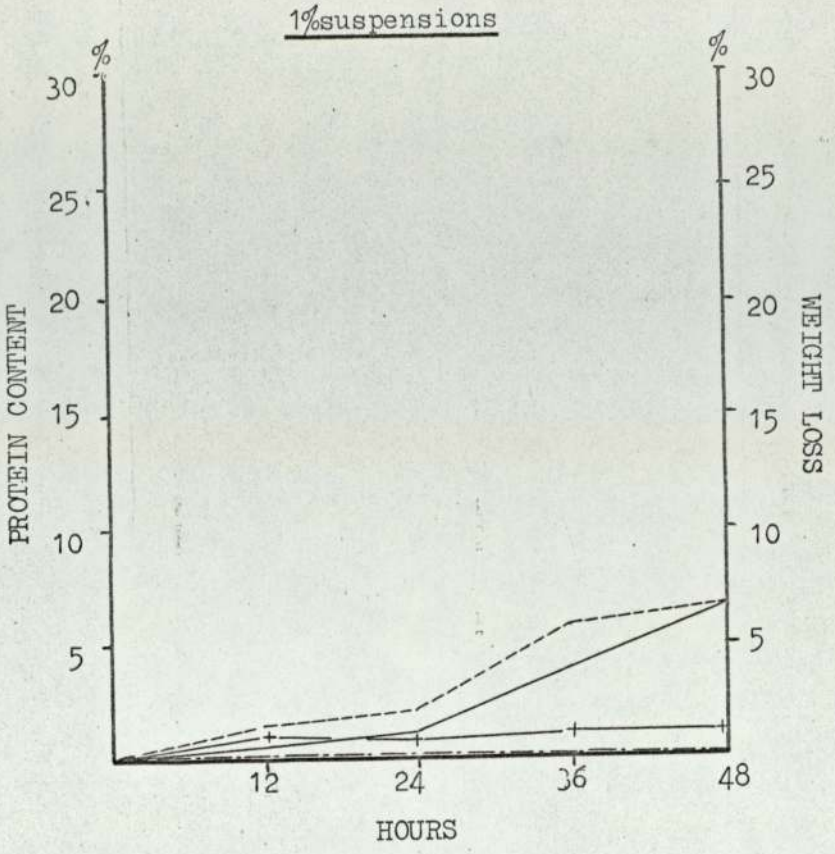


FIGURE 5.3(d) GROWTH OF Humicola insolens ON SUSPENSIONS OF BALL-MILLED NEWSPRINT.



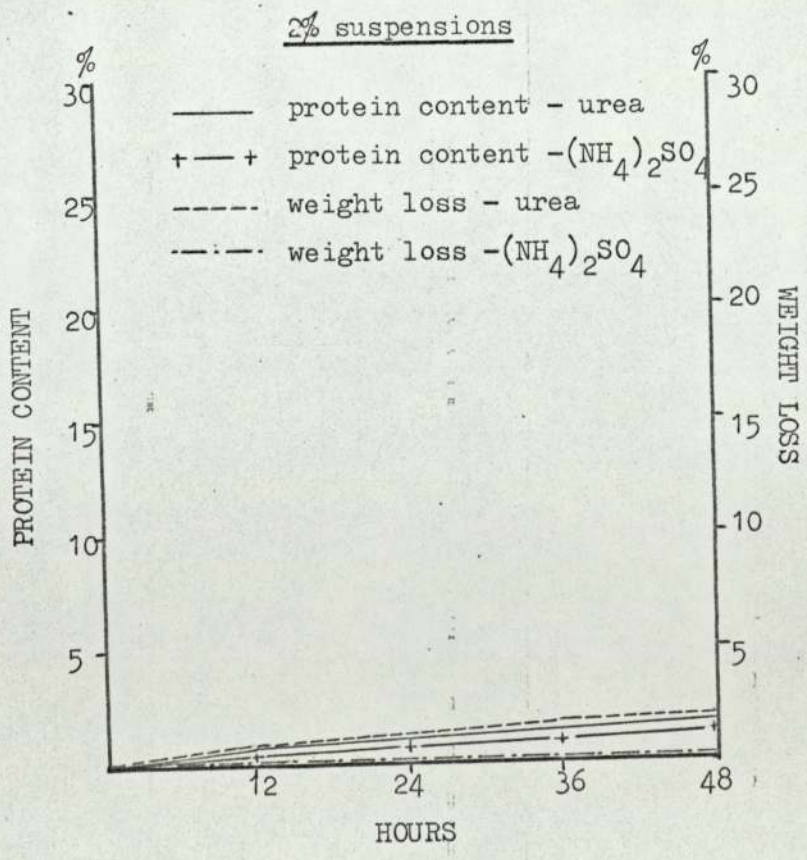
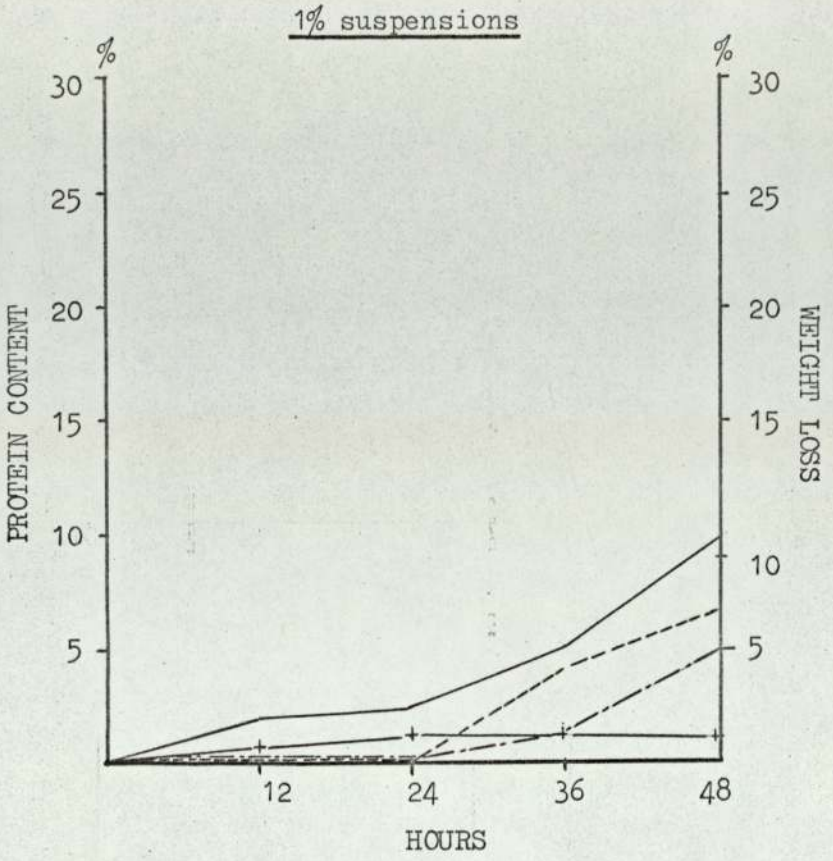


FIGURE 5.3(e) GROWTH OF Sporotrichum thermophile var 1 ON SUSPENSIONS OF BALL-MILLED NEWSPRINT.



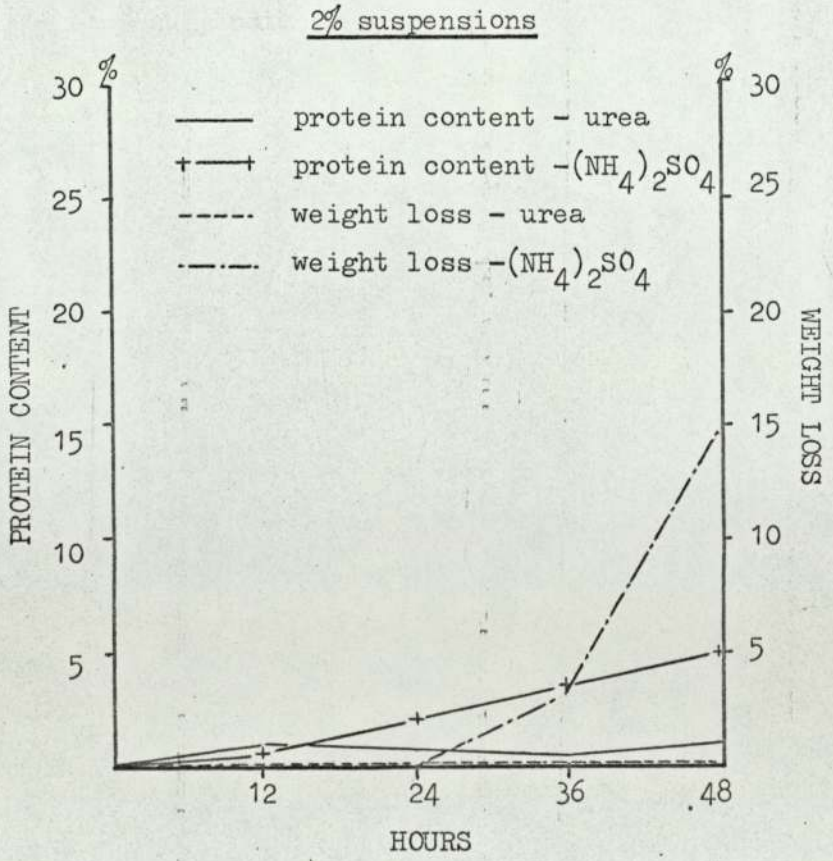
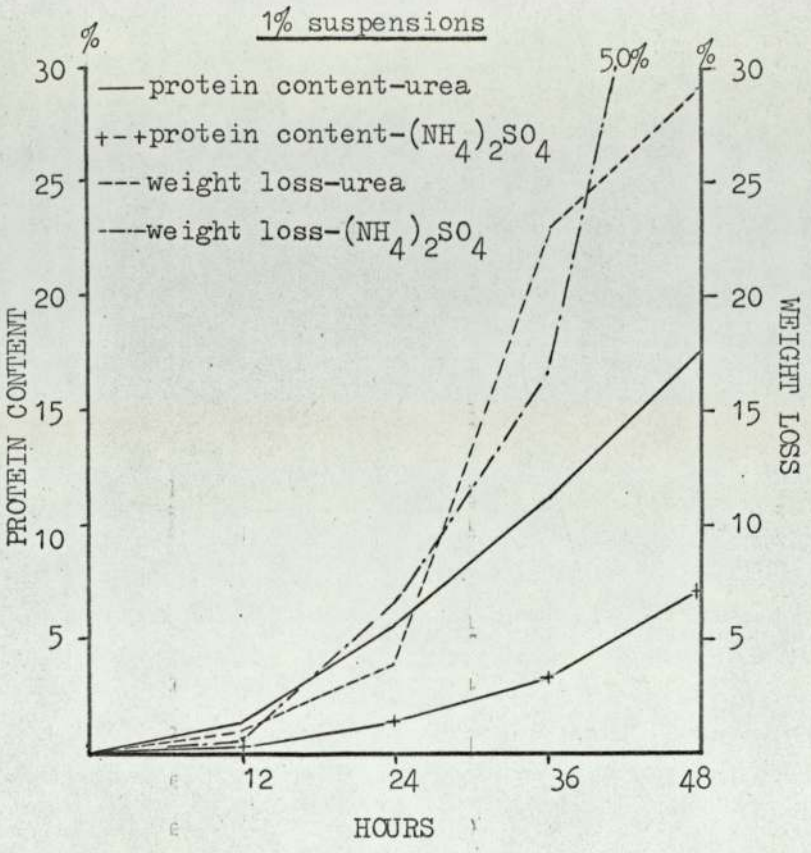


FIGURE 5.3(f) GROWTH OF Sporotrichum thermophile var 2 ON SUSPENSIONS OF BALL-MILLED NEWSPRINT



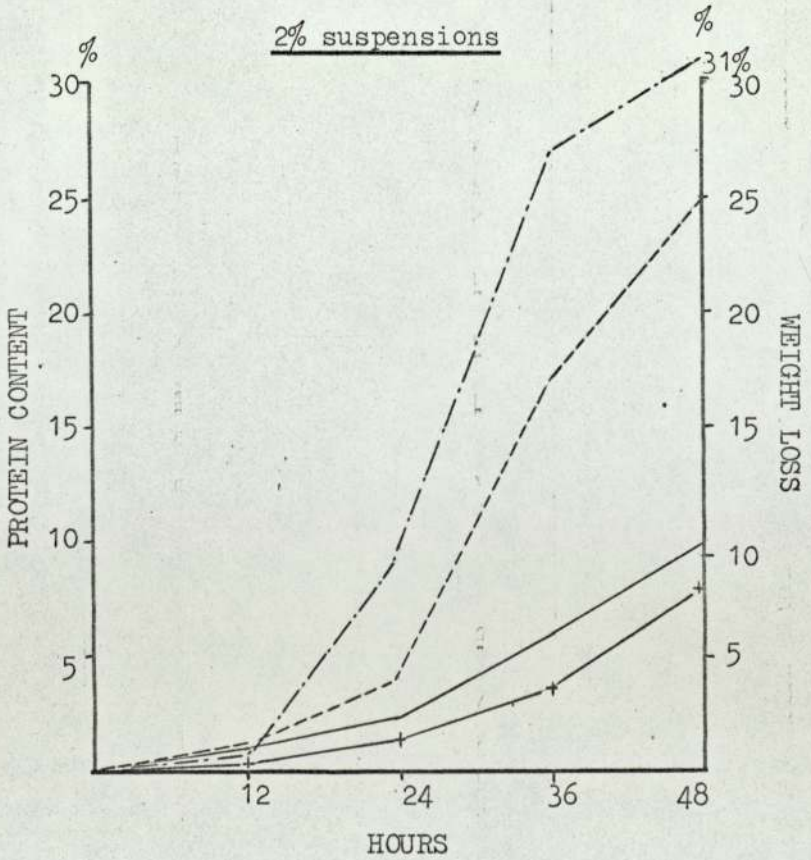
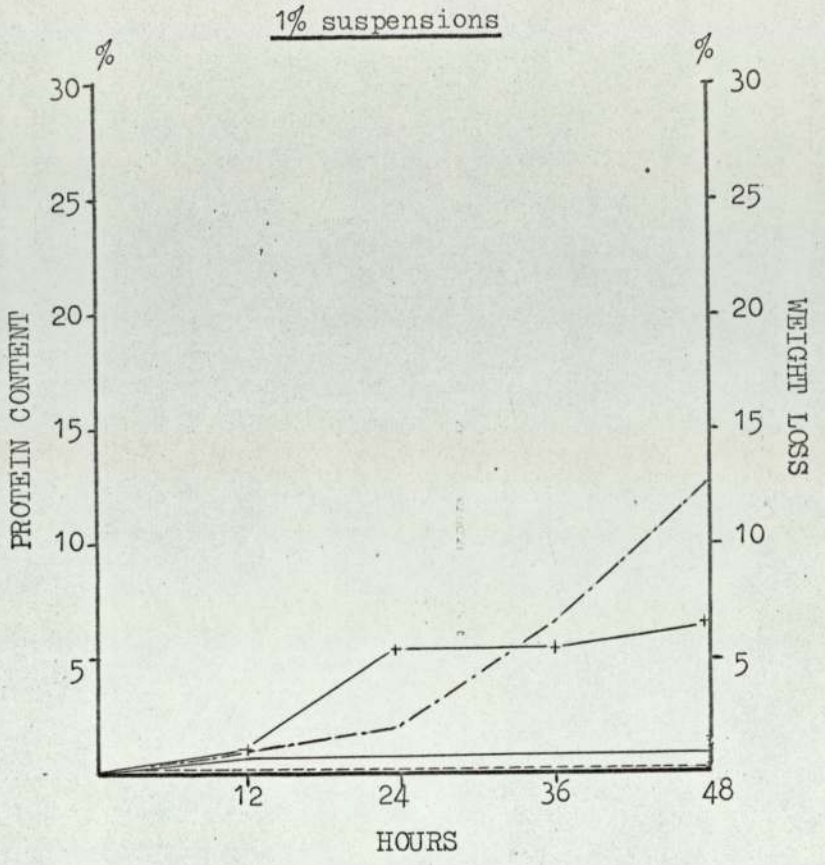


FIGURE 5.3(g) GROWTH OF Thielavia thermophila ON SUSPENSIONS OF BALL-MILLED NEWSPRINT.



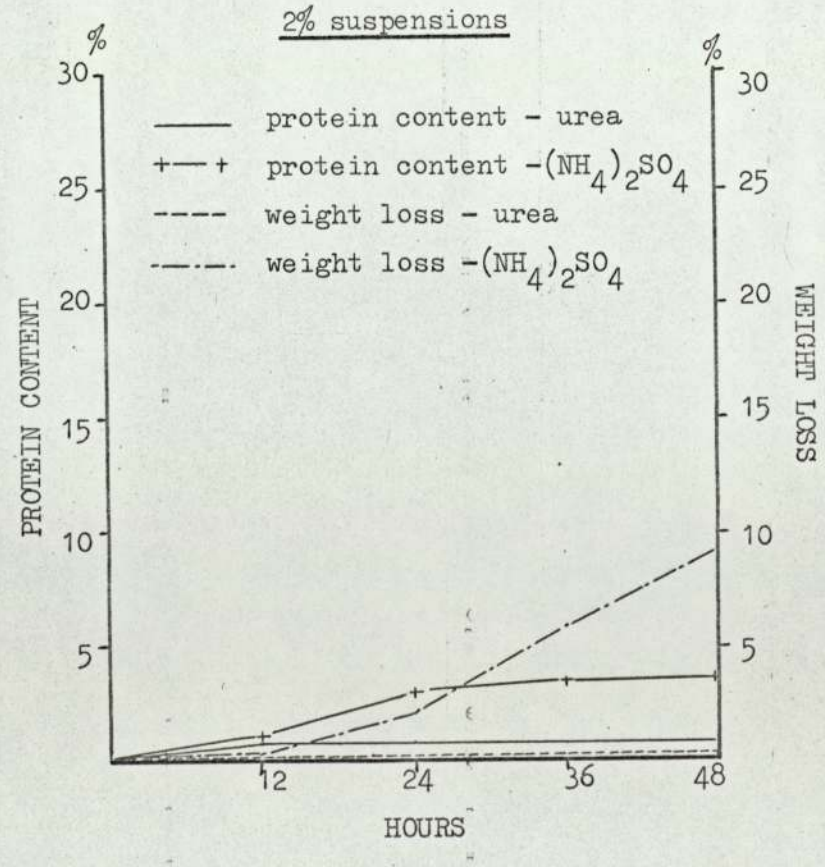
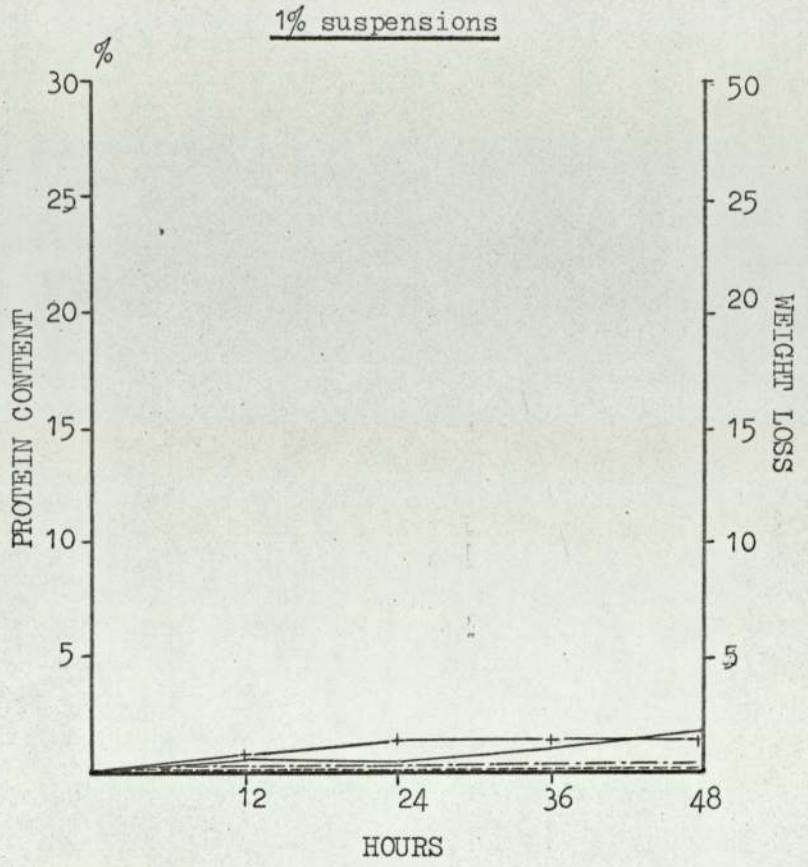
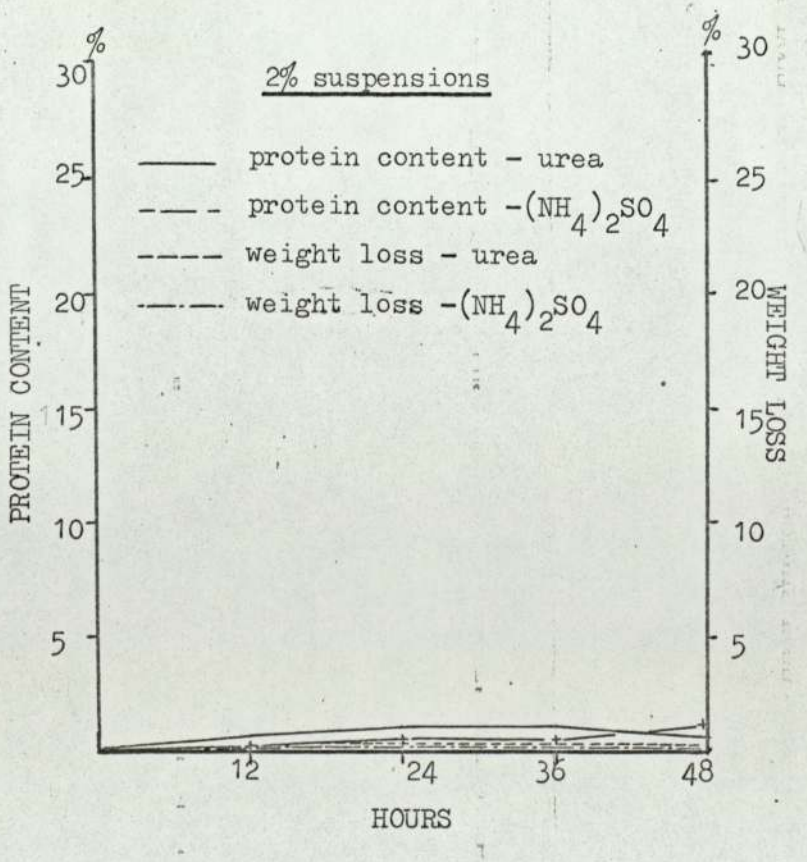


FIGURE 5.3(h) GROWTH OF Torula thermophila ON SUSPENSIONS OF BALL-MILLED NEWSPRINT.





In the shake-flask cultures containing the 1% suspensions, all of the fungi with the exception of Thielavia thermophila produced a much higher percentage of protein when urea was used as the main nitrogen source. In the case of Thielavia thermophila the percentage of protein produced after 48 hours of incubation with urea was 0.5% but when ammonium sulphate was used the percentage of protein produced rose to 6.2%. In the shake-flask cultures with the 2% suspensions only Chaetomium thermophile var coprophile, Sporotrichum thermophile var 1, Thielavia thermophila and Torula thermophila produced a greater percentage of protein and hence better growth when ammonium sulphate was used as the main nitrogen source.

The second parameter used to monitor the growth and the cellulolytic activity of the thermophilic fungi was the extent of the weight loss produced in the suspensions. With the exception of Sporotrichum thermophile var 1 the greatest percentage weight losses were recorded from the shake flasks containing the 1% suspensions. In the 2% suspensions containing ammonium sulphate and inoculated with Sporotrichum thermophile var 1 a 14% loss in weight was recorded compared with the 5% loss recorded from the corresponding shake-flask culture with the 1% suspension. In both the 1 and 2% suspensions, Chaetomium thermophile var dissitum, Humicola grisea, Humicola insolens and Sporotrichum thermophile var 1 (1% suspension only) all produced greater percentage weight losses when urea was the main nitrogen source. Chaetomium thermophile var coprophile, Sporotrichum thermophile var 2 and Thielavia thermophile produced greater percentage weight

losses, in both the suspensions, when ammonium sulphate was used as the main nitrogen source. Torula thermophila did not produce any weight losses in either of the suspensions with either of the nitrogen sources; the small amount of mycelium observed in the shake-flask cultures could have been produced using the small quantity of dextrose in the medium as a carbon source.

With the exception of the two varieties of Sporotrichum thermophile, which both produced a diffuse mycelium, all of the test fungi produced discrete pellets of mycelia usually about 3-5mm in diameter.

Using the parameter of protein production and weight loss as the indicators of growth, the most vigorously growing fungi from the shake-flask cultures were Chaetomium thermophile var dissitum, Chaetomium thermophile var coprophile, Sporotrichum thermophile var 2 and Humicola grisea. Maximum protein production, after 48 hours of incubation, was achieved by Chaetomium thermophile var dissitum which on the 1% suspension with urea gave a product with a protein content amounting to 20% of the total weight. After 36 hours of incubation the protein content had been 24%, and the decrease of 4% during the next 12 hours probably being caused by autolysis. Chaetomium thermophile var coprophile, Sporotrichum thermophile var 2 and Humicola grisea achieved protein productions of 18.6, 17.4 and 13.5% respectively on the 1% suspension with urea as the main nitrogen source. The greatest weight losses were produced by the cultures of Sporotrichum thermophile var 2 on the 1% suspensions, with

urea a weight loss of 29% was recorded and with ammonium sulphate a weight loss of 50% was recorded, but in this case the protein content was only 7%.

The two fungi selected for use in the tower fermenter were Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2. These were the fungi which in the shake-flask cultures achieved the production of the highest protein contents combined with the greatest amounts of degradation of the suspensions of newsprint.

(ii) Submerged fermentations

The rate of evaporation from the fermentation medium in the tower aerated at 1 vol/vol/min. at 48°C is shown in Fig. 5.4 from which it can be seen that the rate of evaporation was constant throughout the duration of the fermentations and after 24 hours just over 4% of the total volume had evaporated. The same aeration rate was used for each fermentation and any errors introduced by evaporation would be constant and therefore no attempt was made to correct the results for this small degree of error.

The results of the growth of Chaetomium thermophile var dissitum on 1% suspensions of ball-milled newsprint with the different concentrations of urea used are presented in Figs. 5.5 - 5.10. It was found that the different concentrations of urea had a marked effect on the progress of the fermentations. On the basis of total protein production and substrate utilisation the optimum concentration of urea was the one yielding 400gm of nitrogen/litre and giving a C:N

FIGURE 5.4 RATE OF EVAPORATION FROM FERMENTATION MEDIUM IN THE TOWER-FERMENTER AERATED AT 1 VOL./VOL./MINUTE.

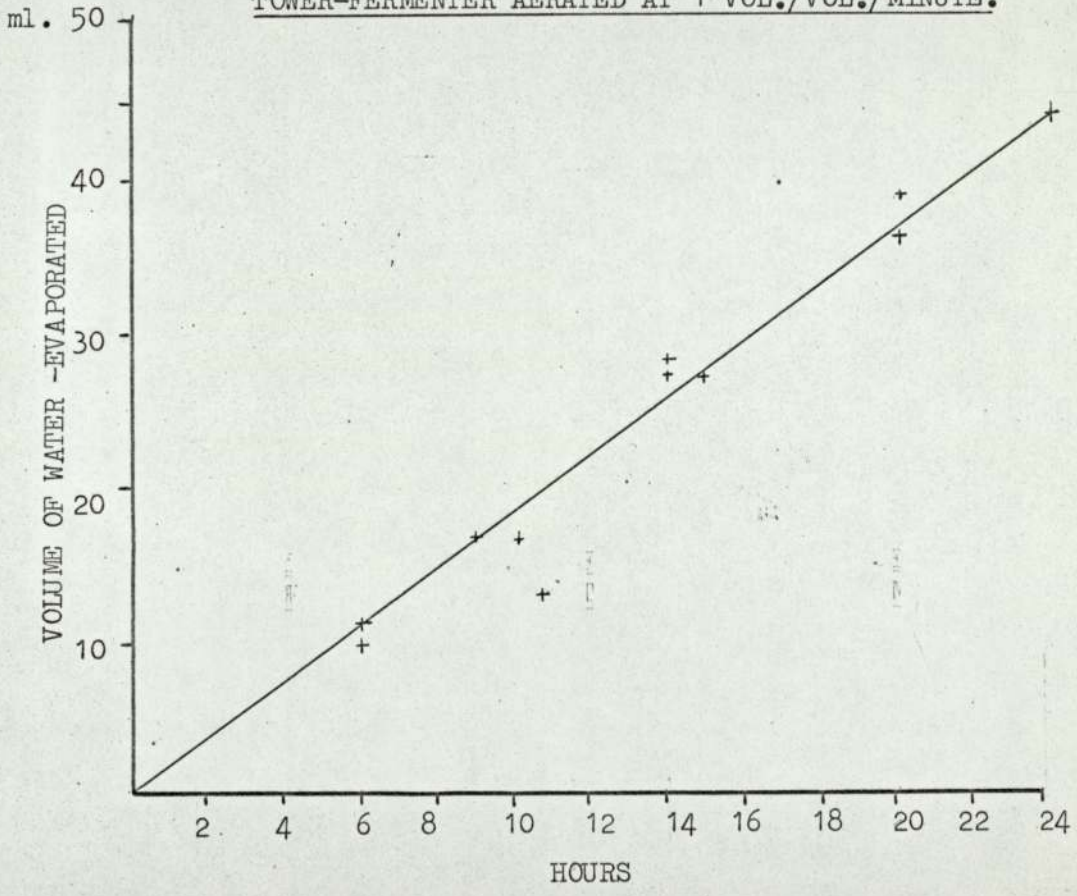
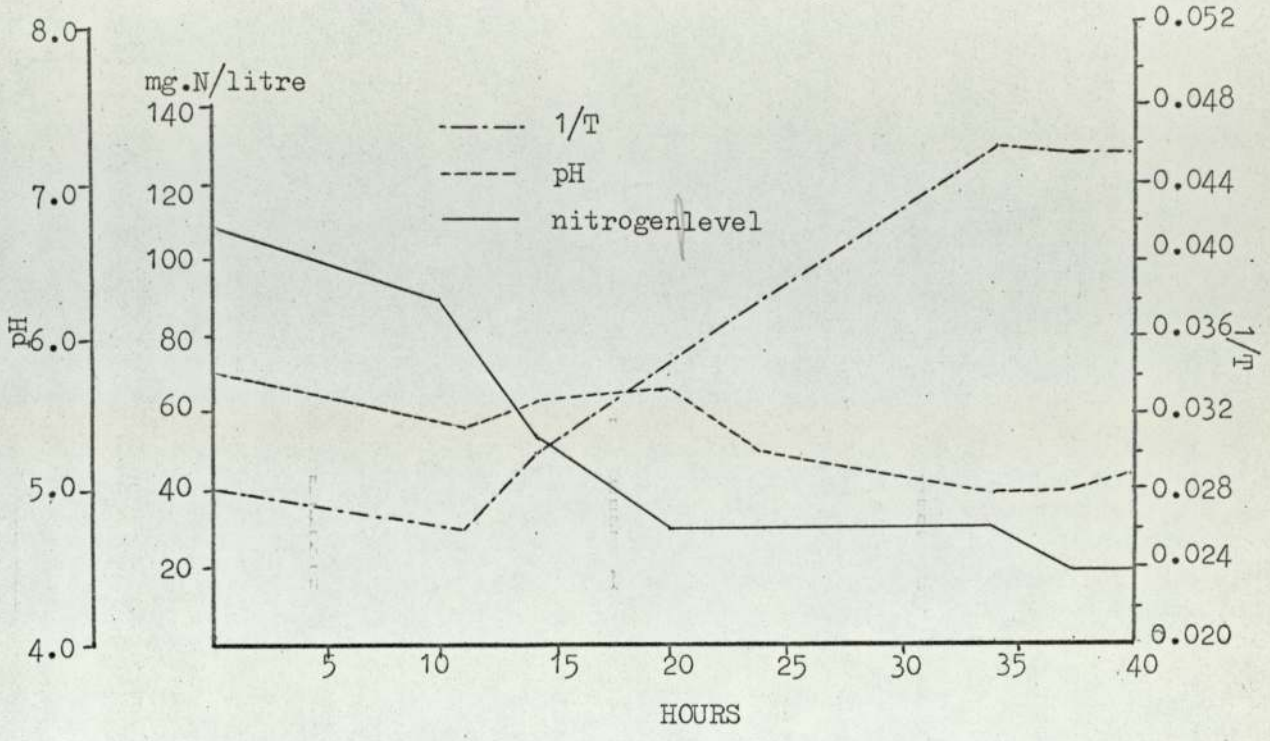


FIGURE 5.5 GROWTH OF Chaetomium thermophile var dissitum ON A 1% SUSPENSION OF B.M.N.P. IN A 1 LITRE TOWER FERMENTER WITH 100mg. OF NITROGEN/LITRE.

Part (A) Changes in pH, rate of flow and nitrogen content of medium.



Part (B) Changes in protein content of mycelium, protein production, G% mycelium and G.% suspension.

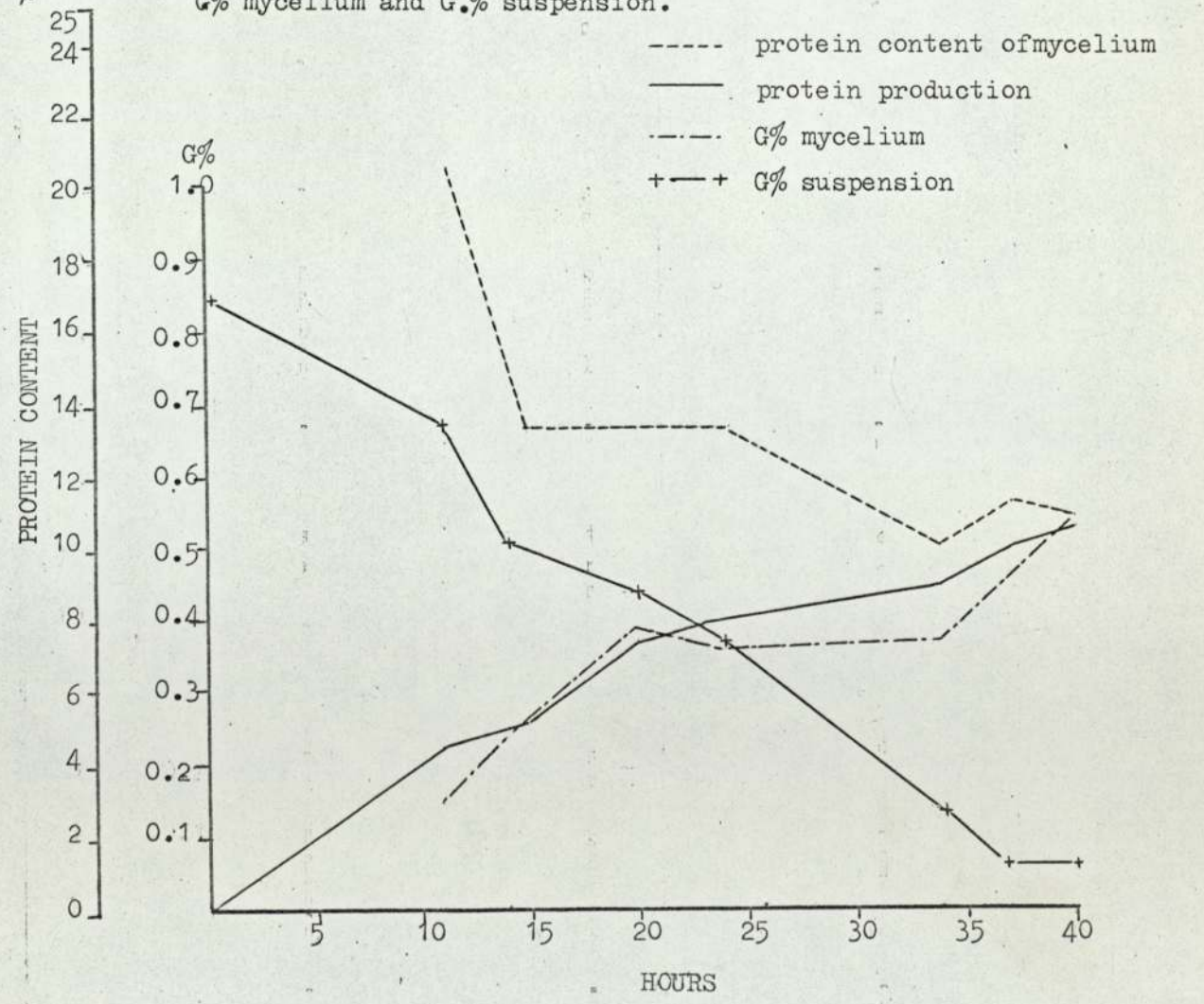
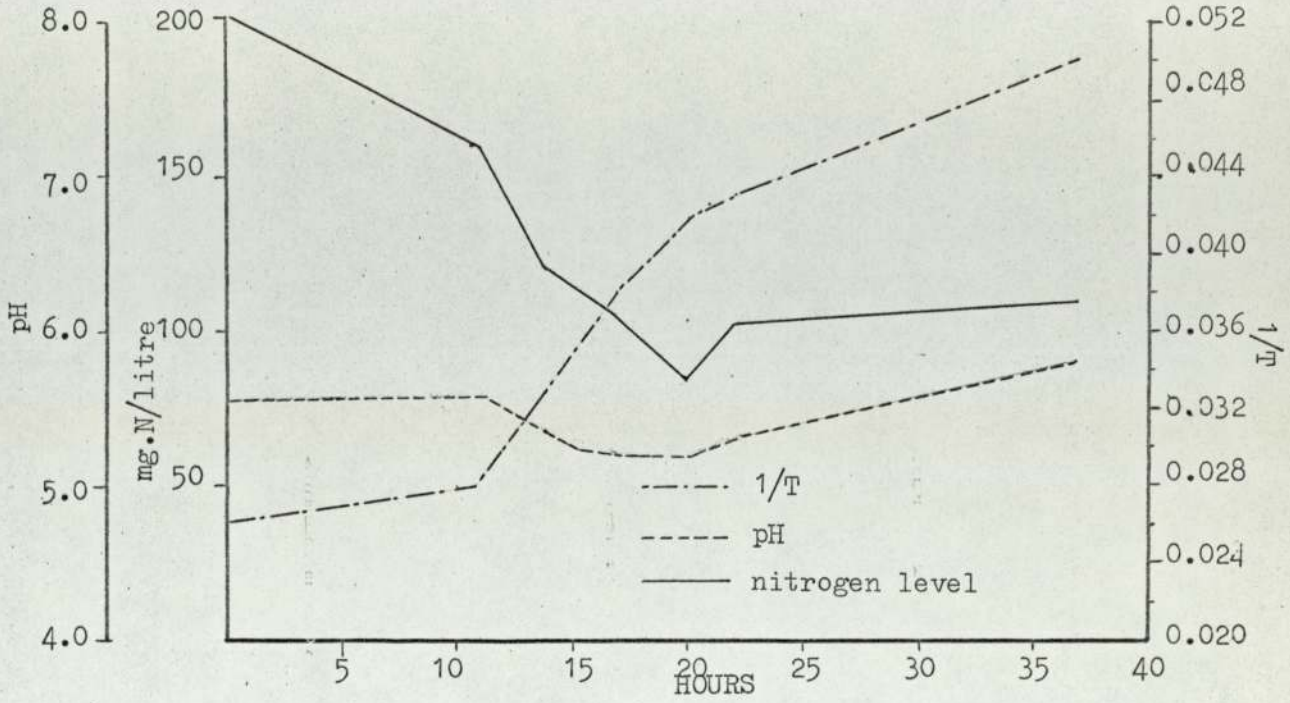


FIGURE 5.6 GROWTH OF Chaetomium thermophile var dissitum ON A 1% SUSPENSION OF B.M.N.P. IN A 1LITRE TOWER FERMENTER WITH 200 mg. OF NITROGEN/LITRE

Part (A) Changes in pH, rate of flow and nitrogen content of the medium



Part (B) Protein content of mycelium, protein production, G% mycelium and G% suspension.

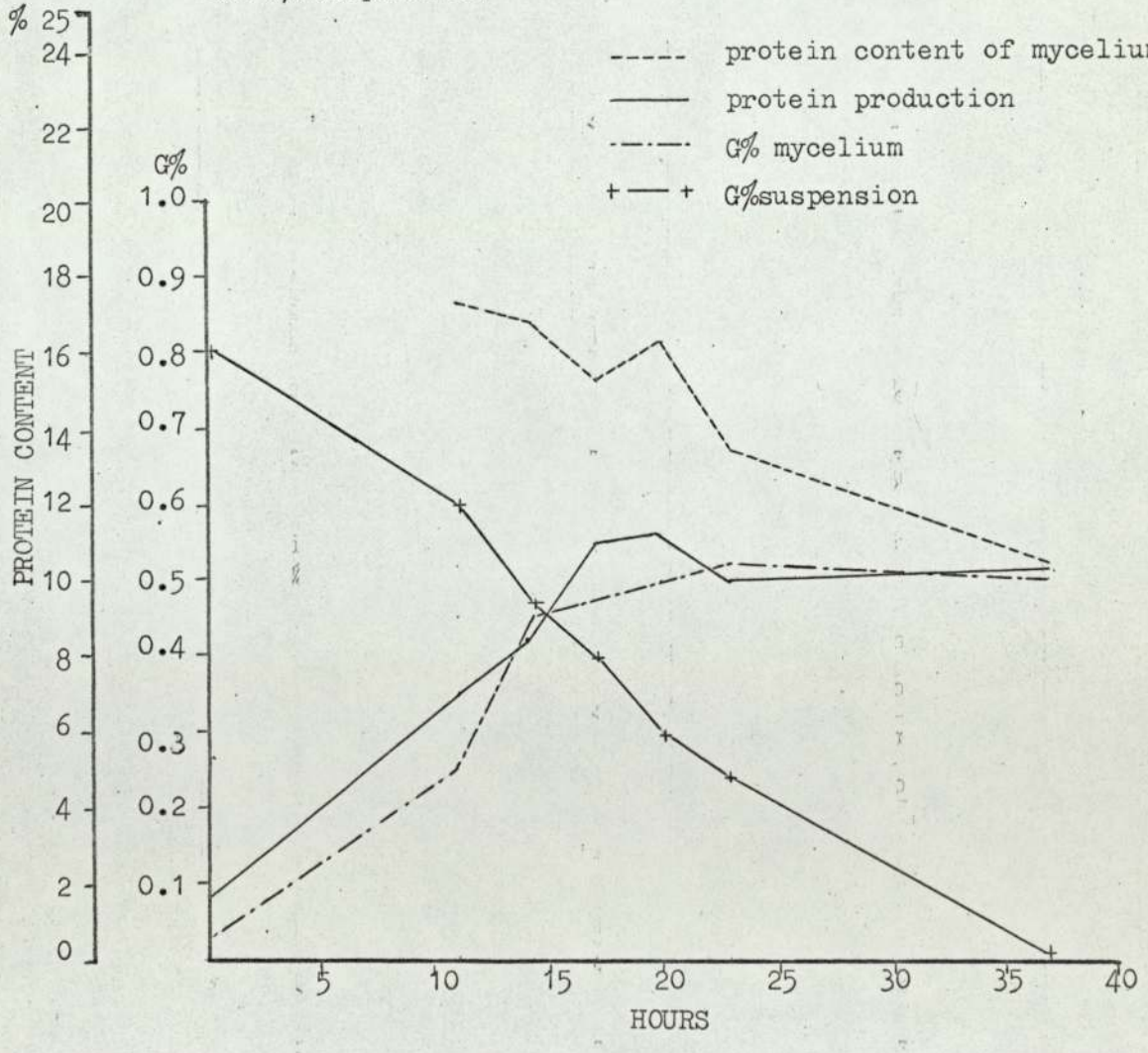
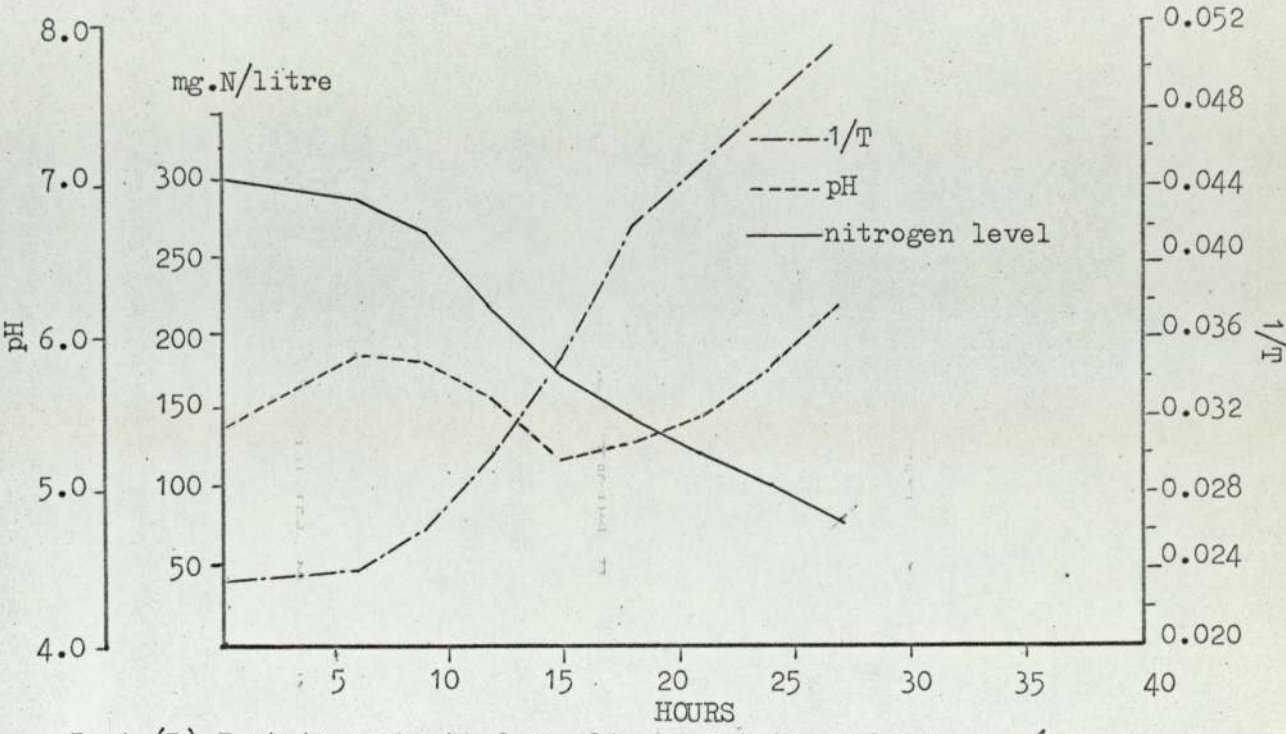


FIGURE 5.7 GROWTH OF Chaetomium thermophile var dissitum ON A 1% SUSPENSION OF B.M.N.P. IN A 1 LITRE TOWER FERMENTER WITH 300mg. OF NITROGEN/LITRE.

Part (A) Changes in pH, rate of flow and nitrogen content of the medium



Part (B) Protein content of mycelium, protein production, G% mycelium and G% suspension.

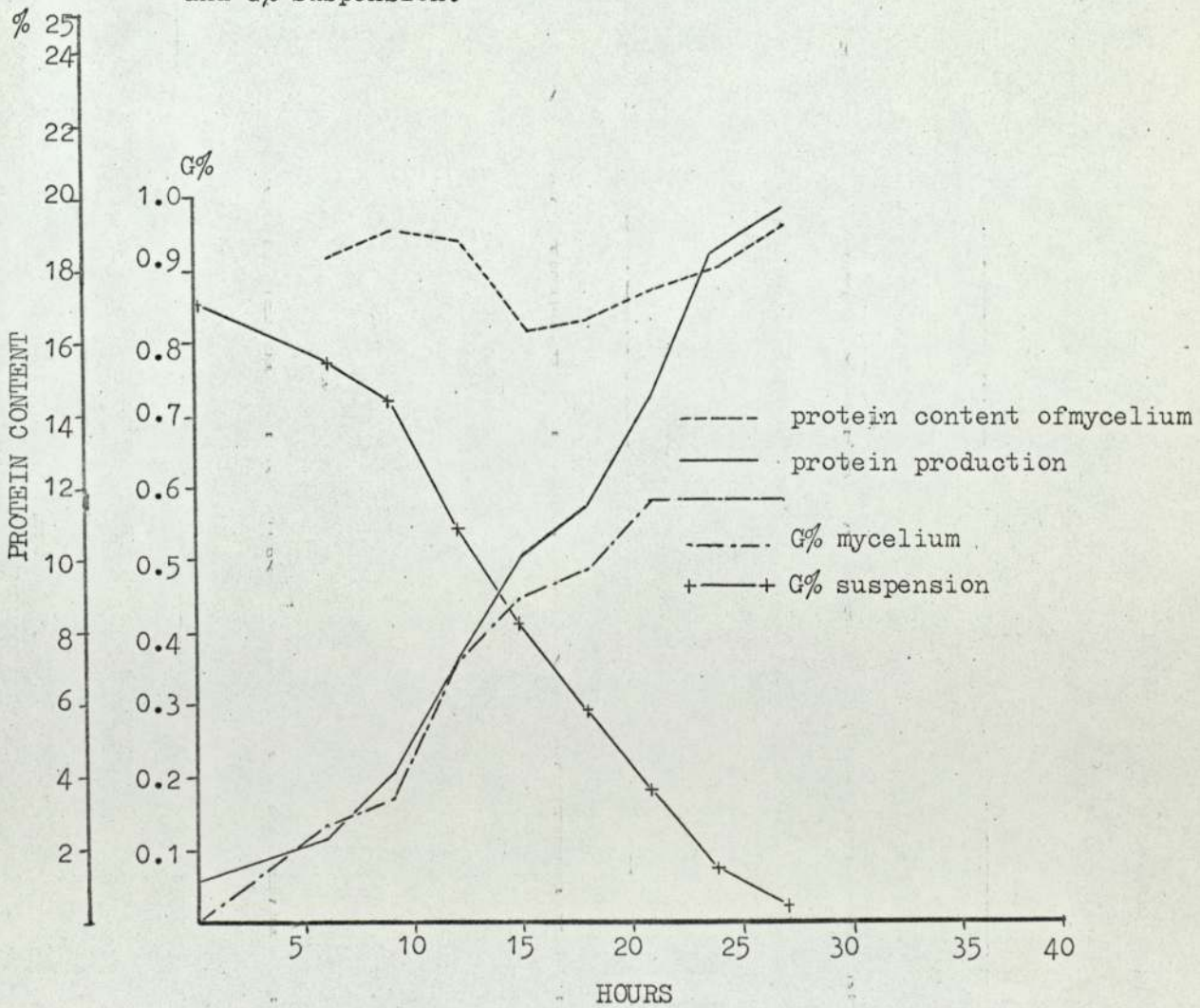
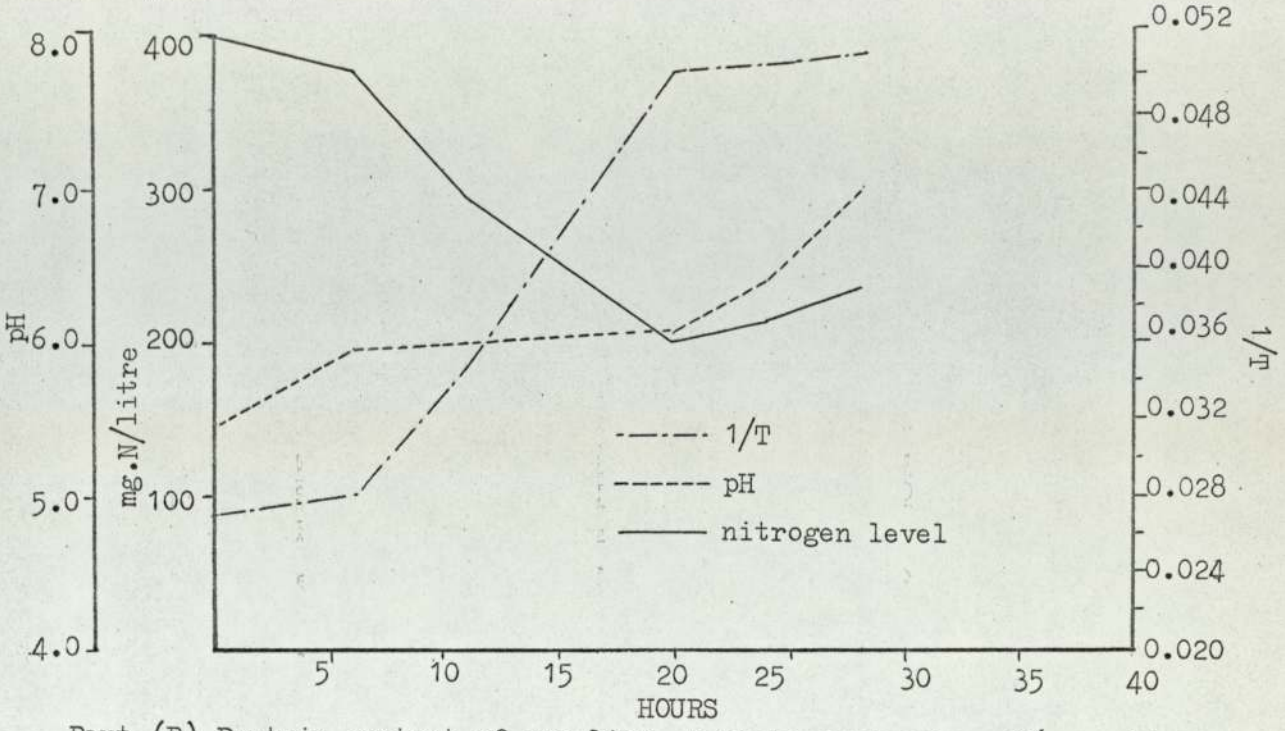


FIGURE 5.8 GROWTH OF Chaetomium thermophile var dissitum ON A 1% SUSPENSION OF B.M.N.P. IN A 1 LITRE TOWER FERMENTER WITH 400mg. NITROGEN/LITRE.

Part (A) Changes in pH, rate of flow and nitrogen content of the medium.



Part (B) Protein content of mycelium, protein production, G% mycelium and G% suspension.

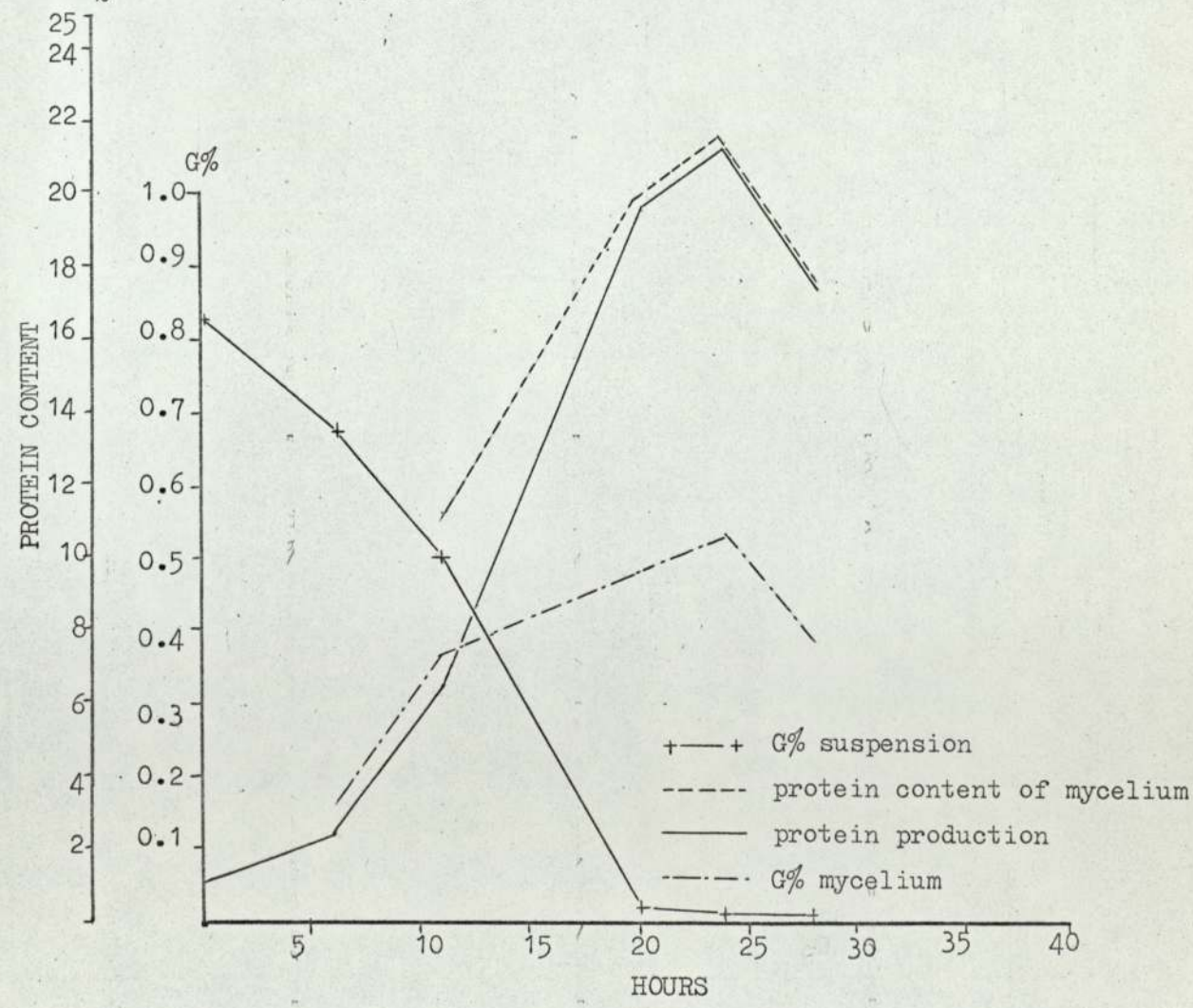
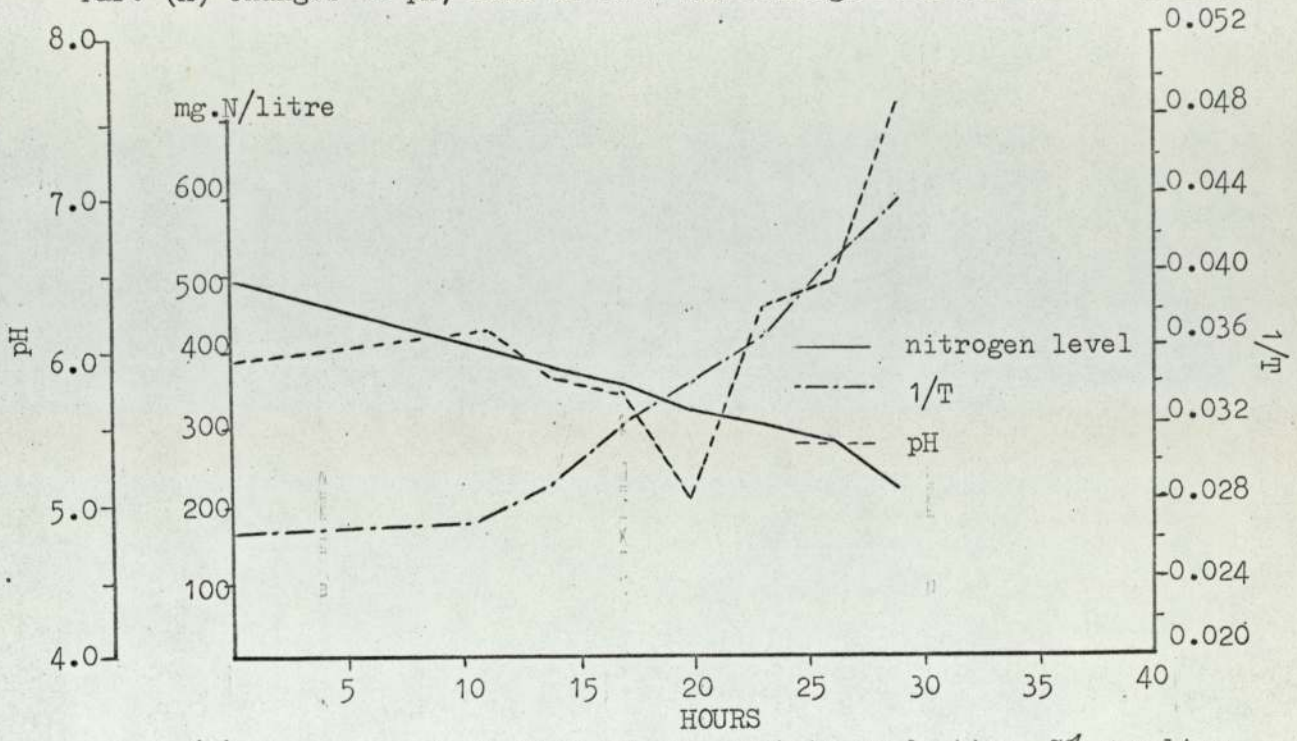


FIGURE 5.9 GROWTH OF Chaetomium thermophile var dissitum ON A SUSPENSION OF B.M.N.P. IN A 1 LITRE TOWER FERMENTER WITH 500 mg. OF NITROGEN/LITRE.

Part (A) Changes in pH, rate of flow and nitrogen content of the medium.



Part (B) Protein content of mycelium, protein production, G% mycelium and G% suspension.

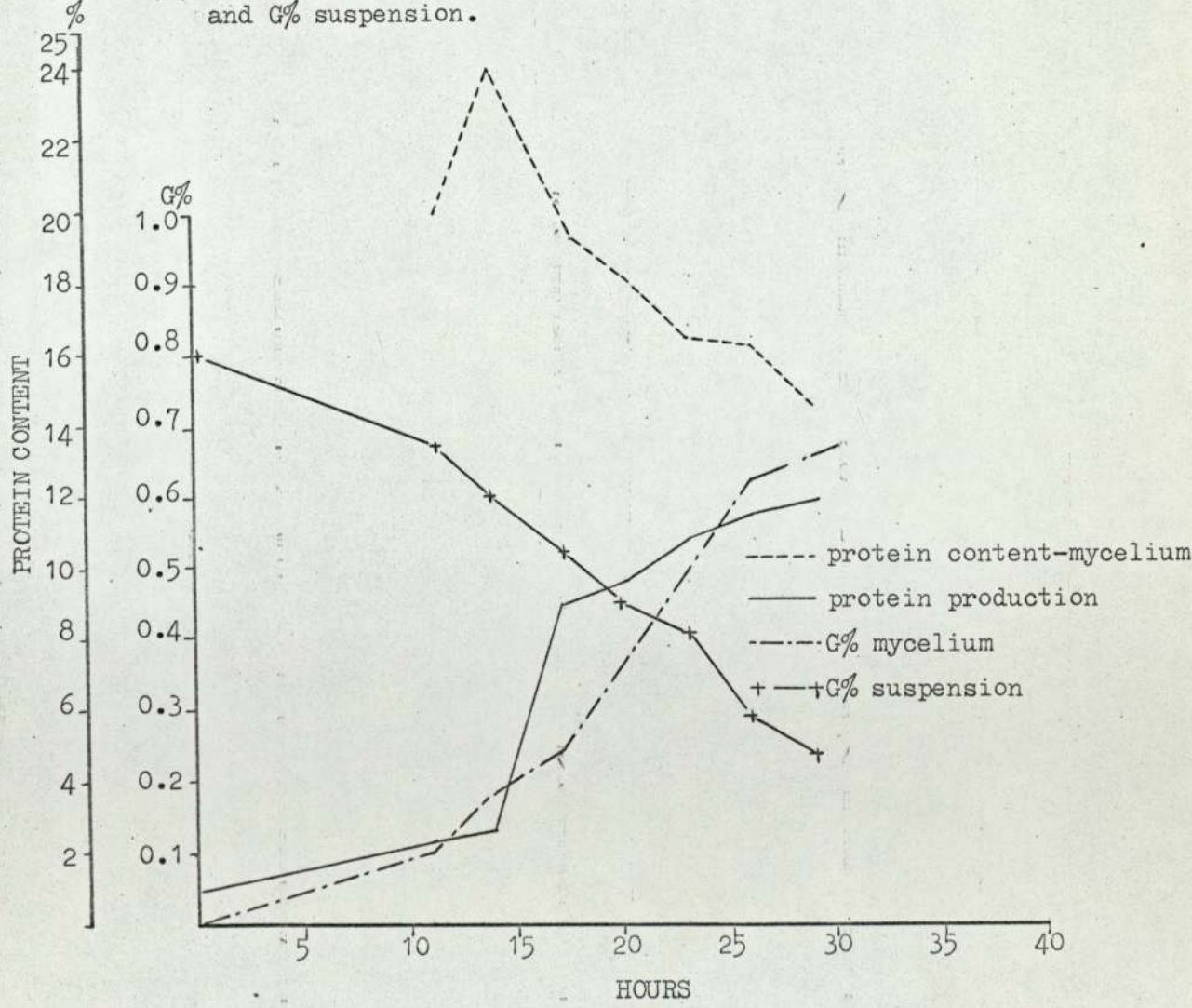
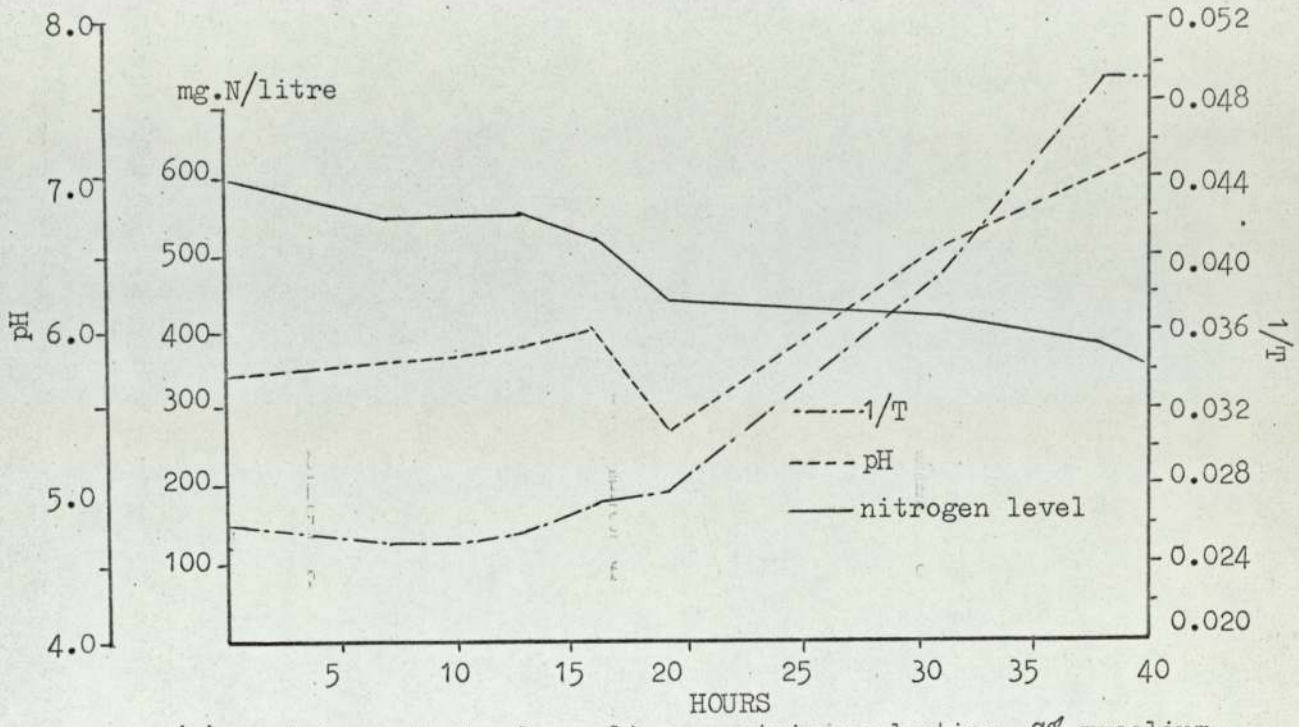
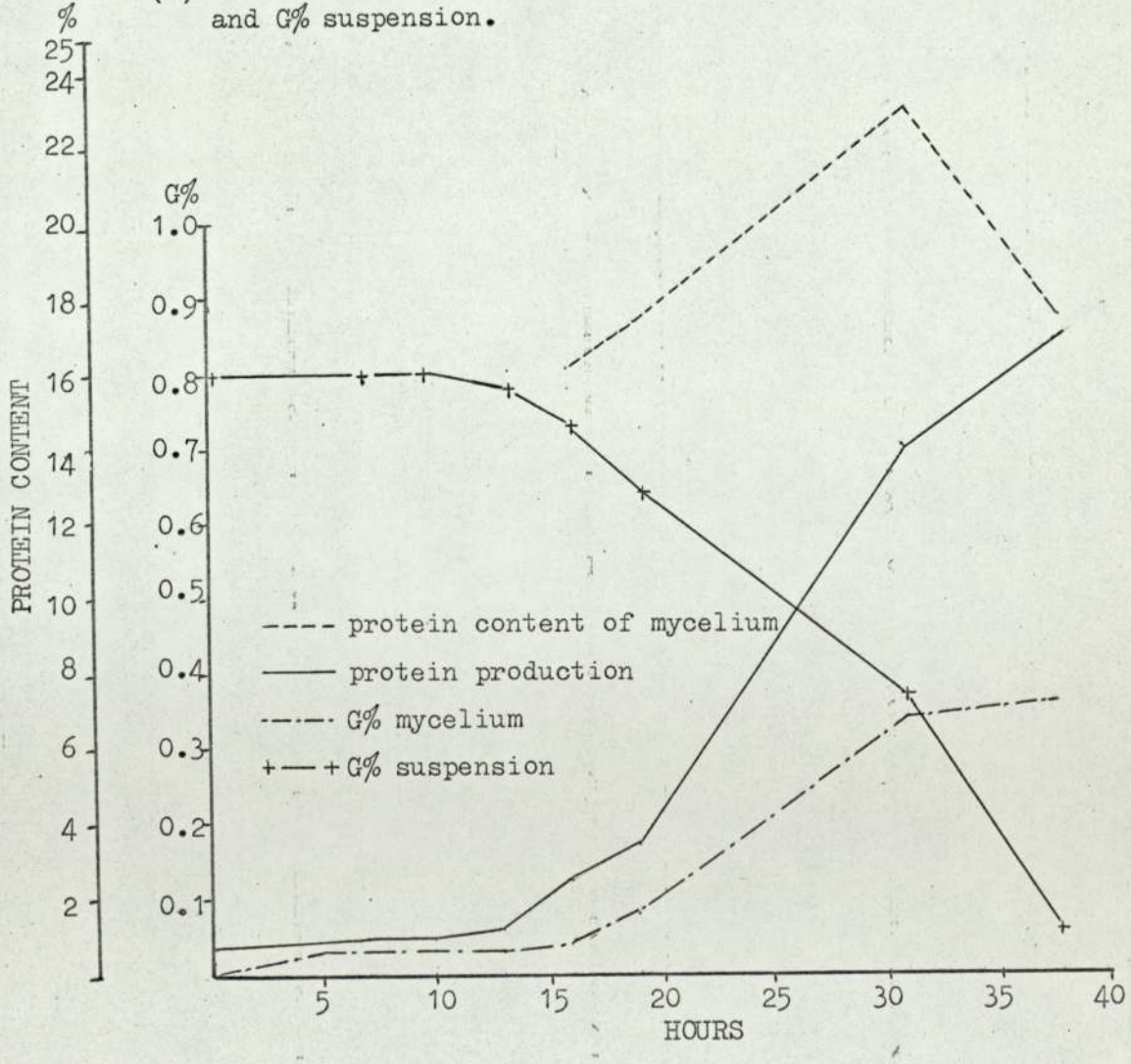


FIGURE 5.10 GROWTH OF Chaetomium thermophile var dissitum ON A 1% SUSPENSION OF B.M.N.P. IN A 1 LITRE TOWER FERMENTER WITH 600 mg. OF NITROGEN/LITRE.

Part (A) Changes in pH, rate of flow and nitrogen content of medium.



Part (B) Protein content of mycelium, protein production, G% mycelium and G% suspension.



ratio of 13:1. The results of the fermentation with 400mg of nitrogen/litre are presented in Fig. 5.8 which indicates that the fermentation was completed after 20 hours giving a total protein production (including mycelium and residual suspension) of 19.7% with the utilisation of 97.6% of the suspension of newsprint. The protein content of the mycelium produced was 20.0% almost identical to that of the whole product, showing that very nearly all of the product consisted of mycelium.

The progress of all the fermentations with Chaetomium thermophile var dissitum, after 20 hours is summarised in Table 5.1 with the consideration of the parameters of G.% mycelium (w/v), G.% suspension (w/v), total protein production, protein content of the mycelium and utilisation of the substrate and nitrogen source.

In all of the fermentations the morphology of Chaetomium thermophile var dissitum was the same as that observed in the shake-flask cultures, that is of discrete mycelial pellets which grew to a maximum size of 3-4mm in diameter. On completion of a fermentation the suspension was usually completely utilised leaving the mycelial pellets in a clear fermentation medium.

It has already been stated that the greatest degree of substrate utilisation and protein production occurred with a nitrogen content of 400mg/litre. However, the greatest production of mycelium occurred when the nitrogen content was 300mg/litre, see Fig. 5.7, equivalent to a C:N ratio of

Table 5.1 Effect of different concentrations of urea on the submerged fermentation of newsprint by Chaetomium thermophile var dissitum after 20hrs of growth

mg N/litre	G.% of Mycelium	G.% of residual suspension	Utilisation of substrate (%)	Total protein production (%)	Protein content of mycelium (%)	Nitrogen utilisation (%)
100 (C:N 53:1)	0.38	0.45	47.6	7.2	13.4	73.6
200 (C:N 26:1)	0.50	0.30	64.2	11.3	16.4	64.4
300 (C:N 18.1)	0.55	0.21	76.0	13.4	17.1	52.0
400 (C:N 13:1)	0.50	0.02	97.6	19.7	20.0	44.9
500 (C:N 11:1)	0.36	0.46	43.9	9.6	18.3	25.0
600 (C:N 9:1)	0.10	0.63	23.4	4.0	17.8	20.0

18:1. In this case the total protein production and the protein content of the mycelium were much lower. When the nitrogen content of the fermentation medium was either raised or lowered from 400mg/litre the fermentation was adversley affected.

When the nitrogen content was very much lower than 400mg/litre, that is at 100mg and 200mg/litre the total protein produced, even after prolonging the duration of the fermentation, decreased by almost a half from 19.7% to 9.9% and 10.56% (after 37 hours) respectively. The protein contents of the mycelium produced from the fermentations with 100 and 200mg of nitrogen/litre were 11.15 and 10.36% respectively, decreases of very nearly half from the maximum observed for the fermentation with 400mg of nitrogen/litre. The total amounts of mycelium produced in these two fermentations after 37 hours amounted to 0.46 G.% and 0.52 G.% and the amount of substrate utilised amounted to 93 and 97.5% respectively. Thus although the yields of protein were very much smaller with these low concentrations of urea, the fermentations did eventually reach completion with the utilisation of very nearly all the substrate. The final protein content of the mycelium produced in these fermentations was very low and was observed to steadily decrease as the fermentation progressed, indicating the probable occurrence of some degree of autolysis.

When the nitrogen content of the medium was higher than 400mg/litre, that is at 500 and 600mg/litre the fermentations again took much longer to utilise the substrate.

With the nitrogen contents of 500 and 600mg/litre the lag and acceleration phases, of the fermentation were very long lasting between 14 and 16 hours compared with 6-8 hours under optimum conditions and 9-11 hours for the lower concentrations of urea used. After 38 hours, the fermentation with 600mg of nitrogen per litre, Fig. 5.10, had utilised 92.7% of the substrate and the fermentation with 500mg of nitrogen per litre after 29 hours had utilised 70% of the substrate. In the latter case the fermentation was stopped prematurely and from Fig. 5.9 it can be seen that a greater degree of utilisation of the substrate would have occurred if the fermentation had been allowed to continue. The final yields of mycelium obtained from these fermentations were for the one with 600mg of nitrogen/litre after 38 hours, 0.46 G% and the one with 500mg of nitrogen/litre after 29 hours, 0.67 G%.

Under optimum conditions of 400gm of nitrogen per litre the generation time (Rhodes and Fletcher, 1966) of Chaetomium thermophile var dissitum in the fermentation of a 1% suspension of ball-milled newsprint (calculated from $P = Po\ e^{rt}$ where P is the cellular mass formed by time 't', Po the cellular mass present at the beginning of the logarithmic phase of growth and r is the rate of increase in cellular matter) was calculated to be 5.7 hours. In this case the plot of the G% mycelium in Fig. 5.8 did not accurately reveal the logarithmic phase of growth and the generation time was calculated from the plot of total protein production.

The maximum utilisation of the available nitrogen occurred in the fermentation with the smallest quantity of

nitrogen that is with 100mg of nitrogen per litre. A plot shown in Fig. 5.11 of the amount of nitrogen utilised against the amount of nitrogen available indicates that the percentage utilised is directly proportional to the total available.

A plot, presented in Fig. 5.12 of the effect of the nitrogen concentration on the production of protein quite clearly shows that a nitrogen content of 400mg/litre was optimum for the synthesis of protein and maximum utilisation of the substrate.

In all the fermentations, despite the lack of a buffer, the pH of the fermentation medium usually remained within the pH range of 5-7. The only exceptions to this occurred with the prolonged fermentations with the two highest concentrations of urea when in the later stages the pH rose above 7. The initial pH of the fermentation medium was usually between pH5.5 and 6.0 but during all of the fermentations, with the exception of the one with 400mg of nitrogen per litre, the pH would drop during the logarithmic phase of growth but rise again in the later stages of the fermentation.

The method of measuring the rate of flow of the separated suspension through a reverse flow viscometer to monitor the progress of each fermentation was found to give results about the state of the fermentation consistent with those of the other parameters monitored. In practice this method was found to give an accurate estimate of the progress of the fermentation.

FIGURE 5.11 RELATIONSHIP BETWEEN THE PERCENTAGE OF NITROGEN UTILISED AND THE TOTAL QUANTITY OF NITROGEN AVAILABLE.

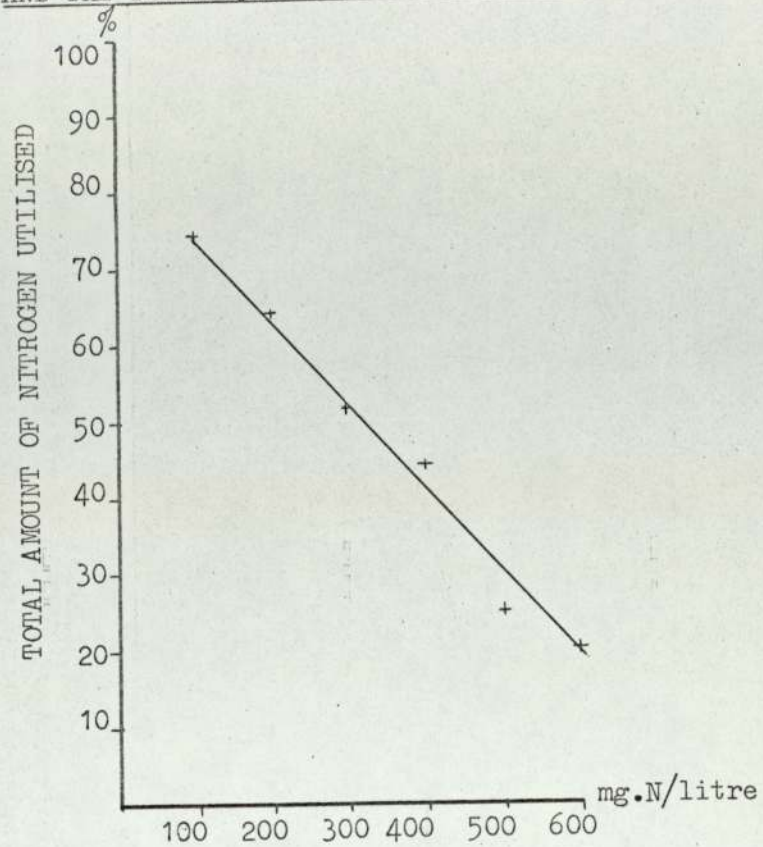
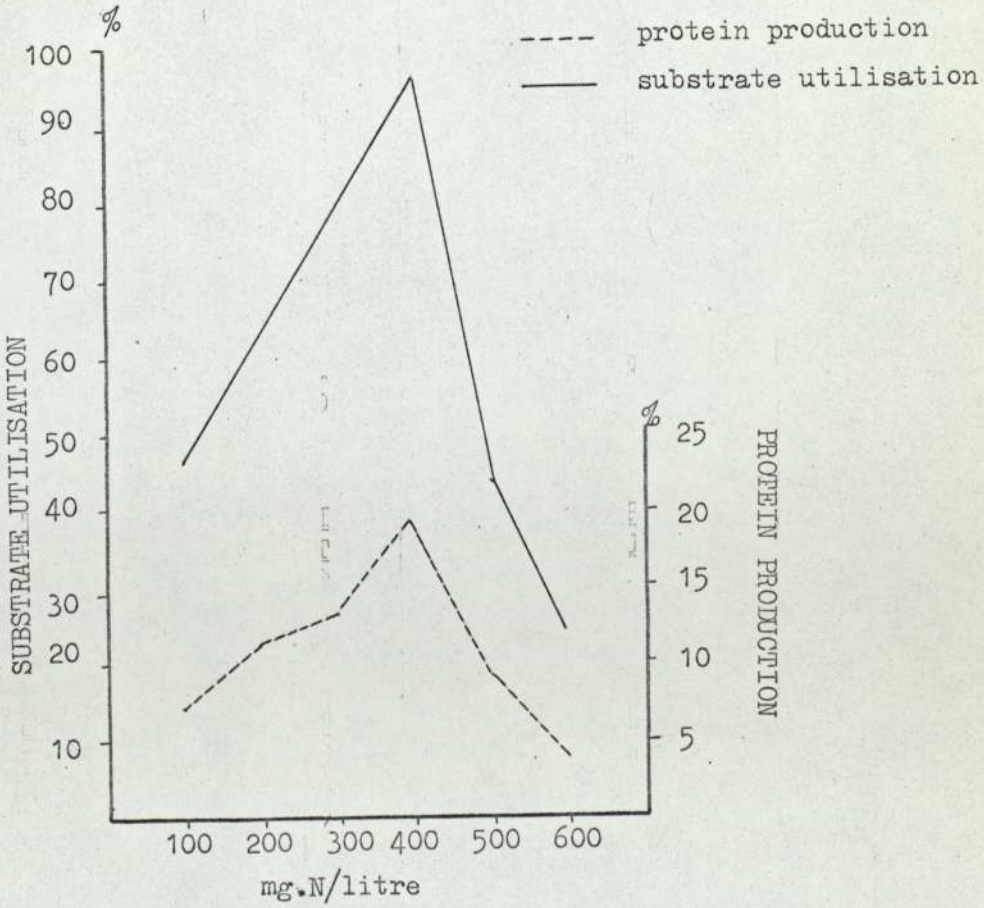


FIGURE 5.12 EFFECT OF CONCENTRATION OF UREA ON THE PRODUCTION OF PROTEIN AND THE UTILISATION OF THE SUBSTRATE.



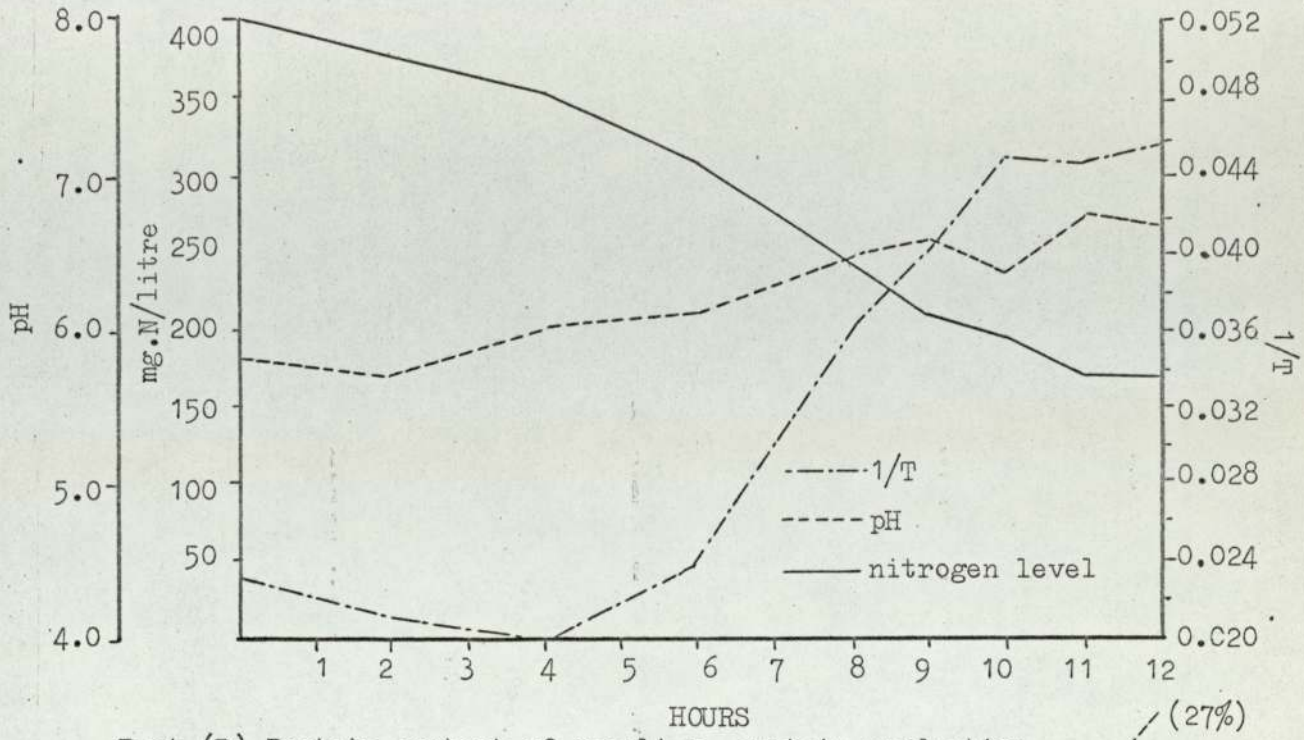
The results of the growth of Sporotrichum thermophile var 2 on the 1% suspensions of ball-milled newsprint and pure cellulose, in the 1 litre tower fermenter with urea as the main nitrogen source at a concentration of 400mg of nitrogen per litre, are presented in Figs. 5.13 and 5.14.

The growth of Sporotrichum thermophile var 2 on both substrates was extremely rapid. The morphology of Sporotrichum thermophile var 2 differed from that of Chaetomium thermophile var dissitum in not producing mycelial pellets but growing in the form of a diffuse and dense mycelium. The fermentation of the cellulose was judged on the basis of the rate of flow of the suspension through the reverse-flow viscometer to have been completed after 10 hours and the fermentation of the newsprint judged on the same basis was taken to have been completed after 11 hours. The results of these two fermentations are summarised in Table 5.2.

From the results presented in Table 5.2 it can be seen that Sporotrichum thermophile var 2 produced better growth on the suspension of cellulose, the yield of mycelium, the utilisation of the substrate and the total protein production all being greater and produced in less time than with the fermentation of the suspension of newsprint. The generation times for Sporotrichum thermophile var 2 were calculated (this time the calculations were made on the basis of the cell mass) and found to be, for the ball-milled cellulose (calculated between 2 and 8 hours) 1.5 hours and for the ball-milled newsprint (calculated between 4 and 8 hours) 1.6 hours. These generation times represent an extremely rapid rate of growth, especially for fungi.

FIGYRE 5.13 GROWTH OF Sporotrichum thermophile var 2 ON A 1% SUSPENSION OF B.M.N.P. IN A 1 LITRE TOWER FERMENTER WITH 400 mg.N/LITRE.

Part (A) Changes in pH, rate of flow and nitrogen content of medium



Part (B) Protein content of mycelium, protein production, G% mycelium and G% suspension.

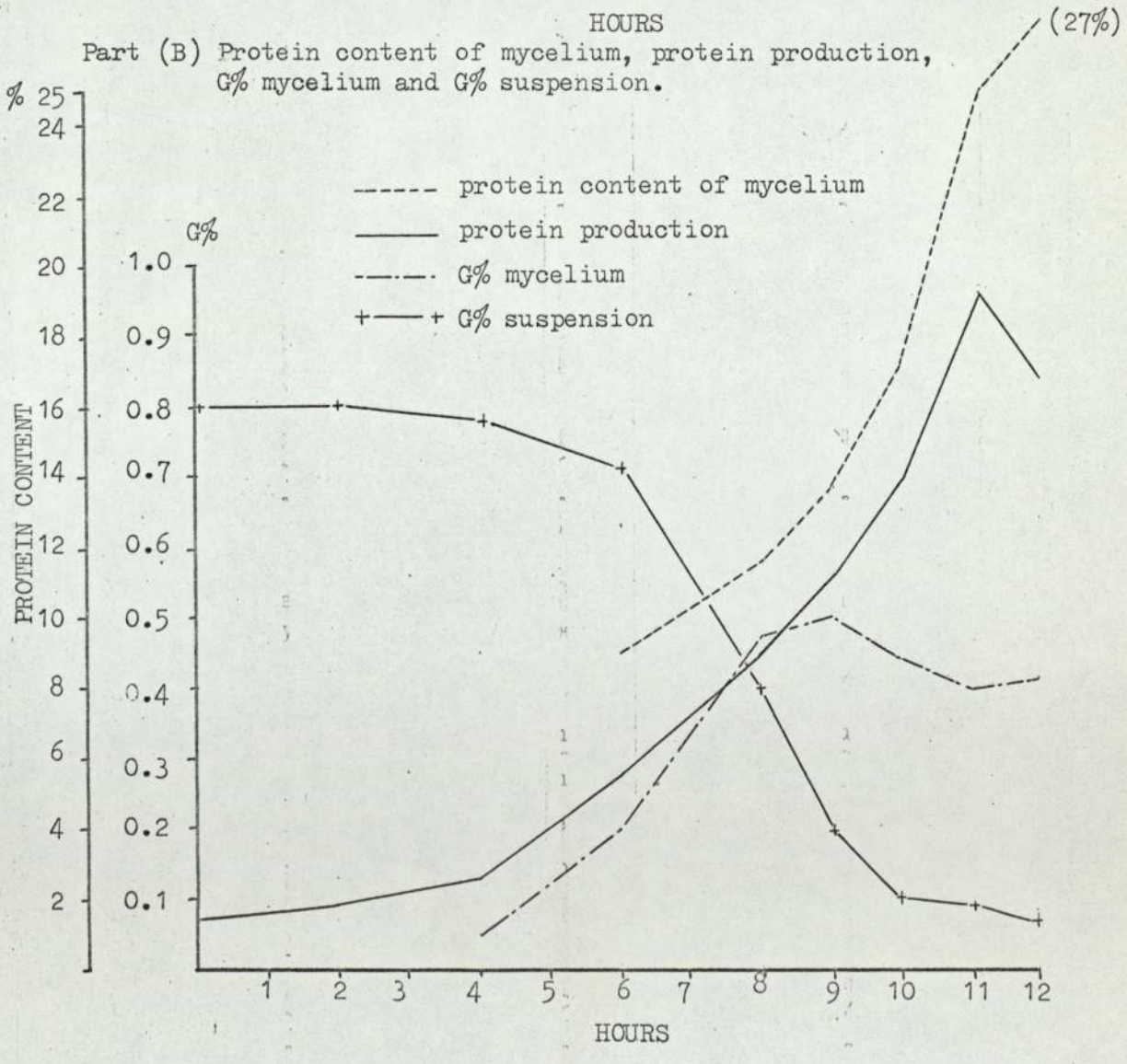
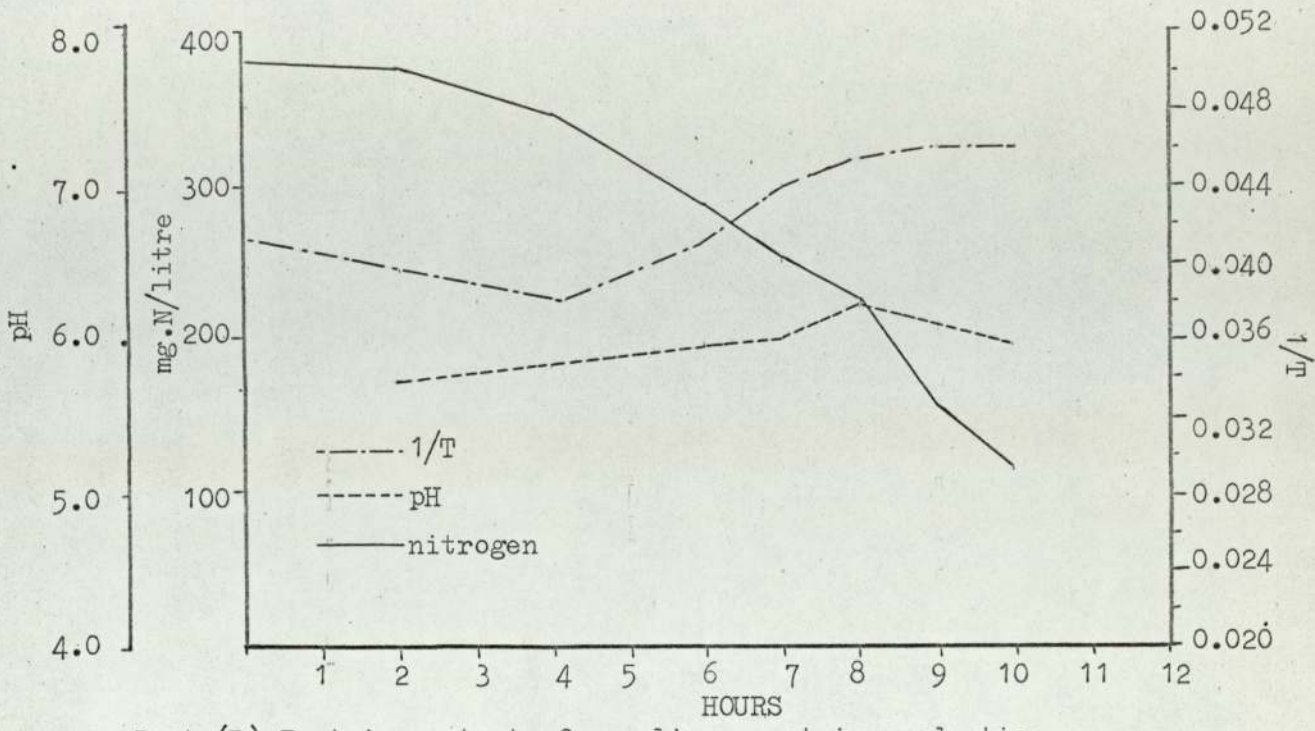


FIGURE 5.14 GROWTH OF Sporotrichum thermophile var 2 ON A 1% SUSPENSION OF BALL-MILLED CELLULOSE IN A 1 LITRE TOWER FERMENTER WITH 400 mg. OF NITROGEN/LITRE.

Part (A) Changes in pH, rate of flow and nitrogen content of the medium



Part (B) Protein content of mycelium, protein production, G% mycelium and G% suspension.

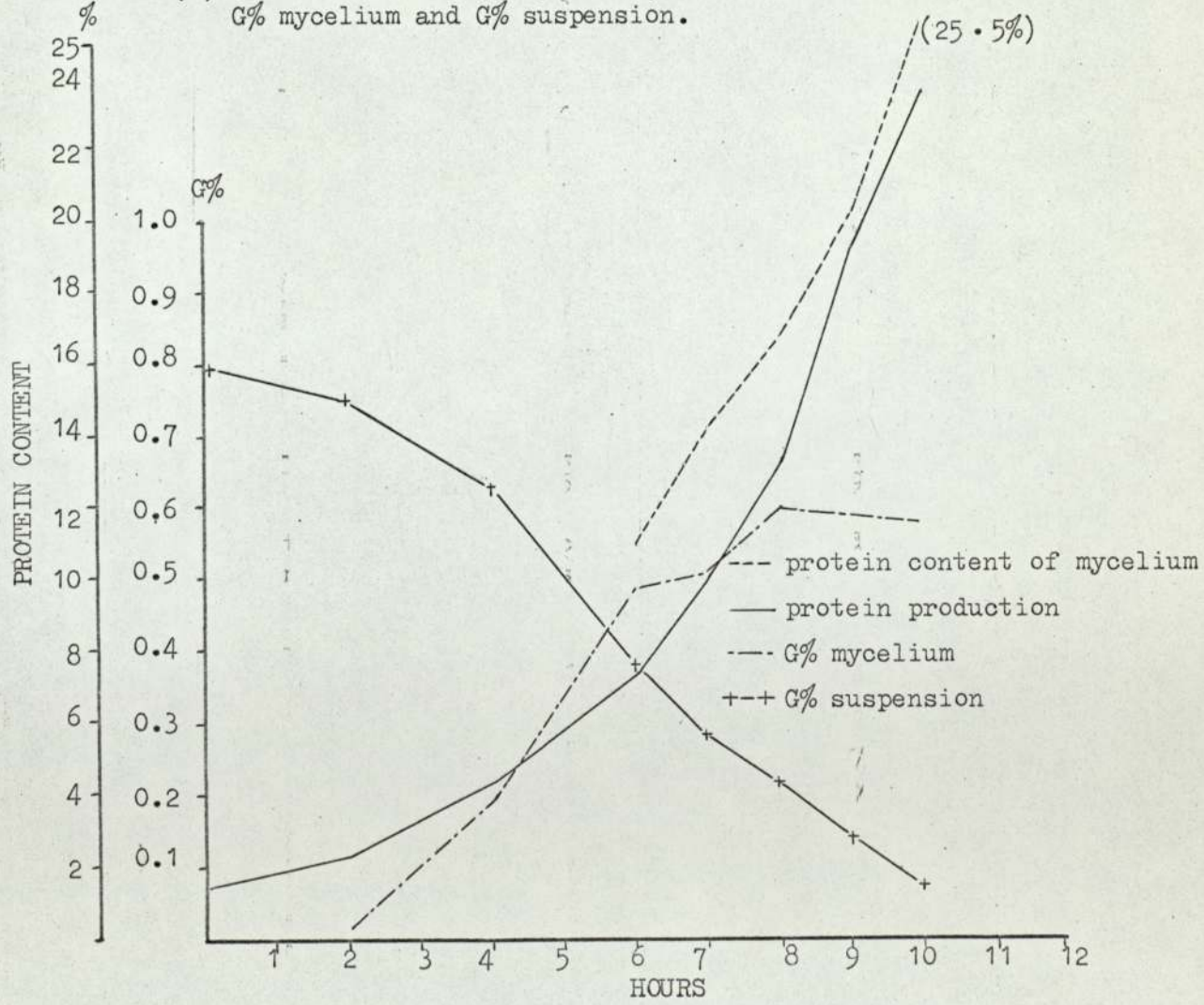


Table 5.2 The growth of Sporotrichum thermophile var 2 on 1 per cent suspensions of ball-milled cellulose and newsprint.

Substrate and duration of fermentation	G.% Mycelium	G.% Suspension (Remaining)	Utilisation of substrate (%)	Total protein production (%)	Protein content of mycelium (%)	Nitrogen utilised (%)
Newsprint 11hrs	0.41*	0.08	90	19.2	25.1	59.4
Cellulose 10hrs	0.57	0.02	97.4	23.5	25.5	64.4

* After 9 hours of fermentation 0.50 G% of mycelium was recorded equalling the yield obtained from Chaetomium thermophile var dissitum.

In the later stages of both fermentations the mycelium produced had become so dense, that individual air bubbles could not pass through it but instead they coalesced forming a large slug of air which passed very rapidly through the fermenter. The maximum concentrations of mycelium were produced, in the cellulose fermentation after 8 hours and the newsprint fermentation after 9 hours. After these times the quantities of mycelia present decreased in both cases until the fermentations were stopped. In these later stages a bright yellowish green pigment was formed by Sporotrichum thermophile var 2.

During the first 4 hours of both these fermentations the rates of flow of the cellulose and newsprint suspensions recorded with the viscometer decreased, inspite of repeated filtrations of the samples through 80 and even 100 mesh sieves to ensure the complete removal of any mycelium present. On close inspection of the suspensions some 'swelling' of the fibres could be observed and the suspensions became slightly gelatinous. After 4 hours the rates of flow of the suspensions began to increase in the normal manner.

In both fermentations, the pH as with the fermentations with Chaetomium thermophile var dissitum remained in the range of pH 5-7.

Discussion

The results from the shake-flask cultures confirmed that some of the thermophilic fungi were highly cellulolytic.

The synthesis of protein and the extent of the degradation of the newsprint achieved by Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2 was particularly noticeable and within 48 hours equalled the results obtained by any of the mesophilic fungi, used by Chahal and Gray (1969, 1970), after 5 days incubation on delignified wood pulp. The growth attained by seven out of the eight thermophilic fungi used in these shake-flask cultures demonstrated that urea is a better nitrogen source than 'ammonium' nitrogen for the fungal synthesis of protein from cellulose substrates and thus agrees with the results of Chahal and Gray (1969) and Jones and Irvine (1972).

In the tower fermenter the utilisation of the newsprint and the growth of Chaetomium thermophile var dissitum and in particular Sporotrichum thermophile var 2 was very good. Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2 had generation times of 5.7 hours and 1.6 hours respectively on the 1% suspension of newsprint. The shortest generation times recorded for fungi grown on any substrate are in the order of 4 to 12 hours (Litchfield, 1968). Clearly then, Chaetomium thermophile var dissitum is a fast growing fungus but Sporotrichum thermophile var 2 exhibited a growth rate comparable to those of bacteria and yeasts. The generation time achieved by Sporotrichum thermophile is even more remarkable when the substrate, a mixture of mainly hemicelluloses, cellulose and lignin, is considered, for it is generally agreed in the literature that these are the most resistant of all naturally occurring substrates. Yield factors (Spicer, 1971), calculated from the weight of the

mycelium formed divided by the weight of the suspension utilised, were for Chaetomium thermophile var dissitum on the 1% suspension of newsprint 0.61 and Sporotrichum thermophile var 2 on the same substrate 0.56 and on the pure cellulose 0.75. Spicer (1971) reported that with fermentations for the production of biomass it is of paramount importance to get the yield factor as high as possible and with carbohydrates this factor does not usually exceed 0.5 because of the energy the organism requires to grow. The high yield factors obtained by the two thermophilic fungi on a carbohydrate substrate demonstrated an unusually high degree of efficiency in converting substrate to mycelium.

The analysis of the newsprint, used in all of these fermentations, revealed that it had a hemicellulose content of 14.58%, a cellulose content of 44.19% and a lignin content of 30.53%. From the submerged fermentation of the 1% suspension of newsprint it was found, under optimum conditions of nitrogen concentrations, that Chaetomium thermophile var dissitum on completion of the fermentation had utilised 97.6% of the substrate and Sporotrichum thermophile var 2 90% of the substrate. These figures quite clearly indicate that both of the thermophilic fungi must have attacked and degraded substantial quantities of the lignin in the newsprint as well as utilising the cellulose and hemicelluloses. The mycelial pellets of Chaetomium thermophile var dissitum were carefully washed to ensure that all traces of the residual suspension were removed from the volume of fermentation medium enclosed by their growth, microscopical examination of these washed pellets revealed that all the residual suspension

had been removed. Sporotrichum thermophile var 2 did not form pellets but grew in the form of a diffuse mycelium and therefore could not have enclosed any residual suspension containing lignin from the newsprint. Foster (1949) reported on the evidence that lignin-like materials have been extracted from the mycelium of fungi. These lignin-like substances are not chemically identical with the lignin of higher plants, like that found in newsprint, but have general properties sufficiently in common to be considered as lignin-like. In fungi the neglible methoxyl (OCH_3) content (which is considered typical of all lignins) of the lignin-like material has been interpreted as verification that true lignin does not exist in the fungi but is an artifact produced by the methods of extraction. In the present investigation it is conceivable that the lignin from the newsprint could be incorporated in the fungal mycelium but this would necessitate its solubilisation by extracellular enzymes in the same way as insoluble cellulose is metabolised by fungi. Trojanowski and Leonowicz (1969) reported that in the biodeterioration of wood by fungi lignin is degraded by the action of fungal enzymes but neither the mechanism of the cleavage of the lignin molecule nor the enzyme systems involved are understood.

The faster growth rate exhibited by Sporotrichum thermophile var 2 on the pure cellulose suspension probably indicated the inhibitory effects of the lignin content of the newsprint.

Merrill and French (1968) reported the occurrence of a lignolytic isolate of Sporotrichum pruinosum which was

found to be abundant in some wood fibre products. In pure culture decay studies it was reported that this fungus could cause rapid and extensive strength losses in fibre-boards accompanied by a consumption of holocellulose, alpha cellulose and lignin. They further state that the decomposition by this fungus resembled that caused by white rotting fungi and that it caused strength losses comparable with Lenzites trabea a brown rotting species.. On investigating the temperature relationships of their isolate, Merrill and French (1968) found that its optimum range for growth was 33-41°C but that it could grow albeit slowly at 50°C. It would appear that their isolate was not a true thermophile because it could grow at temperatures below 20°C. However, it is particularly significant to note the confirmed lignolytic ability of another member of the genus Sporotrichum capable of growth at elevated temperatures usually associated with thermophilic fungi. Merrill and French concluded that Sporotrichum pruinosum is probably the asexual stage of a Basidiomycete but unfortunately they do not include a description of their isolate and a comparison could not be made with the vigorously growing isolate of Sporotrichum thermophile used in the present investigation.

In studies of the types of organisms involved in the deterioration of stored wood chips thermophilic and thermotolerant fungi have been isolated in the areas of the piles where high temperatures were recorded and it has been found that a greater degree of degradation occurred in the wood chips in these areas of the pile than in the cooler regions (Shields, 1967). In a study of the deterioration of soft-

wood chips Shields and Unligil (1968) found that Sporotrichum thermophile (in association with unidentified thermotolerant Basidiomycetes) was chiefly responsible for the microbial deterioration of the wood chips in the hot areas of the pile. Tansey (1971) isolated seven species of thermophilic fungi from wood chip piles including Chaetomium thermophile var dissitum and Sporotrichum thermophile and concluded that the thermophilic fungi contributed to the biodeterioration of the wood chips. Pure culture studies with the thermophilic fungi grown on wood blocks have shown that these fungi can cause the degradation of wood (Ofosu-Asiedu & Smith, 1973). Thus it can be seen that some species of the thermophilic fungi have a widespread occurrence on wood in self-heating environments and can cause significant degradation of the wood. It would seem likely from the evidence presented above and that obtained in the present investigation that some species of the thermophilic fungi have lignolytic ability.

The results of the submerged fermentation of newsprint by Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2 indicate that the thermophilic fungi could prove valuable industrial micro-organisms in fermentations of this type. The very fast growth rate and high yield factor obtained by Sporotrichum thermophile var 2 is of particular significance. The problem of fermenting cellulose in this way in the past has been the time taken to breakdown the cellulose, usually necessitating the use of some form of pre-treatment such as acid hydrolysis which in an open economy has always proved to be a prohibitive expense. The results of this study have shown that Sporotrichum thermophile var 2 can

cause an extremely rapid and complete breakdown of cellulosic substrates, even when substantial quantities of lignin are present, and could therefore be used for the industrial fermentation of cellulose. Spicer, (1971) stated that the difficulties of fermenting cellulose, hemicelluloses and lignin were the probable specialised nature of such fermentations and the long duration which would make them economically unviable. These arguments could not be applied to the fermentation of cellulose by Sporotrichum thermophile var 2 where the costs of using a 'traditional' centralised system of fermentation would be comparable with those for the fermentation of more amenable substrates such as molasses, although this does not necessarily imply that such a process would be economically viable at the present time.

The biggest drawbacks to this type of fermentation are the high capital costs of the sophisticated equipment involved, the high running costs produced by the need for conditions of complete sterility and the labour costs necessitated by the high level of technical ability required to run fermentations of this type. These were the decisive factors in deciding to undertake research and develop a process free from these problems of the traditional fermentation. The shake-flask cultures and tower fermentations have shown that under optimum conditions two species of the thermophilic fungi can almost completely degrade newsprint. Thus in a system developed to overcome the economic disadvantages of the traditional methods of fermentation, but using less than optimum conditions for growth, these fungi should be able to convert newsprint to a feedstuff containing protein, vitamins and digestible carbohydrates.

CHAPTER 6 Pages

Chapter 6

THE SOLID SUBSTRATE FERMENTATION OF A CELLULOSIC SUBSTRATE

Introduction

The technique of solid substrate fermentation has been used in many areas of the world for centuries in the small scale batch fermentation of cereals beans and rice. Many of the fermentations have been such primitive and simple processes, in which mainly fungi are used, that the process can continue only because the operators of the fermentation found by chance, conditions favourable for the growth of the appropriate fungi (Hesseltine, 1965). There are many of these traditional types of food fermentations the better known ones being the preparation of tempeh, ragi, sufu, miso, tea fungus, shoyu and ang-kak. The substrates and organisms involved in these fermentations, along with their geographical locations are presented in Table 6.0.

Most of the traditional food fermentations are of a similiar nature, although some do include two stages of fermentation, with the purpose of producing a palatable, digestible and nutritious food from a substrate of poor nutritional qualities. Fermentations of this type are desirable mainly because of the synthesis of enzymes by the fungi present. For example in the tempeh fermentation the cultures of Rhizopus sp. which are used are strongly proteolytic and this results in the rapid breakdown of the proteins in the soybeans to their constitutive amino acids which are easily digestible by man. In the miso-

*Table 6.Q. Food fermentation based on a process of solid substrate fermentation

Name	Organisms used	Substrate	Nature of Product	Area where article of commerce
Tempeh	Rhizopus oligosporus	Soybeans	Solid	Indonesia and vicinity
Sufu	Principally:- Actinomucor elegans and Mucor sp.	Soybeans	Solid	China, Formosa
Ragi	Mucor, Rhizopus and yeast	Rice	Solid	Indonesia, China
Tea Fungus	Two yeasts and Acetobacter sp.	Extract tea and sucrose	Liquid	Eastern Europe, Russia, parts of the Orient
Miso	Aspergillus oryzae Saccharomyces rouxii	Rice and other cereals plus soybeans	Paste	Japan, China and some other parts of the Orient
Shoyu	Aspergillus oryzae, Lactobacillus, Hansenula and Saccharomyces	Soybeans, wheat	Liquid	China, Japan, Philippines and some other parts of the Orient
Ang-kak	Monascus purpureus	Rice	Deep red pigmented solid	China, Indonesia and Philippines

* Taken from Hesseltine (1965).

fermentation, both the protein of the soybean and the starch of the rice are hydrolysed by the enzymes of the strains of Aspergillus oryzae employed. The digestion of the plant constituents in these food fermentations is reported by Hesseltine (1965) to enhance the digestibility and nutritional qualities of the food consumed. In the preparation and fermentation of soybeans in the production of tempeh, certain undesirable odours, flavours and factors such as trypsin inhibitors are neutralised and increases in the contents of riboflavin, niacin and vitamin B12 have been reported by Hesseltine (1965). Additional advantages of these traditional fermentations include the addition of pleasant flavours to the food and the changes that often occur in the physical state of the substrate, for example in the production of tempeh a solid cake is made from loose particles of soybeans.

This traditional small scale, type of fermentation, has been adopted by the Japanese for the production of enzymes on solid substrates (Hesseltine, 1965). The koji fermentation, a preliminary fermentation for making the enzymes needed in soybean fermentations such as shoyu and miso, in Japan traditionally used wooden trays on which cereals such as rice and wheat were allowed to ferment, after mixing with koji fungi as an inoculum. This preliminary fermentation has been developed and automated to produce the large quantities of enzymes required in traditional soybean fermentations. The solid substrate, rice, is inoculated automatically, fermented in layers and periodically turned whilst passing through fermentation rooms with

moisture and temperature continuously controlled.

Apart from the composting of organic wastes the main applications of solid substrate fermentation are the production of foods, as described above and some enzymes and other fungal metabolites. The production of microbial amylases and proteases by the solid substrate fermentation of wheat bran with Aspergillus oryzae is an example of this type of fermentation. The rate of enzyme production from such solid substrate fermentations has been reported by Meyrath (1965) to be very much higher than with comparable liquid culture techniques.

Hesseltine (1972) has recently developed another type of solid substrate fermentation in which the substrate is continuously agitated. This technique was developed by Hesseltine (1972) for the production of a range of metabolites such as aflatoxin and ochratoxin. After the discovery of aflatoxin and the realisation of its importance in the contamination of ground nuts, a method of producing it in substantial quantities for feeding trials was required. Hesseltine (1972) found that if polished rice kernels, inoculated with Aspergillus parasiticus, were incubated on a reciprocal shaker then very substantial increases in the yields of aflatoxin were obtained when compared with those from submerged cultures and static solid substrate fermentations. The method has since been adopted for producing a number of secondary metabolites from different genera and species of fungi with exceptionally good results (Hesseltine, 1972). This type of fermentation

was found to be characterised by the production of hardly any mycelium and Hesseltine (1972) reported that after 48 hours of incubation only small white areas of mycelium were observed on each kernel and at no time did sporulation occur on the substrate. In the static cultures of the same solid substrate the fungus was reported to sporulate profusely.

Hesseltine's research with solid substrates indicated that the physical characteristics of the substrate are of particular importance, especially those affecting the free circulation of air. The particle size and the moisture content of the substrate were the most important characteristics in this respect. The addition of a limited amount of water in order to obtain a satisfactory moisture content is advantageous in one respect because it lowers the chances of bacterial contamination. Hesseltine (1965) found with the process of tempeh fermentation that the incidence of bacterial contamination was correlated with the substrate moisture content.

The technique of solid substrate fermentation most nearly reproduces those conditions under which fungi grow naturally, that is at a gas/liquid interface on a solid and accordingly this type of technique, when used with fungi, can have several advantages over the more usual methods of fermentation. The physical organisation and mode of growth of the fungi is ideally suited for the process of solid substrate fermentation. The intrusive hyphal growth habit of fungi is a great advantage in the colonisation of a solid substrate when compared with the unicell-

ular morphology of the bacteria. The intrusive growth habit of fungi leads directly to one of the major advantages of solid substrate fermentation, the elimination of the need for absolute sterility. The growth of the desired fungus is promoted by the use of a heavy inoculum and the manipulation of environmental conditions such as the use of a high temperature of incubation. Meyrath (1965) reported that the confinement of infection is characteristic of the technique of solid substrate fermentation because infections remain localised. This statement is especially true of bacterial contaminants and in the case of fungal contaminants these can only become widespread throughout the substrate by growing over the substrate, an eventuality which is unlikely to occur because of the heavy inoculum of the fungus selected for the fermentation.

The space required by solid substrate fermentation is small relative to the yield of the product, much less water being used than in submerged fermentations. During the process of fermentation additional water and ancillary nutrients can be added which because of the evaporation of water from the surface of the substrate will be perfused throughout the substrate replenishing those exhausted by the fungus. This phenomenon is particularly marked in fibrous cellulose substrates and is the same principle used by Malik and Eggins (1970) to study the fungal ecology of cellulose deterioration.

The equipment required by the technique of solid substrate fermentation is minimal and this was illustrated perfectly by the traditional food fermentations, previously

described, which were successfully developed by people without any knowledge of microbiology. In comparison the technique of submerged fermentation often requires sophisticated and expensive apparatus and highly trained personnel for successful operation. Submerged fermentations often require large quantities of mycelial inoculum requiring special steps for its preparation whereas solid substrate fermentations can simply be inoculated with spores or by seeding with part of the product from the previous fermentation. Seeding is more difficult to carry out in submerged fermentations because of the conditions of absolute sterility which must be maintained. The products of solid substrate fermentations in most cases can be dried and then incorporated directly into diets for human or other animal consumption whereas with submerged fermentations expensive steps of harvesting and processing are required before the final product is obtained.

Perhaps the most significant advantage of the technique of solid substrate fermentation is that it can be used for the upgrading of cheap insoluble substrates, such as straw, in simple processes operated by unskilled labour. At the present time large quantities of straw are destroyed by incineration (Fulbrook et al, 1973) when it could be profitably upgraded to an animal feedstuff by a process of solid substrate fermentation, similar to the traditional food fermentations outlined above. A majority of the developing countries, where the need for feedstuffs is most acute, produce large quantities of insoluble cellulosic wastes such as grain stalks and hulls and bagasse etc., which

could also be upgraded to feedstuffs. Before such substrates could be used in submerged fermentations expensive pre-treatments to either solubilise or finely grind the substrate would be required.

Although the technique of solid substrate fermentation is simple and economical to operate, as demonstrated by the traditional food fermentations, it does have one major drawback compared with the technique of submerged fermentation of particulate suspensions which can be illustrated by reference to the present investigation. With the submerged fermentation of suspensions of ball-milled newsprint, the substrate was very finely divided and presented a large surface area for microbial attack. With a system of solid substrate fermentation there is a limit to the minimum particle size which can be used because of the need for interstitial air spaces between the particles to allow aerobic fermentation to take place. This restriction regarding particle size also limits the surface area which can be exposed to microbial attack and ultimately controls the rate and degree of degradation of the substrate.

It has previously been stated that the purpose of the traditional food fermentations is to improve local diets through their action as nutritional supplements and as stimulants to the consumption of rice or other carbohydrate rich staples. Stanton and Wallbridge (1969) reported that these fermentations do not produce any overall enrichment of the staple but instead supply a specific enrichment by improving digestibility, destroying toxins

and causing favourable changes in taste. Stanton and Wallbridge (1969) developed a process of solid substrate fermentation derived mainly in concept from the traditional food fermentations for the protein enrichment of cassava, a low protein starch rich root crop. The process developed differs from the traditional food processes by essentially being a synthesis of protein from mineral nitrogen in contrast to the modification of the original vegetable protein, which occurs in the traditional processes. For the process developed by Stanton and Wallbridge (1969) the cassava is reduced to a coarse flour, pasteurised, mixed with a solution of mineral salts and a source of nitrogen and inoculated by the mycelium and spores of the appropriate fungus, usually Rhizopus oligosporus or Rhizopus stolonifer previously blended into the solution of mineral salts. The subsequent fermentation of the moist paste formed was described by Stanton and Wallbridge (1969) as being dominant because conditions of absolute sterility were not required, the success of the fermentation being dependent on the type of micro-organism used, size of the inoculum and the control of the additives and the environment. The process of cassava fermentation takes 40-80 hours for completion, maturation being dependent on the extent of mycelial growth and protein synthesis from the original simple nitrogen source, raising the level of protein to 3 per cent a thirty fold increase over that of the original material.

The process of solid substrate fermentation developed by Stanton and Wallbridge (1969) is an example of a new type of development in which microbial growth is stimulated in

the aerobic fermentation of a solid into which nutrients essential for microbial growth have been incorporated. This process has been successfully used to upgrade a material with a low protein content, dispensing with the need for expensive processing equipment necessary in most of the current methods used for the production of microbial biomass.

In the introduction to this thesis, the successful feeding trials which were carried out with beef and dairy cattle fed on newsprint and other types of waste paper, were mentioned. An account of the digestibility of cellulosic materials, such as newsprint, by ruminants and herbivores, based on the analysis of feeds and faeces, has been presented by Crampton and Maynard (1937). They found that for nutritional purposes the carbohydrates could be divided into three fractions; a practically non-digestible portion, lignin; a highly digestible fraction, the hemicellose and cellulose. The digestibility of cellulose was found by Crampton and Maynard (1937) to be linked with the degree and nature of its lignification.

The economic advantages of solid substrate fermentation have been briefly described and it would seem that as waste paper is already digestible by beef and dairy cattle, a process of solid substrate fermentation similar to that developed by Stanton and Wallbridge (1969) could increase its value as a feedstuff.

The results obtained from the submerged fermentations of newsprint indicated that the thermophilic fungi Chaetomium thermophile var dissitum and Sporotrichum thermophile

var 2 have lignolytic ability as well as being highly cellulolytic. Thus if these fungi were used in a system of solid substrate fermentation of finely divided newsprint, a significant proportion of the less digestible components such as lignin and cellulose would be degraded, with added disruption of the ligno-cellulose matrix and the formation of more easily digestible carbohydrates and some protein and vitamins, as in the process of tempeh production by the fermentation of soybeans. The results of the tempeh fermentations and the other solid substrate fermentations carried out by Hesseltine (1972) indicated that only small quantities of the substrate were utilised as energy sources by the micro-organisms, this was reflected in only a slight loss in weight. This finding indicates that while the substrate is 'altered', so that it becomes palatable, digestible and nutritious only a very small amount is consumed by the micro-organisms responsible for the fermentation leaving most of it available as a food for human or animal consumption.

A process of solid substrate fermentation developed for the upgrading of newsprint or other waste cellulosic materials could be used on a small scale by farmers, similiar to the manner in which tempeh and miso fermentations are carried out by villagers in Indonesia, on their farms using their own or local production of cellulosic wastes.

Materials and Methods

In the previous chapter the importance of disrupting the ligno-cellulose matrix of woody tissues in order to facilitate the biodegradation of cellulose, by micro-organisms, was emphasised. Thus for a process of solid substrate fermentation of such tissues, that is newsprint, it is imperative to expose the maximum surface area of the substrate to microbial attack by grinding. This also causes some disruption of the ligno-cellulose matrix.

On a laboratory scale the newsprint was reduced to the fibrous state illustrated in Fig. 6.1. by passing it through a document shredder, pulping in a Waring Blender, oven drying and finally grinding it dry in a multi-purpose mixer (Griffin 533-330). A second, more efficient, method was subsequently developed which involved feeding small pieces of a roll of newsprint directly into an 8 inch laboratory grinding mill manufactured by Christy and Norris Limited. This method eliminated the need for pulping and drying and was much more convenient, producing a more finely divided product.

After the newsprint had been prepared in this manner it was pasteurised at 100°C for 24 hours after which time the appropriate nutrients and inoculum were mixed into it in a large clean (surface sterilised) plastic beaker, stirring by hand with a large spatula. This was found to be an efficient method of mixing the paper with the inoculum and nutrients and depending on the final moisture content of the mixture small spherical particles of paper

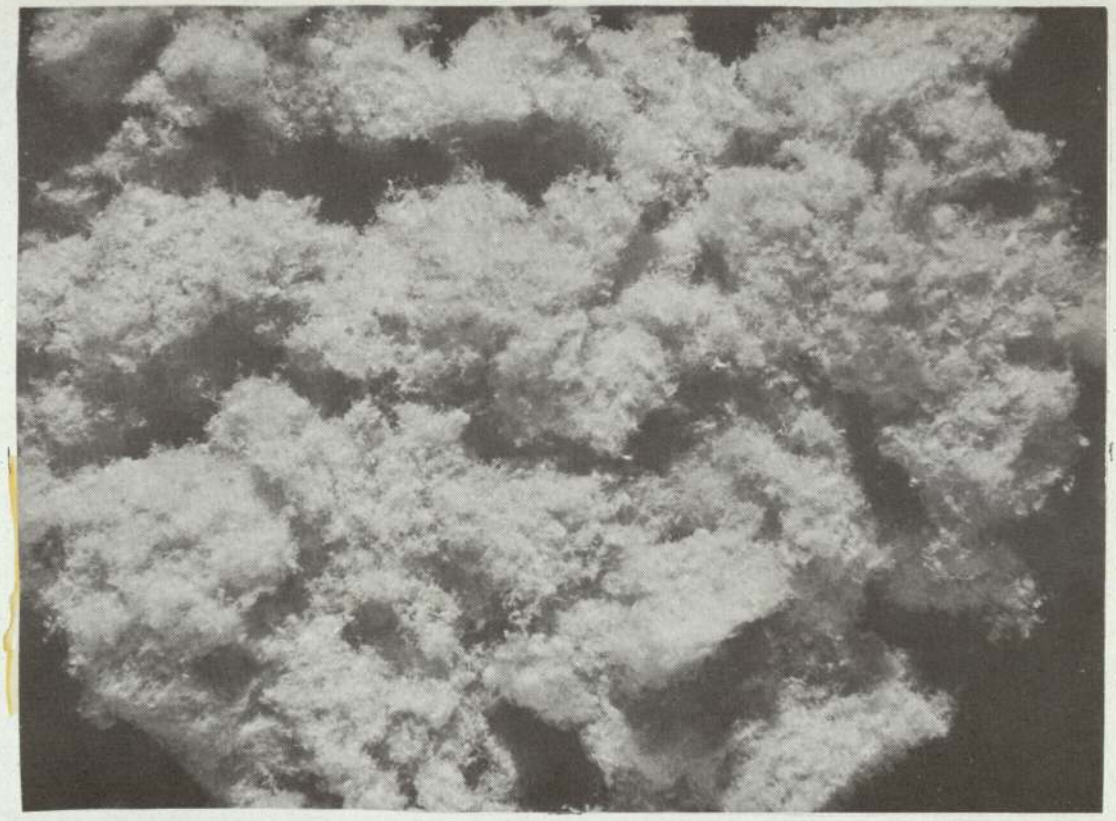


FIGURE 6.1 FIBROUS MASS OF PAPER BEFORE THE ADDITION OF THE NUTRIENT SOLUTION.

were formed which had sufficient interstitial spaces between them to allow good aerobic growth. The resultant mixture was incubated in a layer approximately ½ inch thick at 48°C, for each of the fungi used in this system, in a small tray fermenter with a capacity for 30gms of newsprint (dry weight). The tray fermenter consisted of a small aluminium tray with a false bottom of stainless steel to give increased aeration and is illustrated in Fig. 6.2.

The nutrients used were the same as those used in Eggins and Pugh's (1962) cellulose agar. Attempts were made at fermentation of the newsprint with the nutrient solution developed by Chahal and Gray (1969) and used successfully in the system of submerged fermentation described in the previous chapter. The nutrient solution of Chahal and Gray (1969) was used with urea as the nitrogen source, Stanton and Wallbridge (1969) reported that in their system of cassava fermentation, urea above certain levels of concentration became inhibitory to the growth of the fungi used and that below this level increases in urea concentration were not reflected in increased yields of protein. The concentration of the Eggins and Pugh nutrients and the resultant volume of the solution were calculated to give a suitable C:N ratio (in this case preliminary trials had indicated that a C:N ratio of 20:1 was adequate) and a moisture content, for the growth of some of the thermophilic fungi. The pH of the nutrients was adjusted to pH 5.6-5.8. For every 10gms of substrate used in this system of fermentation a set of Eggins and Pugh's (1962) mineral salts, as detailed in Appendix I, were added; thus for 30gms of substrate three times the normal quantities of mineral salts were used.

Inocula of the fungi were prepared by taking the mycelium and spores from 7-10 day old cultures grown on plates of YpSs agar (Cooney and Emerson, 1964). The mycelium and spores, were removed from the surface of the plates by gently scraping an alcohol flamed spatula across the surface of the agar and then added to the sterile nutrients in a Waring Blender which was used to disrupt the mycelium and spores producing an inoculum with a very large number of growing points. Two agar plates of the culture were used for every 10 gm of substrate to be fermented.

Using the small-tray fermenter the effect of the moisture content of the substrate on the fermentation was investigated with both Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2. During these investigations the C:N ratio was kept constant at 20:1. The success of each fermentation was quantitatively estimated on the basis of protein production which was determined as usual by the method of micro-Kjeldahl and using the conversion factor 6.25xN. The residual nitrogenous nutrients were however first removed by washing with distilled water and the substrate was then dried to constant weight at 60°C. The protein content of the substrate was expressed as a percentage of the dry weight.

After the optimum moisture content of the substrate had been determined the effect of a range of C:N ratios on the growth of Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2 was determined. The different C:N ratios were produced by varying the concentration of the ammonium sulphate, the concentrations of the other two

nitrogen sources, L-asparagine and yeast extract, in Eggins and Pugh's mineral salts were kept constant. The C:N ratios of 25:1, 20:1, 15:1 and 10:1 were investigated.

On the determination of the optimum parameters for C:N and substrate moisture content for Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2 the growth of the remaining six of the eight thermophilic fungi, which were used in the shake flask cultures, in this system of solid substrate fermentation was examined.

In the introduction of the chapter it was mentioned that control of the environmental conditions in solid substrate fermentations could prevent the growth of contaminants and in this system of solid substrate fermentation, for newsprint, this was partially achieved by using an elevated temperature of incubation at which only thermophilic fungi could grow (this of course did not exclude the possibility of contamination by thermophilic bacteria and actinomycetes but it was thought that the low moisture content and the pH of 5.6-5.8 would be effective in suppressing the growth of these groups of micro-organisms). There are however two known pathogenic fungi which can grow at the elevated temperature used in these fermentations and they are the thermotolerant Aspergillus fumigatus Fresenius and the thermophilic Mucor pusillus. Raper and Fennell (1965) described Aspergillus fumigatus as a major pathogen of animals, particularly birds, and it is particularly well known in association with infections of the respiratory system. Outbreaks of this type of disease are particularly prevalent in poultry where the reported

mortality has been as high as 90 per cent of the population. Mucor pusillus has been isolated from body tissues of animals, it has been associated with animal mycoses on a number of occasions and it has also been known as a cause of bovine mycotic abortion (Cooney and Emerson, 1964). Humicola lanuginosa, Malbranchea pulchella and Mucor miehei have also been reported as pathogens but Cooney and Emerson (1964) failed to find any pathogenic tendencies in their isolates of Humicola lanuginosa and Malbranchea pulchella. It was therefore important to establish that the growth of pathogenic thermotolerant or thermophilic fungi, especially Aspergillus fumigatus, was unlikely to occur in the system of solid substrate fermentation.

The effectiveness of the heavy inoculum of the desired organism, in this case Sporotrichum thermophile var 2, to suppress the growth of any pathogenic contaminants was determined by the deliberate introduction of known quantities of spores of Aspergillus fumigatus. Cultures of Aspergillus fumigatus were grown on YpSs agar at 48°C. An 8mm agar plug was taken from the periphery of a 7-10 day old colony and placed in a known volume of water to which a small quantity of 'tween 80' amounting to a concentration of 0.05 per cent was added to produce an even dispersion of the spores. The number of spores per ml. of the suspension were counted by means of a haemocytometer slide and the number of spores on the 8mm agar plug could therefore be estimated. After estimating the number of spores present on several agar plugs the assumption was made that agar plugs removed from adjacent areas of the colony would have approximately the

same number of spores thus providing a basis for the quantitative estimation of spore ratios between Sporotrichum thermophile var 2 and Aspergillus fumigatus. The inoculum of Sporotrichum thermophile var 2 was prepared in the normal way, blending the mycelium and spores with the nutrient solution, the number of spores/ml. of nutrient solution were also estimated by means of the haemocytometer slide. Solid substrate fermentations were then carried out in the normal manner but with various concentrations of spores and mycelium of Aspergillus fumigatus which were added in the form of 8mm agar plugs. The comparative growth of Sporotrichum thermophile var 2 and the Aspergillus fumigatus in these fermentations were monitored by means of the dilution plate technique and visual observations. A sample of substrate of knownweight was added to a sterile 100ml blank of distilled water and dilutions were prepared as described in chapter 3 by the method of Collins and Lyme (1970) with the exception that the initial dilution was prepared with 1gm of sample and not 10gm as with the samples of compost. The dilution plates were prepared with 1ml aliquots of the suspensions with YpSs agar and incubated at 48°C. The number of colonies of Sporotrichum thermophile var 2 and Aspergillus fumigatus on these plates were counted after 24 hours and rechecked after 48 hours. The number of colonies were expressed per gm of dry weight of the substrate which had been determined from a replicate sample. The procedure was repeated with spore ratios of Sporotrichum thermophile var 2 to Aspergillus fumigatus of 28:1, 15:1, 9:1 and 5:1.

The above method of solid substrate fermentation involved the use of relatively large concentrations of

mineral salts in a small volume of water and it was thought quite probable that these concentrations could affect the growth of the fungi. Thus the effects of increasing concentrations of the mineral salts on the growth of three genera of thermophilic fungi namely Chaetomium thermophile var dissitum, Humicola insolens, and Sporotrichum thermophile var 2 were determined based on colony diameters (Brancato and Golding, 1953) produced on the cellulose agar of Eggins and Pugh (1962). Cellulose agars were prepared with increasing concentrations of mineral salts up to 20 times higher than normal. Three replicate plates were prepared for each fungus at each concentration, the plates were incubated at 48°C and the colony diameter was measured every 12 hours by the procedure previously outlined in chapter 2.

Results

In spite of repeated attempts Chaetomium thermophile var dissitum failed to produce any growth in the fermentation with C:N ratios of 20:1, designed to evaluate the effects of the moisture content of the newsprint on the fermentation process. Sporotrichum thermophile var 2, however, produced dense growth in this system of fermentation and the effects of the initial moisture content of the substrate on its growth are presented in Fig. 6.3. Each point on this graph represents the mean of the results obtained from three separate fermentation trays. In all cases very little growth of Sporotrichum thermophile var 2 occurred during the first two days, none being visibly detectable. At 4 days growth was clearly visible in all of the fermentations with moisture contents of 300 per cent or more (moisture contents were

FIGURE 6.2 A TRAY FERMENTER.

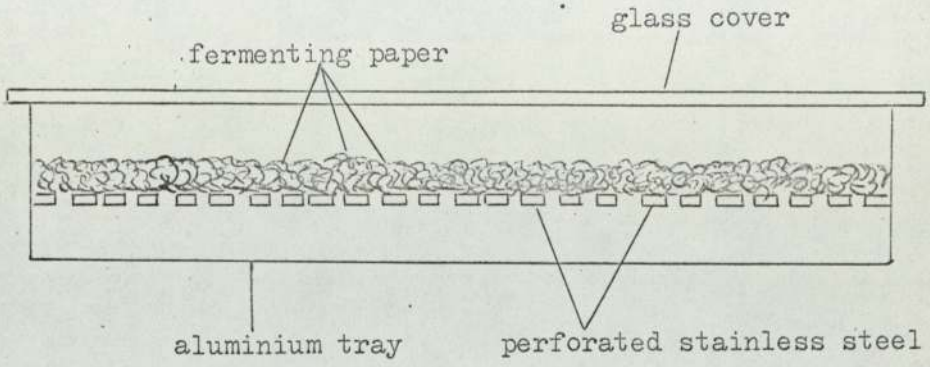
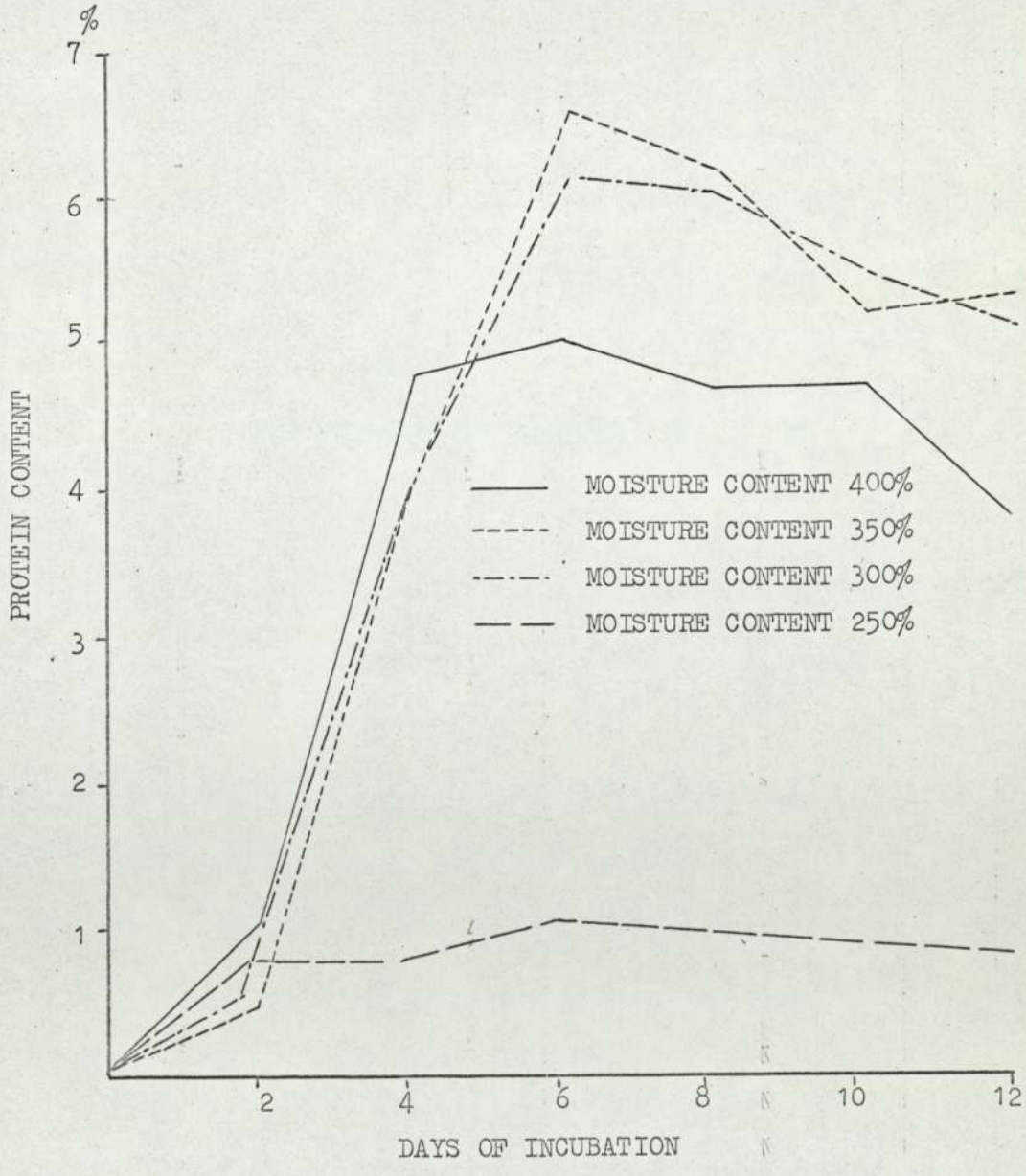


FIGURE 6.3 EFFECT OF MOISTURE CONTENT OF THE SUBSTRATE.



based on the dry weight of the substrate). At 4 days growth was still not visible in the fermentation with a substrate moisture content of 250 per cent, the protein content only increasing marginally during this period. After 4 days the protein content of the substrates with moisture contents of 300 and 350 per cent continued to increase reaching their maximum level at 6 days. The maximum protein content was in fact 6.5 per cent of the total dry weight and was achieved in the fermentation with a substrate moisture content of 300 per cent. During the same period of time the substrate with a moisture content of 400 per cent only showed a slight increase in the protein content. In all cases maximum growth based on protein content was attained after 6 days and decreased gradually from this point onwards. The dense growth of mycelium produced in the fermentations with substrate moisture contents of 300 per cent, after 6 days, is illustrated in Fig. 6.4.

The results of the effects produced by the C:N ratios 25:1, 20:1, 15:1 and 10:1 on the fermentations with Sporotrichum thermophile var 2 and with initial substrate moisture contents of 300 and 350 per cent are presented in Fig. 6.5, which shows that the optimum C:N ratio for Sporotrichum thermophile var 2 in these fermentations lies between 20:1 and 15:1. Chaetomium thermophile var dissitum again failed to produce any growth in any of the fermentations. The protein contents produced by Sporotrichum thermophile var 2 were comparable with the maximum observed for the earlier fermentations in which moisture content was the variable. The weight losses produced in this second set of fermentat-

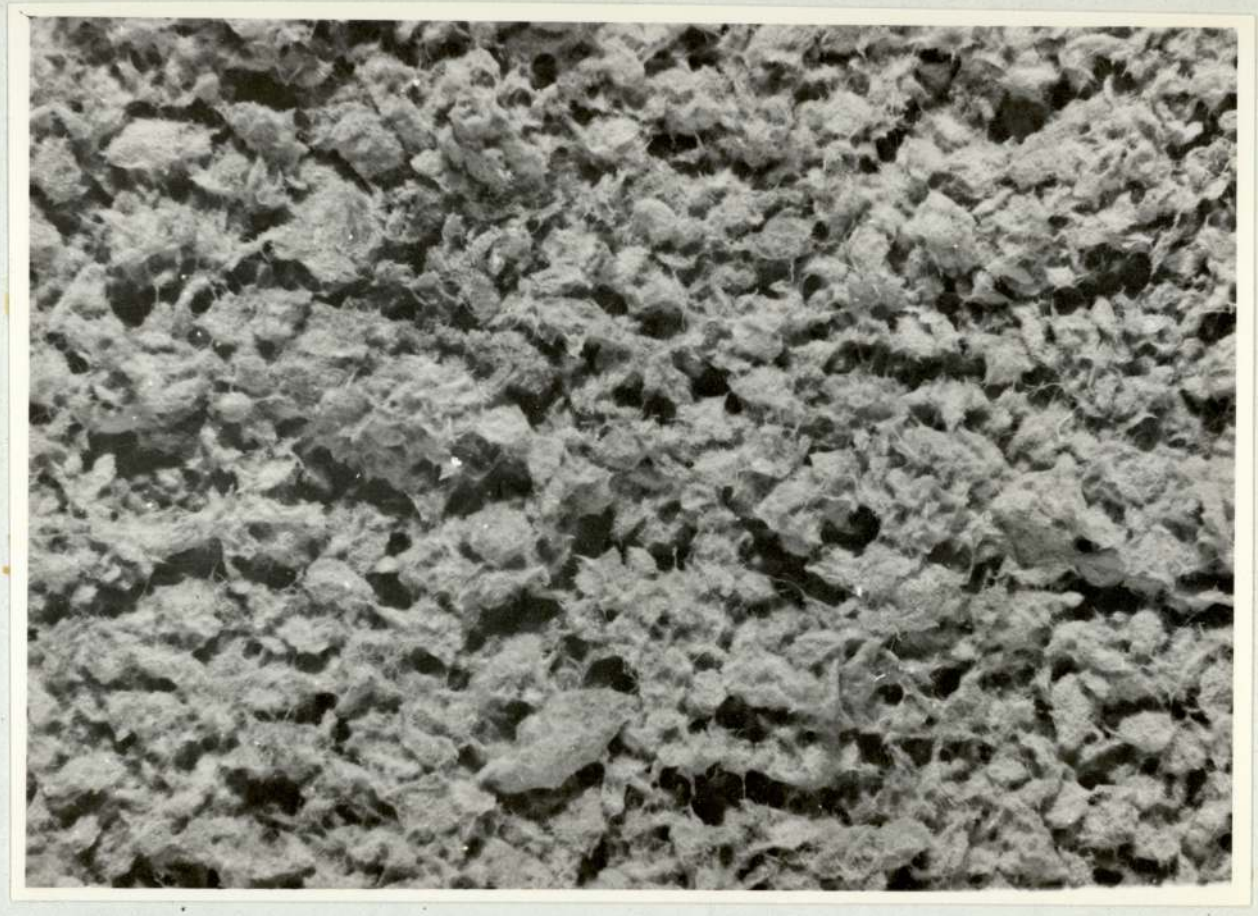
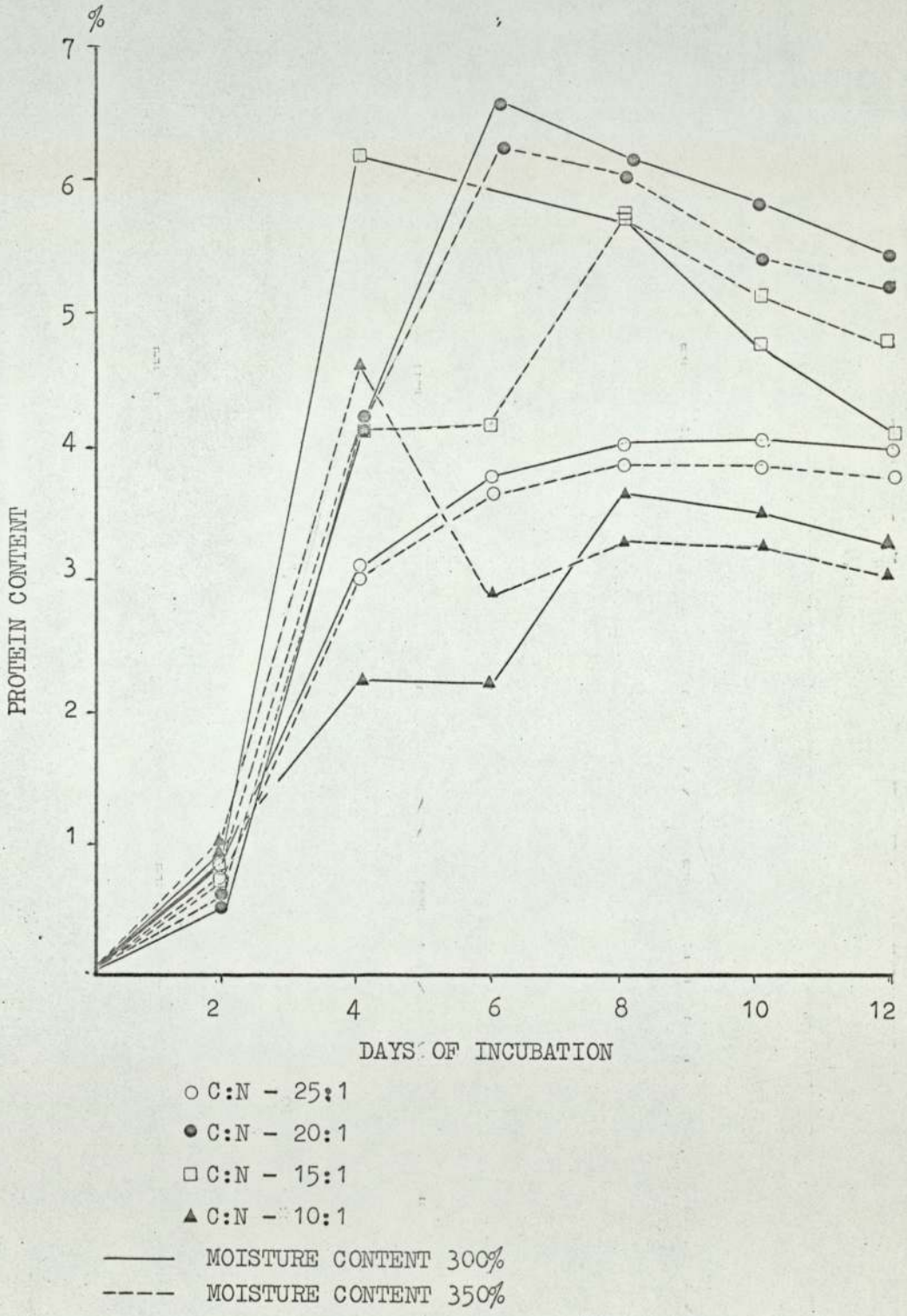


FIGURE 6.4 GROWTH OF Sporotrichum thermophile var 2 ON NEWSPRINT AFTER 6DAYS OF INCUBATION AT 48°C.

FIGURE 6.5 THE EFFECT OF THE C:N RATIO ON PROTEIN PRODUCTION IN SOLID SUBSTRATE FERMENTATION.



ions was determined by, subtracting the total dry weight of the samples taken during the fermentation, from the initial dry weight of the substrate, thus estimating the weight of substrate which should have been present if no degradation had occurred. The weight losses produced at the end of 12 days fermentation are presented in Table 6.1.

Table 6.1. Weight losses produced in the substrates after 12 days fermentation with C:N ratios of 20:1, 25:1 15:1 and 10:1 by Sporotrichum thermophile var 2.

	Moisture content of substrate	
C:N	300 per cent	350 per cent
25:1	20	17
20:1	34	30
15:1	30	27
10:1	8	21

The greatest weight loss of 34.0 per cent of the dry matter was produced in the fermentation with the most nearly optimum C:N ratio of 20:1 with a substrate moisture content of 300 per cent. The smallest weight loss of 8 per cent was produced in the fermentation with the smallest C:N ratio of 10:1 with an initial substrate moisture content of 300 per cent.

The attempts at the solid substrate fermentation of newsprint, using the optimum conditions for substrate moisture content and C:N ratio evaluated above, with the remaining six species of thermophilic fungi were unsuccessful, all of

the fungi failing to produce any visible signs of growth. Sporotrichum thermophile var 2 was the only thermophilic fungus to grow in this system of solid substrate fermentation.

Control fermentations which were set up without any incubation gave nitrogen contents identical with those from unfermented newsprint showing that all of the nitrogenous nutrients were removed by washing.

The results of the experiment to determine the efficiency of the heavy inoculum in suppressing the growth of the pathogenic fungus Aspergillus fumigatus are presented in Figs. 6.6(a) and 6.6(b). Fig. 6.6(a) shows the number of propagules of Sporotrichum thermophile var 2 present on each gram of dry newsprint during the fermentation, as estimated by the dilution plate technique. The results of the fermentations with spore ratios (number of spores of Sporotrichum thermophile var 2 divided by number of spores of Aspergillus fumigatus) of 28:1, 15:1 and 9:1 are presented in Fig. 6.6(a) and in all of these fermentations Aspergillus fumigatus failed to produce any visible growth or any viable propagules detectable by the dilution plate technique. The number of viable propagules of Sporotrichum thermophile var 2 increased during the fermentation, slowly during the first two days but much more rapidly after this time, reaching a maximum on day 8. After this the number of viable propagules decreased, probably due to autolysis. In the previous fermentations the protein content of the substrate had decreased after the sixth day of incubation, also indicating the autolysis of the mycelium and spores present. Fig. 6.6(b) shows the

FIGURE 6.6 (a) NUMBER OF VIABLE PROPAGULES OF Sporotrichum thermophile var 2 PER GRAM OF DRY PAPER, RECORDED DURING SOLID SUBSTRATE FERMENTATION.

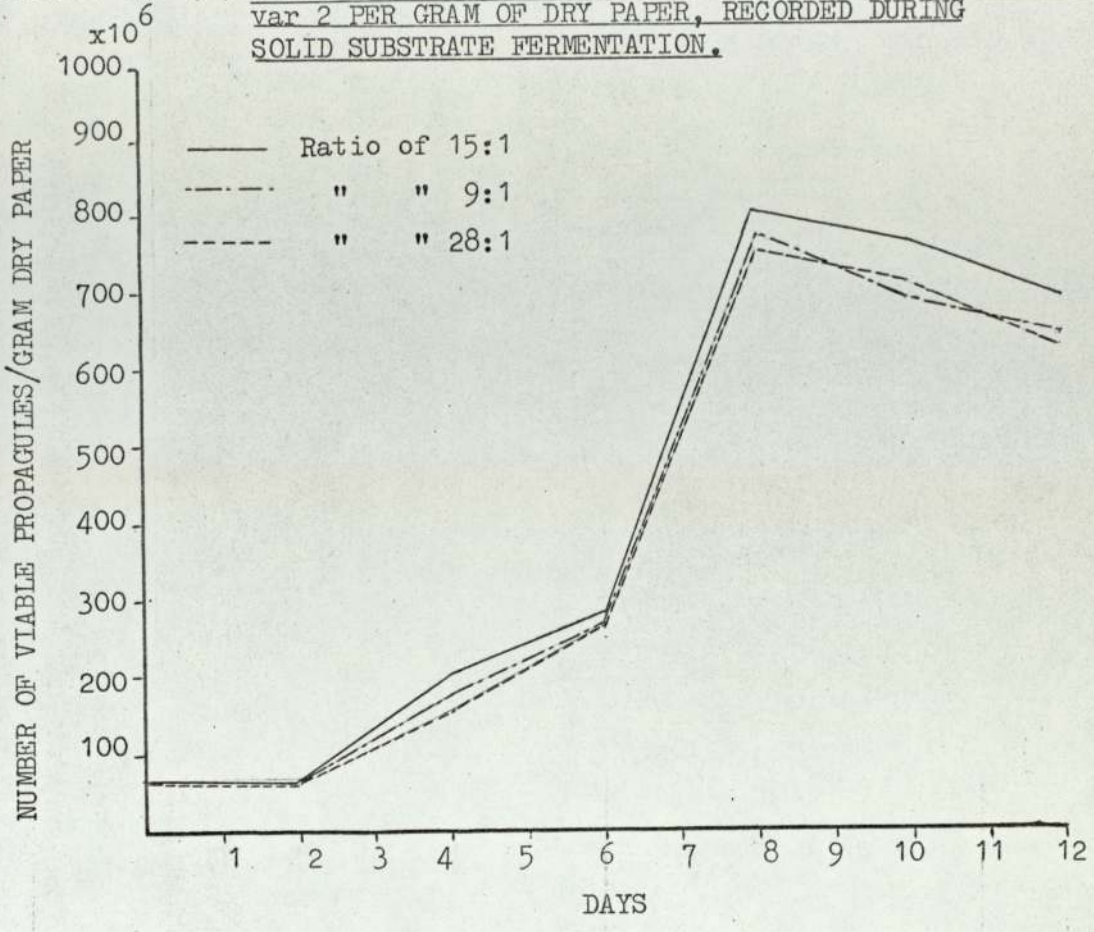
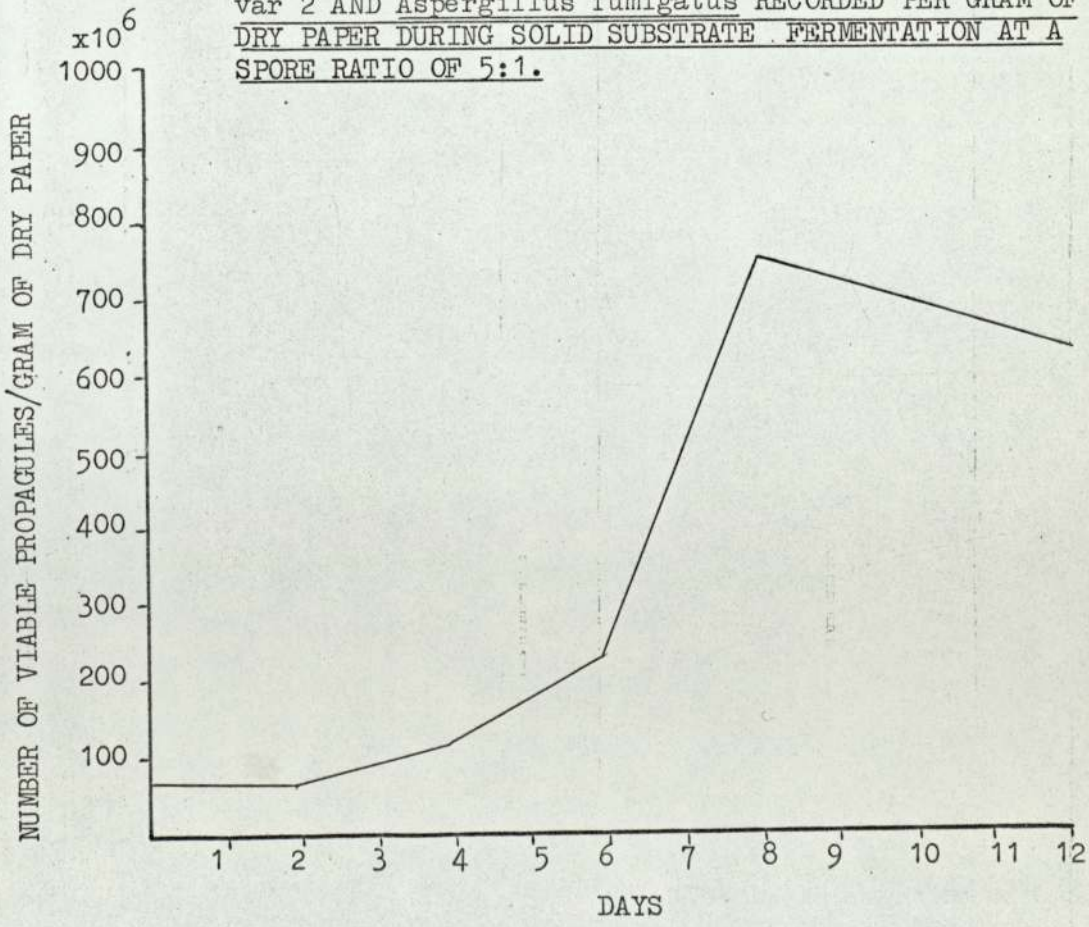


FIGURE 6.6 (b) NUMBER OF VIABLE PROPAGULES OF Sporotrichum thermophile var 2 AND Aspergillus fumigatus RECORDED PER GRAM OF DRY PAPER DURING SOLID SUBSTRATE FERMENTATION AT A SPORE RATIO OF 5:1.



results of the fermentation with a spore ratio of 5:1 and in this case slight growth of Aspergillus fumigatus was observed on some regions of the fermenting newsprint during the first 4 days of the fermentation and a small number of viable propagules of Aspergillus fumigatus were recorded on the dilution plates. After day 4 the small colonies of Aspergillus fumigatus were completely overgrown by Sporotrichum thermophile var 2 and viable propagules of Aspergillus fumigatus were not detected on the dilution plates.

The determinations of the number of spores present in the prepared inocula of Sporotrichum thermophile revealed spore counts of between 21 and 23 million spores/ml of nutrient solution. The total volume of nutrient solution used to obtain a moisture content of 300 per cent with 30gms of dry substrate was 90ml and this gave a total spore count in the region of 2×10^9. The number of spores of Aspergillus fumigatus present on an 8mm agar plug of a culture grown on YpSs agar varied between 60 and 80 million giving spore counts in the 90ml of nutrient solution of the order of $0.6 - 0.9 \times 10^6$ spores/ml. The numbers of spores of Aspergillus fumigatus per ml of nutrient solution were increased by the addition of more agar plugs.

The results of the effects of increasing mineral salt concentration on the growth of Chaetomium thermophile var dissitum, Humicola insolens and Sporotrichum thermophile var 2 are presented in Tables 6.2, 6.3 and 6.4 and Figs. 6.7(a), (b) and (c) respectively.

Table 6.2 Colony diameters (cm) attained by Chaetomium thermophile var dissitum on cellulose agars with increasing concentrations of mineral salts incubated at 48°C.

Days/ Conc.	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7
1	0.9	2.9	5.0	7.1	M									
2	1.6	5.5	7.4	M										
3	1.6	6.0	7.4	M										
4	1.5	5.8	7.2	M										
5	1.3	5.4	6.7	M										
6	1.3	3.3	6.3	M										
7	1.4	3.3	6.4	M										
8	1.2	2.0	4.7	6.3	7.7	M								
9	1.3	2.3	4.6	5.9	7.7	M								
10	1.2	2.0	4.6	6.0	7.4	M								
11	0.9	2.5	4.1	5.5	6.7	7.8	M							
12	1.0	2.5	4.2	5.5	6.5	7.5	M							
13	1.0	1.9	3.6	4.4	5.7	6.6	7.6	M						
14	1.0	1.7	2.8	3.8	4.5	5.2	6.0	6.7	7.5	8.2	M			
15	1.0	1.8	2.9	4.0	4.7	5.4	6.0	6.7	7.6	8.2	M			
16	0.9	1.5	2.6	3.6	3.9	4.7	5.3	6.1	7.0	7.6	8.2	M		
17	0.9	1.6	2.4	3.1	3.5	4.2	4.8	5.5	6.1	6.9	7.3	7.8	M	
18	1.0	1.6	2.0	2.5	3.2	3.6	4.3	4.9	5.3	5.9	6.4	6.9	7.4	8.0
19	0.9	1.5	1.9	2.4	3.0	3.5	4.2	4.7	5.0	5.5	5.8	6.4	6.7	7.3
20	0.9	1.4	1.9	2.4	2.9	3.5	4.2	4.5	4.7	5.0	5.3	5.8	6.1	6.5

M = Maximum Colony Diameter of 8.5cm.

Table 6.3 Colony diameters (cm) attained by Humicola insolens on cellulose agars with increasing concentrations of mineral salts incubated at 48°C.

Days/ Conc.	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7
1	1.6	2.0	4.1	5.3	6.4	7.6	M							
2	1.5	2.1	4.3	5.5	6.6	7.8	M							
3	1.3	2.5	4.4	5.9	6.8	7.9	M							
4	1.2	2.8	4.7	6.2	7.5	M								
5	1.2	2.7	4.5	6.0	7.3	8.4	M							
6	1.0	2.5	4.4	5.8	7.1	8.4	M							
7	1.0	1.8	3.6	4.8	6.3	7.8	M							
8	0.9	1.4	3.0	4.0	5.5	7.0	8.4	M						
9	0.9	1.4	2.9	4.0	5.7	7.1	8.4	M						
10	0.9	1.6	3.0	4.0	5.5	7.2	8.4	M						
11	0.9	2.2	3.4	4.7	5.9	7.2	8.3	M						
12	0.9	2.3	3.6	4.9	6.0	7.2	8.4	M						
13	0.9	2.1	3.4	4.7	5.9	7.2	8.4	M						
14	1.0	2.3	3.4	4.7	5.9	7.2	7.6	8.0	M					
15	0.9	2.1	3.5	4.7	6.0	7.2	8.0	M						
16	0.9	1.9	3.1	4.0	5.0	5.8	6.7	7.5	8.2	M				
17	0.9	1.7	2.7	3.4	4.0	4.4	5.1	5.8	6.7	7.4	8.1	M		
18	0.9	1.6	2.4	2.8	3.6	4.3	5.0	5.6	6.2	6.8	7.4	7.9	M	
19	0.9	1.6	2.2	2.3	3.5	4.3	4.8	5.4	5.9	6.4	7.0	7.5	8.0	M
20	0.9	1.4	2.1	2.8	3.4	4.2	4.6	5.1	5.6	6.1	6.6	7.1	7.6	8.1

M = Maximum Colony Diameter of 8.5cm.

Table 6.4 Colony diameters attained by Sporotrichum thermophile var 2 on cellulose agars with increasing concentrations of mineral salts, incubated at 48°C.

Days/ Conc.	0.5	1	1.5	2	2.5	3	3.5	4
1	1.3	2.0	3.5	5.6	6.7	7.8	M	
2	1.3	2.1	3.5	5.7	6.8	8.0	M	
3	1.3	2.0	4.0	6.5	7.5	M		
4	1.4	2.1	4.2	6.6	7.6	M		
5	1.4	2.1	4.3	7.1	7.8	M		
6	1.5	2.5	4.5	6.0	7.6	M		
7	1.5	2.4	4.7	5.8	6.9	8.1		
8	1.5	2.3	4.4	5.8	7.1	M		
9	1.5	2.3	4.4	6.2	7.7	M		
10	1.5	2.6	4.5	5.8	6.8	7.9	M	
11	1.4	2.2	3.7	5.3	6.9	8.4	M	
12	1.4	1.8	3.8	5.8	7.4	M		
13	1.3	2.2	3.8	5.2	6.7	8.2	M	
14	1.3	2.0	3.4	4.8	6.5	8.4	M	
15	1.3	2.0	3.5	4.7	6.6	7.7	M	
16	1.3	2.0	3.5	4.6	6.6	7.6	M	
17	1.3	2.0	3.3	4.4	6.5	7.7	M	
18	1.3	1.9	3.2	4.3	6.4	7.6	M	
19	1.3	1.8	3.2	4.3	6.3	7.5	M	
20	1.3	1.7	3.1	4.1	6.2	7.5	M	

M = Maximum Colony Diameter of 8.5cm.

FIGURE 6.7 (a) GROWTH OF Chaetomium thermophile var dissitum AT 36 HOURS ON CELLULOSE MEDIA OF INCREASING CONCENTRATIONS.

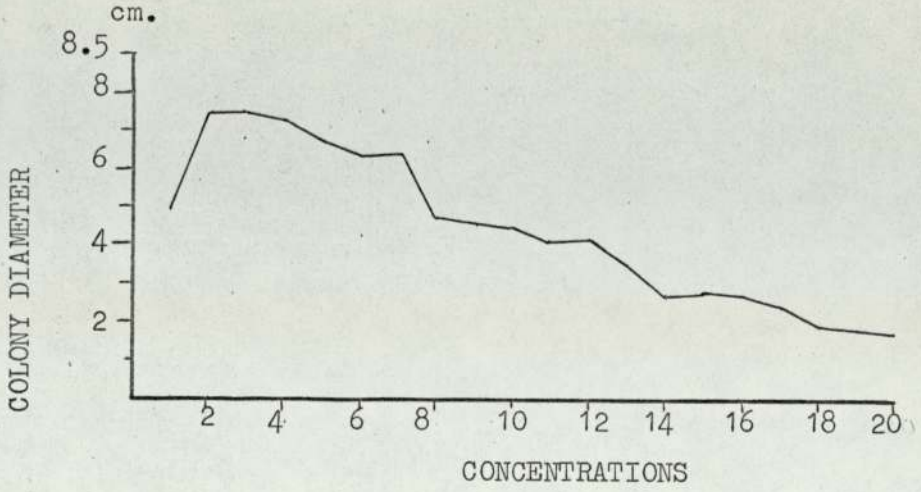


FIGURE 6.7 (b) GROWTH OF Humicola insolens AT 72 HOURS ON CELLULOSE MEDIA OF INCREASING CONCENTRATIONS.

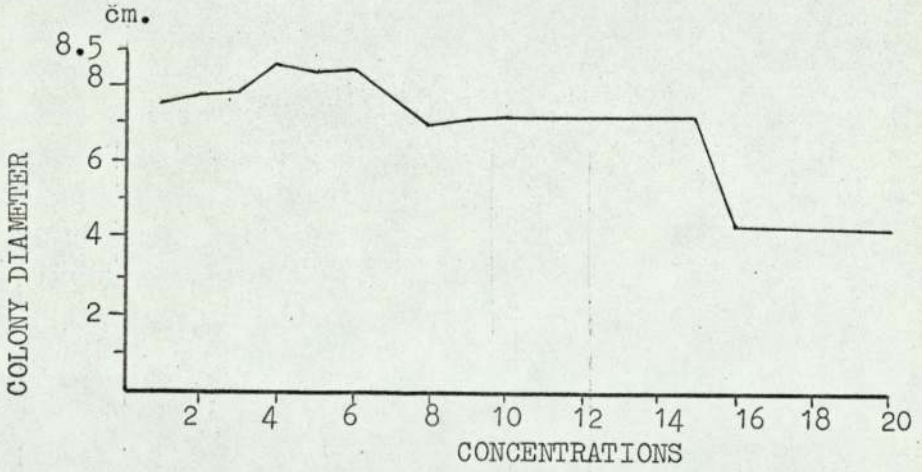
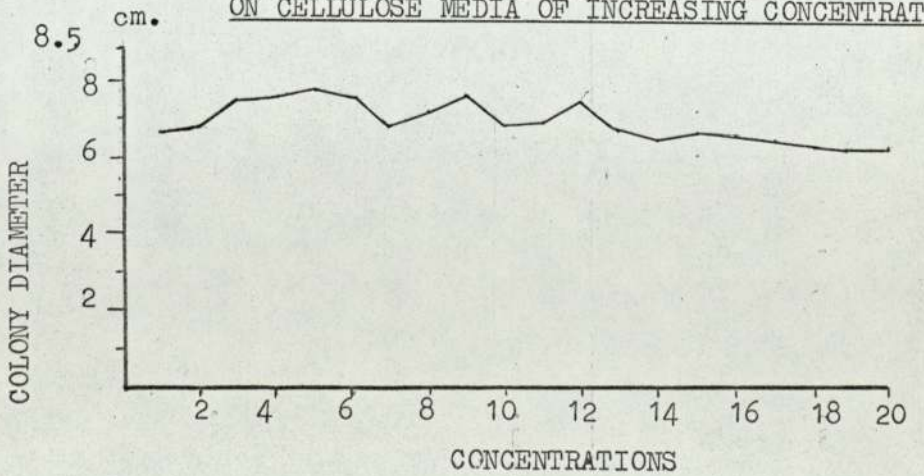


FIGURE 6.7 (c) GROWTH OF Sporotrichum thermophile var 2 AT 60 HOURS ON CELLULOSE MEDIA OF INCREASING CONCENTRATIONS.



Sporotrichum thermophile var 2 was the fungus least affected by the increasing concentrations of mineral salts. Once again there was this slight stimulation in the growth rate as the concentration of mineral salts increased up to 5 times the normal quantity. At concentrations higher than 50 times normal the growth rate unexplainedly fluctuated, as shown after 60 hours of incubation in Fig. 6.7(c). From Table 6.4 it can be seen that between concentrations 3 and 9 the maximum colony diameter was usually attained at approximately the same time, 3 days, and that between concentrations 10 and 20 the maximum diameter, was reached after 3-5 days of incubation showing that the growth rate was not significantly affected by further increases in the concentrations of the mineral salts.

Discussion

The results of the investigations described above indicate the importance of the effect of the moisture content on the physical form of the substrate and hence the fermentation process itself. When materials similar to paper are used in this type of fermentation their moisture content could prove to be one of the more critical factors affecting aerobic growth of the desired organism. The importance of a low substrate moisture content in solid substrate fermentation, in maintaining aerobic conditions for the fermentation and suppressing the growth of bacterial and yeast contaminants, has been stressed by Hesseltine (1965, 1972) and Brookes, Stanton and Wallbridge (1969).

Table 6.2 and Fig. 6.7(a) shows that Chaetomium thermophile var dissitum remained almost unaffected by increasing concentrations of nutrients until the concentration had increased to eight times the normal concentration. Indeed increasing the mineral salt concentration had initially stimulated the growth rate, the maximum rate being obtained when the mineral salts were 2-3 times as concentrated as normal. From this stage the growth rate decreased with increasing concentrations of mineral salts and at 7-8 times the normal concentration, the growth rate was approximately the same as when a normal concentration was used. Fig. 6.7(a) shows that after 36 hours of growth the effect of increasing the concentration of mineral salts was to gradually depress the growth rate until concentrations 18, 19 and 20 times the normal were reached at which stage the growth rate became constant in spite of further increases in concentration.

Humicola insolens displayed a similar reaction to the increasing concentrations of mineral salts, with a slight initial stimulation in the growth rate as the concentrations increased to 4 times the normal level. After this stage the growth rate declined in a series of plateaus, from concentrations 8 times normal until 15 times normal the growth rate remained constant, as recorded at 72 hours, but between concentrations 15 and 17 it dropped sharply and then between concentrations 17 and 20 it remained approximately constant. At the higher concentrations of mineral salts, the inhibitory effect which was produced appeared to increase with the incubation time, that is the growth rate decreased in the later stages of incubation.

Adding the nutrient solution to the fibrous mass of newsprint caused it to form small spherical particles, which if the volume of solution was correct, would have sufficient interstitial air spaces between them to allow good aerobic growth. When an excess volume of the solution was used, the particles formed tended to adhere to one another effectively closing these interstitial air spaces and resulting in the conditions of anaerobiosis and the growth of bacteria producing an obnoxious odour.

Thus if good aerobic growth is to be obtained with this method only a limited amount of water can be used in which to dissolve the required nutrients. This gives rise to a nutrient solution which is much more concentrated than those used in submerged fermentations. Such concentrated nutrient solutions may have been the reason why, of all the thermophilic fungi tested Sporotrichum thermophile var 2 was the only one to produce dense mycelial growth of normal type. The experiment where, Chaetomium thermophile var dissitum, Humicola insolens and Sporotrichum thermophile var 2, were grown on cellulose agars with increasing concentrations of mineral salts, clearly indicated that Sporotrichum thermophile var 2 was the fungus most tolerant of these higher concentrations. The growth of Chaetomium thermophile var dissitum and Humicola insolens were much more markedly affected by the higher concentrations of mineral salts and above concentrations of 8 times and 15 times normal respectively, their growth rates decreased sharply, with further increases in concentration. All three of the fungi tested displayed an initial increase in their growth rates as the

concentration of the mineral salts was increased above the normal level, and for Chaetomium thermophile var dissitum and Humicola insolens this increase became depressed by further increases in concentration.

Brancato and Golding (1954) had noted that some fungi showed a marked initial increase in colony diameter when supplementary concentrations of glucose were added to a medium commonly accepted as adequate. In a further investigation into the cause of this phenomenon Brancato and Golding (1954) cultured six fungi on malt agar variously concentrated with glucose, sucrose, glycerol, ethylene glycol or sodium chloride. Apart from ethylene glycol which proved toxic to all of the fungi, increasing concentrations of the additives to the malt agar initially stimulated the growth rates of at least some of the fungi and with further increases in the concentrations of the additives, the growth rates decreased as the fungi were gradually inhibited. Brancato and Golding (1954) put forward the following possible explanations for the characteristic growth of the six fungi tested on such concentrated substrates; the oxygen in solution was depressed to a level more favourable for growth, the additional glucose served as an added source of nutrients, the oxidation-reduction potential of the medium was altered to a more favourable level, the pH of the basal medium was changed to a more favourable level, the growth was influenced by osmotic concentration changes, increased quantities of deficient trace elements as impurities in the concentrating substances and changes in the growth promoting potential of the medium produced by sterilisation with the concentrating substrates and the effect of these

concentrating substances on cell permeability. The investigation by Brancato and Golding (1954), into the effects on the growth rate, brought about by such changes, indicated that osmotic pressures are an important factor in shaping these growth curves but they also concluded that no single explanation can account completely for the growth stimulation resulting when certain substances are added to an adequate medium.

In the present case it is conceivable that the increases in osmotic pressure, brought about by using a high concentration of mineral salts in a small volume of water, could have suppressed the growth of all the thermophilic fungi with the exception of Sporotrichum thermophile var 2, which has been shown to possess a high degree of tolerance to such concentrations. The high concentration of mineral salts could also have served to suppress the growth of contaminants providing a selective environment for the growth of Sporotrichum thermophile var 2.

At the present stage of development the concentrated solutions of mineral salts used have been necessary to promote the growth of Sporotrichum thermophile var 2, especially in providing the appropriate C:N ratios. However for the successful economic development of this process the amount of mineral salts used will have to be considerably decreased and efficient substitutes found for the expensive ingredients such as L-asparagine and yeast extract. Most of this work will be empirical and thus was not undertaken in the present investigation which was primarily concerned

in proving the feasibility of the technique of solid substrate fermentation for the microbial upgrading of waste cellulosic materials such as newsprint.

The optimum C:N ratio for the growth of Sporotrichum thermophile var 2 was found to be between 20:1 and 15:1. In the system of submerged fermentation of a suspension of ball-milled newsprint the optimum C:N ratio for this fungus was found to be 13:1. The discrepancy between the optimum C:N ratios for the two different systems of fermentation could have been caused by the use of different nitrogen sources or more probably by the effect of the high concentrations of mineral salts used in the system of solid substrate fermentation producing an inhibitory effect at the smaller C:N ratios.

Levi and Cowling (1966) investigated the effect of varying the carbon to nitrogen ratio between 10:1 and 1000:1 on the cellulase synthesis in some cellulolytic (wood degrading) fungi. They found that the soft-rot fungi, consisting of Ascomycetes and Fungi Imperfecti, could only clear cellulose at the lower C:N ratios. Such results indicate that members of the Fungi Imperfecti such as Sporotrichum thermophile var 2 need added nitrogen sufficient to produce C:N ratios of 15:1 to 20:1 to cause rapid decay of woody substrates such as newsprint.

The failure of Aspergillus fumigatus to establish itself in any of the solid substrate fermentations, even when added in large concentrations of up to one-fifth of the total inoculum, on the basis of spore counts, indicates

that the system of heavy inoculation with the desired fungus is efficient in suppressing the growth of contaminants. This suppression of pathogenic contaminants by the heavy inoculum eliminates the need for absolute sterility and this will be of obvious importance in reducing the operating costs. The level of inoculation used in the system of solid substrate fermentation was an arbitrary one, the main concern being the complete suppression of chance contaminants. Hesseltine (1972), in the solid substrate fermentation of rice kernels by Aspergillus parasiticus for the production of aflatoxin, believed that he had used excess conidia for inoculation purposes in most of his experiments but he stated that for the inoculation of solid substrate fermentations it is critical that a heavily sporulating strain be used. Sporotrichum thermophile var 2, used in the present investigations always sporulated profusely and is an ideal fungus for the preparation of inocula for solid substrate fermentations. In an industrial process of solid substrate fermentation inoculation would be done, by simply mixing a portion of the product of a completed fermentation with the fresh substrate for the next fermentation.

The yields obtained in the solid substrate fermentation of newsprint by Sporotrichum thermophile var 2 were found to be reproducible and its growth predictable.

Processes of solid substrate fermentations, like that developed for the upgrading of newsprint, (and other waste cellulosic materials) are particularly well suited to be carried out at low cost since they are an extension of

many traditional methods of food fermentation. The labour and capital required for this type of process would be much less than that required for the operation of the sophisticated apparatus necessary for the majority of submerged fermentations. The use of a thermophilic fungus requiring a temperature of 48°C should not hinder the development of the process because simple growth chambers which provide these high temperatures have been used for many years in the mushroom industry, for phase II (described in chapter 3) of the production of mushroom compost.

The maximum protein content obtained with this method of solid substrate fermentation of newsprint amounted to 6.5 per cent of the total dry weight of the substrate and was produced within 6 days with a corresponding weight loss in the substrate of 30 per cent. The efficiency of the process in terms of protein synthesis and biodegradation of the substrate should be improved when the parameters governing the growth of fungi in such a system are better understood, for example, nutrient concentration and composition, the effect of periodically agitating the substrate, pH of the nutrients, humidity, aeration and the level of inoculation. The results to date show that the product of the fermentations contain, at least, cellulose and hemicelluloses and possibly other digestible carbohydrates, produced by the action of Sporotrichum thermophile var 2 on the newsprint, in addition to the protein and (almost certainly) vitamins from the mycelium and spores of this fungus.

Thus the process of solid substrate fermentation of newsprint appears capable of producing a palatable, digest-

ible and nutritious feedstuff. The quality of the product can however, only be determined by feeding trials which would also indicate whether the fungus Sporotrichum thermophile var 2 is toxic. The production of aflatoxin outside the Aspergillus flavus group has been reported by Wilson, Campbell, Hayes and Hanlin, (1968) and feeding trials carried out on the product from the solid substrate fermentations would thus also detect the production of this or other mycotoxins from Sporotrichum thermophile var 2.

CHAPTER 7 Pages

Chapter 7

PRELIMINARY TOXICITY AND FEEDING TRIALS

Introduction

Newsprint has successfully been fed to beef cattle as a roughage substitute at levels of 5 - 12 percent of their rations (Dinius and Oltjen, 1971). However, when 16 - 24 percent newsprint was fed in complete rations to beef cattle, as energy constituents, the cattle decreased their voluntary feed intake even though molasses was fed to increase palatability (Dinius and Oltjen, 1971). Dinius and Oltjen (1971) also observed cattle consume the grain but leave the newsprint of complete feeds indicating that at high concentrations newsprint is unpalatable. The reduced feed intake by cattle fed on newsprint was found to be caused by 'ruminoreticular fill' as a result of the large quantity of digestible dry matter, approximately 30 percent, present in newsprint (Dinius and Oltjen, 1972).

Newsprint is essentially a mixture of hemicelluloses, cellulose and lignin. Crampton and Maynard (1938) investigated the relation of cellulose and lignin contents to the nutritive value of animal feeds and found that the digestibility of cellulose was linked with its degree of lignification. The newsprint used, in the present studies, for the solid substrate fermentations, was reduced, by grinding,

to the fibrous state illustrated in Photograph 6.1. Such processes of grinding disrupt the ligno-cellulose matrix of woody tissues, which has been shown to be primarily a physical combination, thus enhancing the digestibility of the cellulose fraction of the newsprint. In the feeding trials conducted by Dinius and Oltjen (1972) the newsprint was not finely ground, only being hammer-milled to pass through a 1cm. screen. Thus the physical treatment of the newsprint for the solid substrate fermentations of the present investigations should increase its digestibility above the levels reported by Dinius and Oltjen (1972).

The extent of the utilisation of the substrate in the submerged fermentations of suspensions of newsprint indicated that Sporotrichum thermophile var. 2 may be lignolytic. Thus the process of solid substrate fermentation of newsprint could bring about the decomposition of the lignin present in the newsprint further enhancing its digestibility in addition to supplementing it with a substantial proportion of fungal protein and vitamins, which are usually present in the mycelium of most fungi. For example, Litchfield (1968) reported that all of the B vitamins are usually present in fungal mycelium. The upgraded newsprint still contains large quantities of cellulose and the hemicelluloses and will therefore be most suitable for feeding either to ruminants or herbivores. At the present time it is not envisaged that the upgraded newsprint could act as a complete feedstuff but as an additive in the diet

providing roughage and an energy source from the carbohydrates and essential protein and vitamins from the fungal mycelium.

The nutritive value of the upgraded newsprint could only be fully evaluated from, a knowledge of the amino acid composition of the protein fraction and the degree of utilisation and acceptance of it by ruminants or herbivores. Unfortunately feeding trials involving the use of ruminants or herbivores were outside the scope of the present investigation simply because the laboratory scale of operations could not produce sufficient quantities of the feedstuff. However, if the method of solid substrate fermentation, described for newsprint in the present investigation, is to become accepted such feeding trials will have to be carried out after increasing the level of production.

One of the major factors to be considered with regard to the evaluation of microbiologically derived feedstuffs is that of toxicity which is initially more important than the nutritional qualities of the feedstuff. In undertaking toxicological studies with these types of feedstuffs it is important to have as much information as possible about its intended use, for example, will it be consumed by old or young animals, will it be used frequently and at what level in the diet will it be used. At the present time the basic objective of conventional chronic feeding trials is to ascertain the highest dietary level of the feedstuff that can be fed continuously for a 2 year period without adverse affect (Oser, 1968). Test dosages are usually

set quite high so as to permit a large margin of safety between the minimum, no effect dose level in the experimental animals and the intended use levels in the diet of man or other animal consumers (Oser, 1968). In the case of microbiologically derived feedstuffs feeding levels may be limited by the inadequacy of the protein content from the nutritional standpoint because animals can not be maintained throughout their life cycle on less than the full complement of their amino acid requirements. The toxicological appraisal of a proteinaceous food is not intended to be a nutritional evaluation and the test feedstuff should not be relied upon as the sole source of protein or other nutrients.

Most toxicological studies may be criticised on the grounds that they are usually conducted with healthy animals reared on normal diets. Until recently little attention had been paid to the effects of physiological stress conditions such as pregnancy and lactation and to toxicological effects induced in newborn animals. Oser (1968) reported that animals fed on marginal or sub-optimal diets are less able to withstand the toxic stress of exposure to drugs and chemicals. The animals used in toxicological feeding trials are usually observed for, effects on food consumption, efficiency of food utilisation, physical appearance and behavioural changes.

Special care must be taken in the evaluation of feedstuffs derived from 'new' species of fungi because of

the chance of the presence of mycotoxins. Mycotoxins comprise a group of toxic metabolites which can cause acute and chronic toxicity syndromes in animals and man (Wogan,1966). Mycotoxins are especially important from spoilage fungi contaminating food commodities and Wogan (1966) expresses the opinion that mycotoxin contamination can present real and potential health hazards and therefore impair maximum utilisation of available protein sources and other feedstuffs. These factors will be of particular importance to the exploitation of new feedstuffs of fungal origin and it is important with such feedstuffs that toxicological feeding trials are carried out.

The advantages of using mice as test animals in feeding trials have been fully described by Munck (1968). The main advantages of using mice as test animals in nutritional experiments are that; well defined inbred strains are available, thus eliminating genetic variation as a source of error and mice are small and therefore suitable for feeding and analysis on a mass scale (this point is particularly important when research is carried out on the laboratory scale as in the present investigation).

Accordingly mice were used in the present investigation for a preliminary toxicological and nutritional evaluation of the product obtained from the solid substrate fermentation of newsprint by Sporotrichum thermophile var. 2. Mice, although they possess a vestigial caecum are not

able to digest cellulose and therefore would not be able to completely digest the upgraded newsprint. However, it was thought that if the upgraded newsprint was fed to mice, at different concentrations, as part of their normal diet that it would be acceptable and definitely show whether the mycelium of Sporotrichum thermophile var. 2 was acutely toxic.

Materials and Methods

For the purposes of the feeding trials 25 male white mice, all from the same inbred strain and 20 days old were obtained from the University's animal house. At an age of 20 days the mice had already been weaned onto a diet known by the code '41B'; the composition of this diet is given in Table 7.1. The diet 41B was used in various admixtures with both the product of solid substrate fermentation and newsprint.

The 25 mice were divided into 5 groups of 5 mice each; these groups were kept in identical cages placed in a quiet warm (20 - 22°C) location, continuously illuminated, where they were only subjected to the minimum amount of disturbance, which was thought to be of importance because it has been reported by Munck (1968) that inbred strains of mice are particularly sensitive to stress. Each of the five groups of mice were fed on one of the following diets:-

Table 7.1 Composition of laboratory animal diet '41B'

Crude oil	%	2.73
Crude protein	%	16.95
Crude fibre	%	5.87
Digestible crude oil	%	2.26
Digestible crude protein	%	13.68
Digestible crude fibre	%	3.00
Digestible carbohydrates	%	45.07
Gross energy	Cals/Kg	3849.00
Metabolisable energy	Cals/Kg	3064.00
Saturated fatty acids	%	0.52
Linoleic acid	%	1.13
Other unsaturated acids	%	1.08
Calcium	%	1.10
Phosphorous	%	0.85
Sodium chloride	%	1.19
Magnesium	%	0.35
Potassium	%	0.86
Sulphur	%	0.24
Iron	mg/Kg	181.00
Copper	mg/Kg	16.00
Manganese	mg/Kg	115.00
Cobalt	ug/Kg	97.85
Zinc	mg/Kg	45.30
Iodine	ug/Kg	338.00
Arginine	%	1.00
Lysine	%	0.71
Methionine	%	0.24
Cystine	%	0.25
Tryptophan	%	0.21
Glycine	%	1.13
Histidine	%	0.36
Threonine	%	0.49
Isoleucine	%	0.61
Leucine	%	1.07
Phenylalanine	%	0.63
Valine	%	0.80
Tyrosine	%	0.55
Aspartic acid	%	1.15
Glutamic acid	%	3.31
Proline	%	1.24
Serine	%	0.81
Vitamin A	i.u./Kg	53455.00
Carotene	mg/Kg	37.56
Vitamin B_1	mg/Kg	10.50
Vitamin B_2	mg/Kg	9.20
Vitamin B_6	mg/Kg	10.40
Vitamin B_{12}	ug/Kg	12.94
Vitamin E	mg/Kg	80.00
Vitamin K	mg/Kg	2.62
Folic acid	mg/Kg	0.84
Nictonic acid	mg/Kg	110.00
Pantothenic acid	mg/Kg	33.50
Choline chloride	mg/Kg	2.04
Biotin	mg/Kg	0.12
Vitamin D_3	i.u./Kg	300.00

i. the normal diet of 41B

ii. a mixture of 50 percent of the product from the solid substrate fermentations and 50 percent 41B

iii. a mixture of 50 percent newsprint and 50 percent 41B

iv. the product of solid substrate fermentation (protein content 6.5 percent)

v. newsprint.

The normal diet, 41B was supplied in the form of pellets ⅜" in diameter and approximately 1" long. The other four diets were prepared by, reducing all the constituents to a powder by dry grinding in the 'Christy and Norris' 8 inch laboratory mill, mixing the powdered constituents in the correct proportions, adding a little water to form a paste and pelletising this paste by compressing it in an adapted 10 ml syringe, the resulting pellets were oven dried at 60°C. The procedure outlined above produced pellets with a diameter of approximately ½ inch and these were readily accepted by the mice.

The weight of food and volume of water consumed by each group of mice was recorded daily and the body weights of the individual mice were checked every two days and recorded to the nearest quarter of a gram.

The feeding trials were continued for 22 days. At the beginning of the feeding trials after the mice had been

divided into the five groups in their new cages they were allowed 24 hours to 'settle down' before being fed the experimental diets.

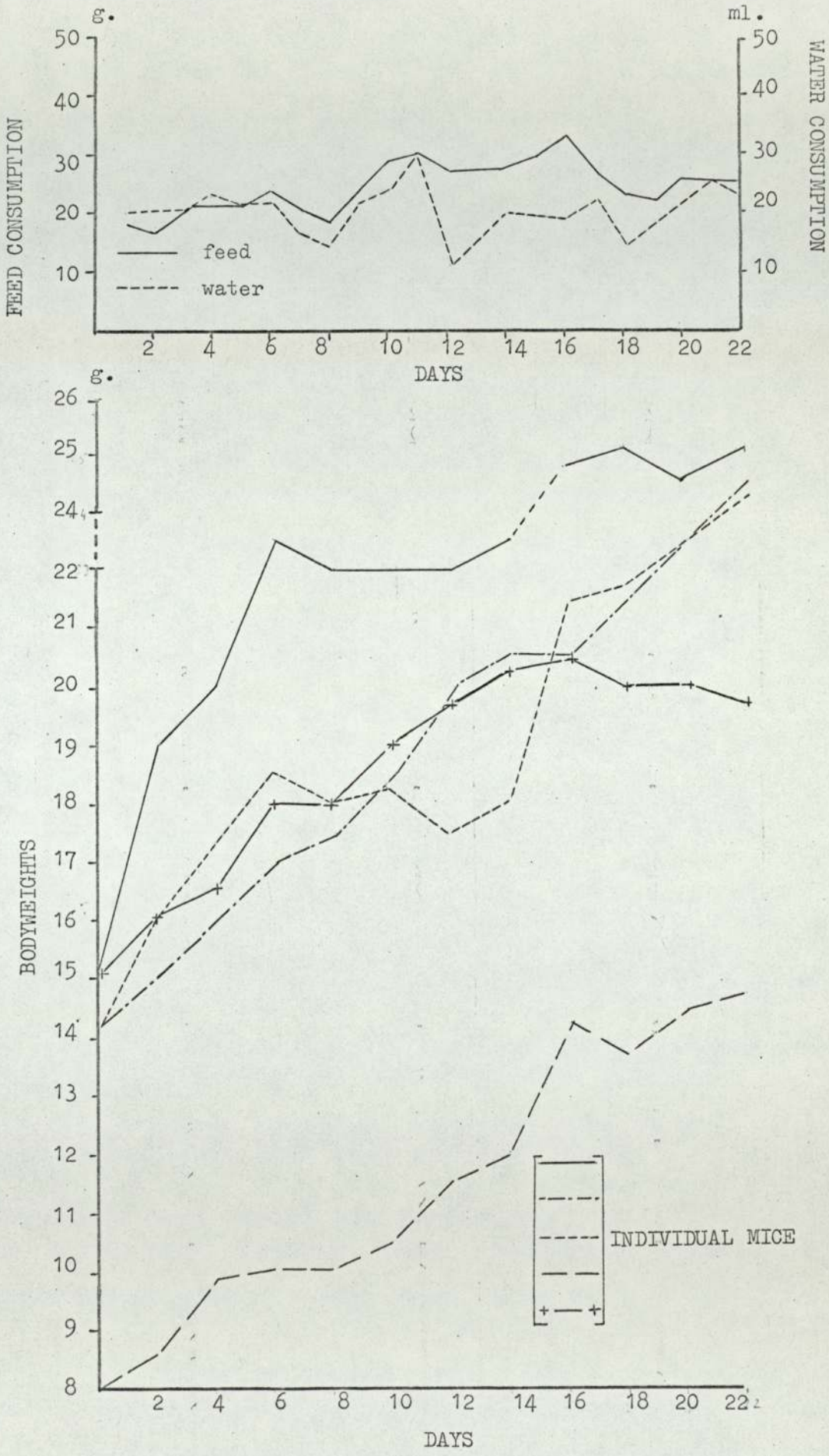
The age of the mice selected for the feeding trials and the duration of the feeding trials were both based on the feeding trials carried out by Munck (1968) in a comparison of the characteristics of inbred and outbred mice for use in feeding trials.

Results

The weight of food and the volume of water consumed each day, by each of the five groups of mice together with their individual body weights are shown in Figures 7.1 to 7.5.

Figure 7.1 shows the changes in body weights and the weight of food and volume of water consumed each day by group of mice fed on the normal diet of 41B. These mice were taken as the controls and their initial body weights varied between 8 and 15g and their final weights after 22 days varied between 14.75 and 25 g. All of the mice in this group showed constant substantial increases in weight throughout the duration of the feeding trials. The graph of the body weights, presented in Figure 7.1, indicates that during the first 8 days the mice consumed between 17 and 24 g of food and 14 - 20 ml of water daily but between days 10 and 16 the

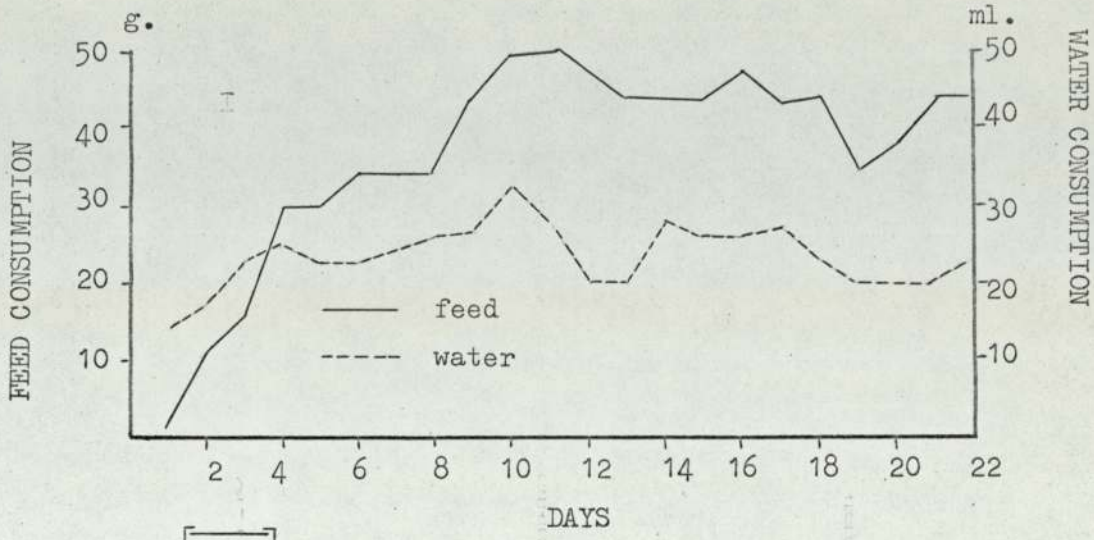
FIGURE 7.1 BODYWEIGHTS OF THE MICE FED ON THE CONTROL DIET OF '41 B' AND THE WEIGHTS OF FOOD AND VOLUMES OF WATER CONSUMED.

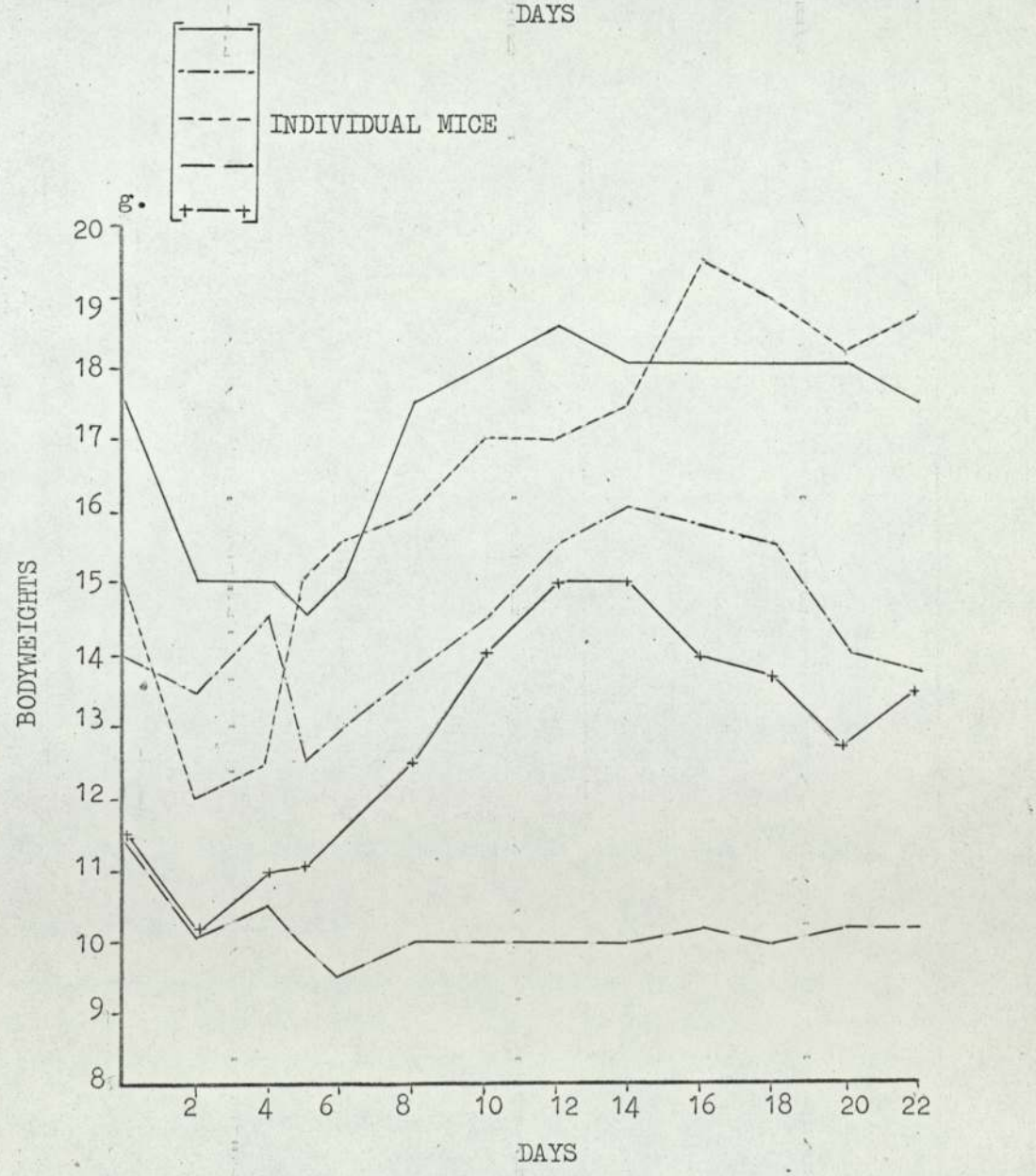


weight of food consumed varied between 30 and 33 g and the volume of water consumed was on average about 20 ml. During this period, on the 11th day, there was a sharp fall in the volume of water consumed and this was reflected in a corresponding fall, during the same period, in the body weights of two of the mice. In the final stages between days 16 and 22 the weight of food consumed dropped to between 22 and 26 g and the volume of water consumed varied between 14 and 20 ml.

Figure 7.2 shows the changes in body weights and the weight of food and volume of water consumed by the group of mice fed on the diet of 50 percent 41B and 50 percent of the product of the solid substrate fermentation. The initial body weights of the mice varied from 11.5 to 17.5 g and the final weights from 10.25 to 18.75 g. During the first two days all of the mice lost weight but from day 2 until day 4 the mice either remained at the same weight or gained weight. Between days 4 and 14 four of the mice showed gains in weight, averaging 3 - 4 g, and the weight of the remaining mouse was constant at 10 g. However, between days 14 and 16 the body weights of 3 of the 4 mice, which had earlier shown gains, gradually declined but by day 20 the rate of decline had slowed down and 2 of the mice once more showed gains in body weight. During the first 8 days the weight of food consumed increased from 2g, on the first day, to 34 g and the volume of water consumed varied from 14 - 23 ml. Between days 8 and 11 there was a further increase in the

FIGURE 7.2 BODYWEIGHTS OF THE MICE FED ON A DIET OF 50% '41B' AND 50% OF THE PRODUCT OF THE SOLID SUBSTRATE FERMENTATIONS AND THE WEIGHT OF FEED AND VOLUME OF WATER CONSUMED.

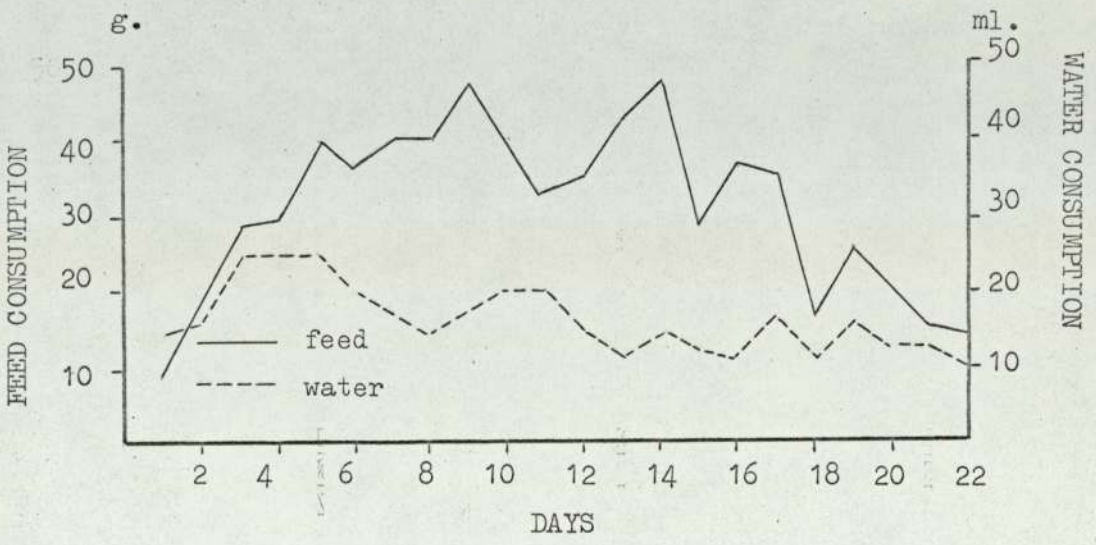


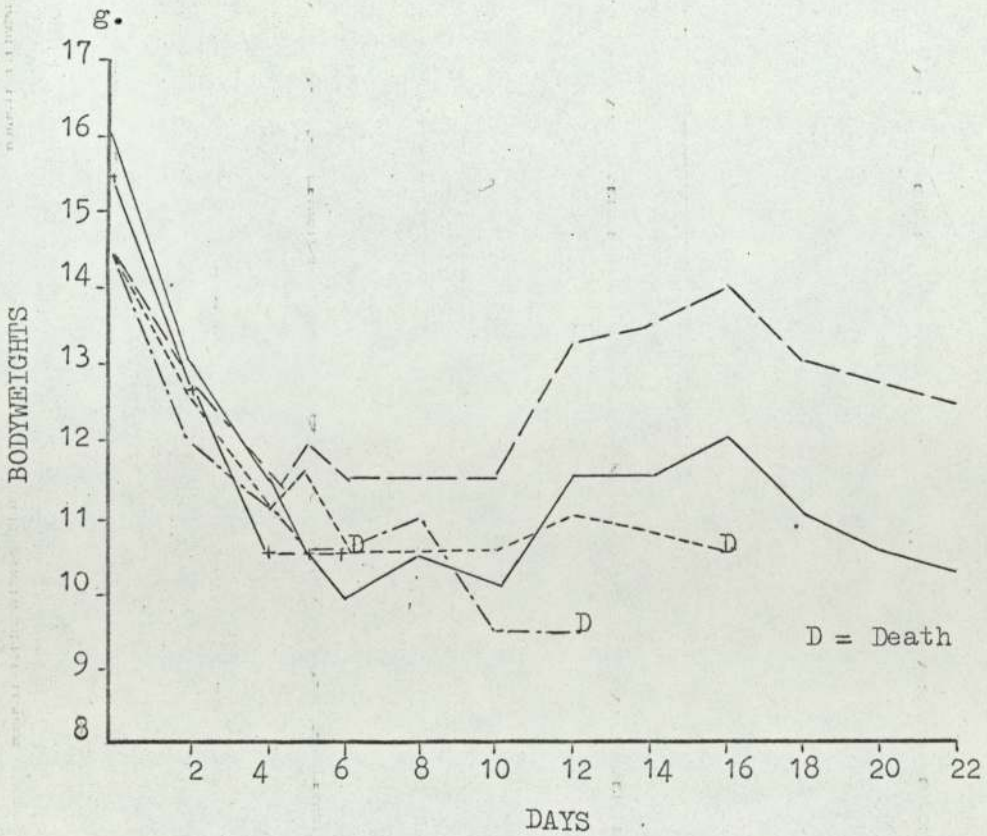


daily consumption of food from 34 to 50 g and the volume of water consumed increased from 23 ml to 32 ml. For the remainder of the feeding trials the daily food consumption fluctuated between 34 and 44 g and the consumption of water between 20 and 27 ml. The behaviour of this group of mice was comparable with that of the control group and could therefore be described as normal at all stages during the feeding trials.

Figure 7.3 shows the changes in body weights and the weight of food and volume of water consumed by the mice fed on a diet of 50 percent 41B and 50 percent newsprint. During the first 6 days all of the mice, which weighed between 14.5 g and 16 g lost weight and on the sixth day the first of the mice died. Between days 6 and 10 the body weights of 3 of the 4 remaining mice remained approximately constant between 10 and 11.5 g. On day 10 the second of the mice died; it had lost 1.5 g in weight in 2 days. After day 10 2 of the remaining 3 mice started to show gains in weight but the weight of the other mouse remained constant and it died on day 16. After day 16 the body weights of the 2 remaining mice began to decline and this trend was continued until the completion of the feeding trials. During the first 9 days the weight of food consumed increased from 9g, after the first day, to 47 g. From day 9 to day 11 food consumption fell from 47 g to 34 g, but by day 14 it had again increased to 47 g. After day 14 until the completion of the feeding trials the weight of food consumed, steadily decreased,

FIGURE 7.3 BODYWEIGHTS OF THE MICE FED ON A DIET OF 50% '41 B' AND 50% NEWSPRINT AND THE WEIGHTS OF FEED AND VOLUME OF WATER CONSUMED.

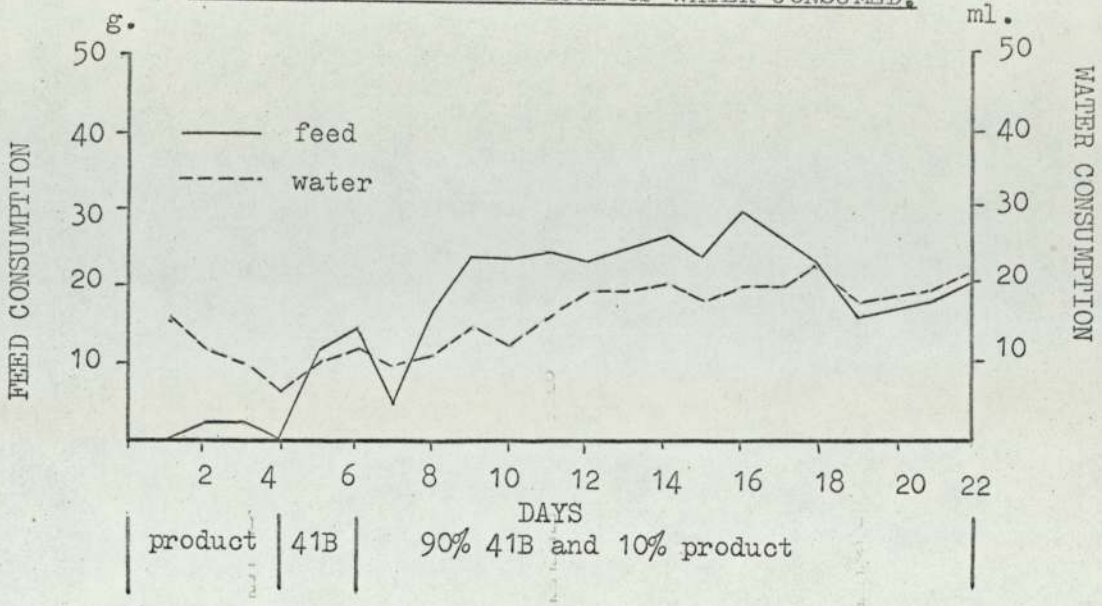


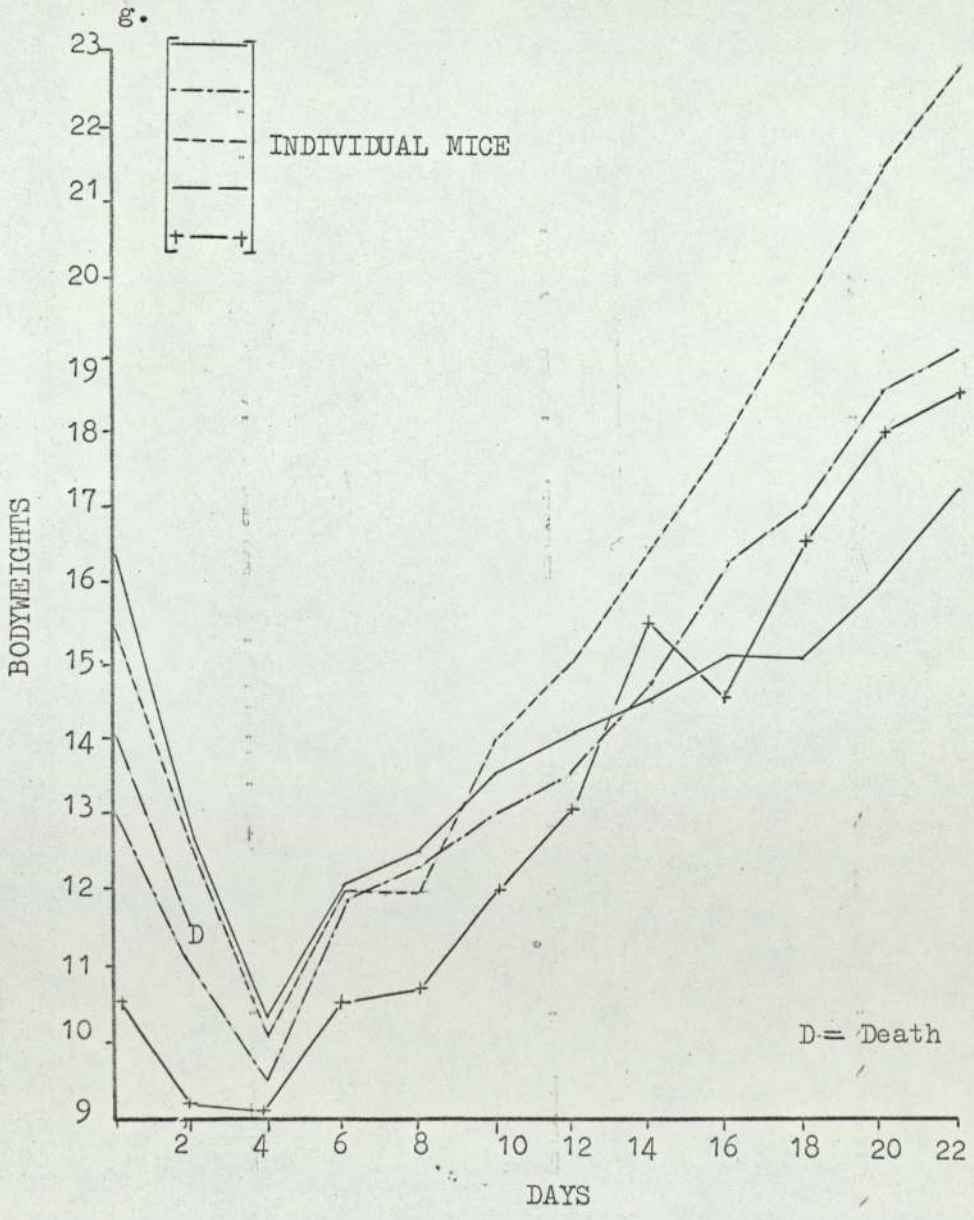


although temporary increases in consumption were observed during this period. The daily consumption of water gradually decreased from 25 ml at days 4 and 6 to 11 ml by day 22 on the completion of the feeding trials. The weight of food consumed by this group of mice was substantially more than that consumed by the control group which at the most only consumed 30 g of food a day. In this case the group of mice, even after the death of two of its members were consuming more than 40 g of food each day for the first 15 days. This enhanced food consumption is explained by the rejection by the mice of most of the newsprint in the food pellets and consumption of only the 41B portion of these pellets. During the feeding trials the behaviour of this group of mice was very sluggish when compared with the mice of the control group. For long periods of time the mice would remain huddled together in the centre of the cage and it was noticeable that they were shivering. On the whole the mice in this group were much less active than those of the control group.

Figure 7.4 shows the changes in body weights and the weight of food and volume of water consumed each day by the mice fed initially with the product obtained from the solid substrate fermentations. In the first 4 days all of the mice, which initially weighed between 10.5 and 16.5 g lost weight and at the end of this time their weights varied from 9 - 10.25 g. After day 2, 1 of the mice died and during the first 4 days hardly any of the food was consumed, the

FIGURE 7.4 BODYWEIGHTS OF THE MICE FED ON A DIET OF THE PRODUCT OF THE SOLID SUBSTRATE FERMENTATION OF NEWSPRINT AND THE WEIGHTS OF FEED AND VOLUME OF WATER CONSUMED.



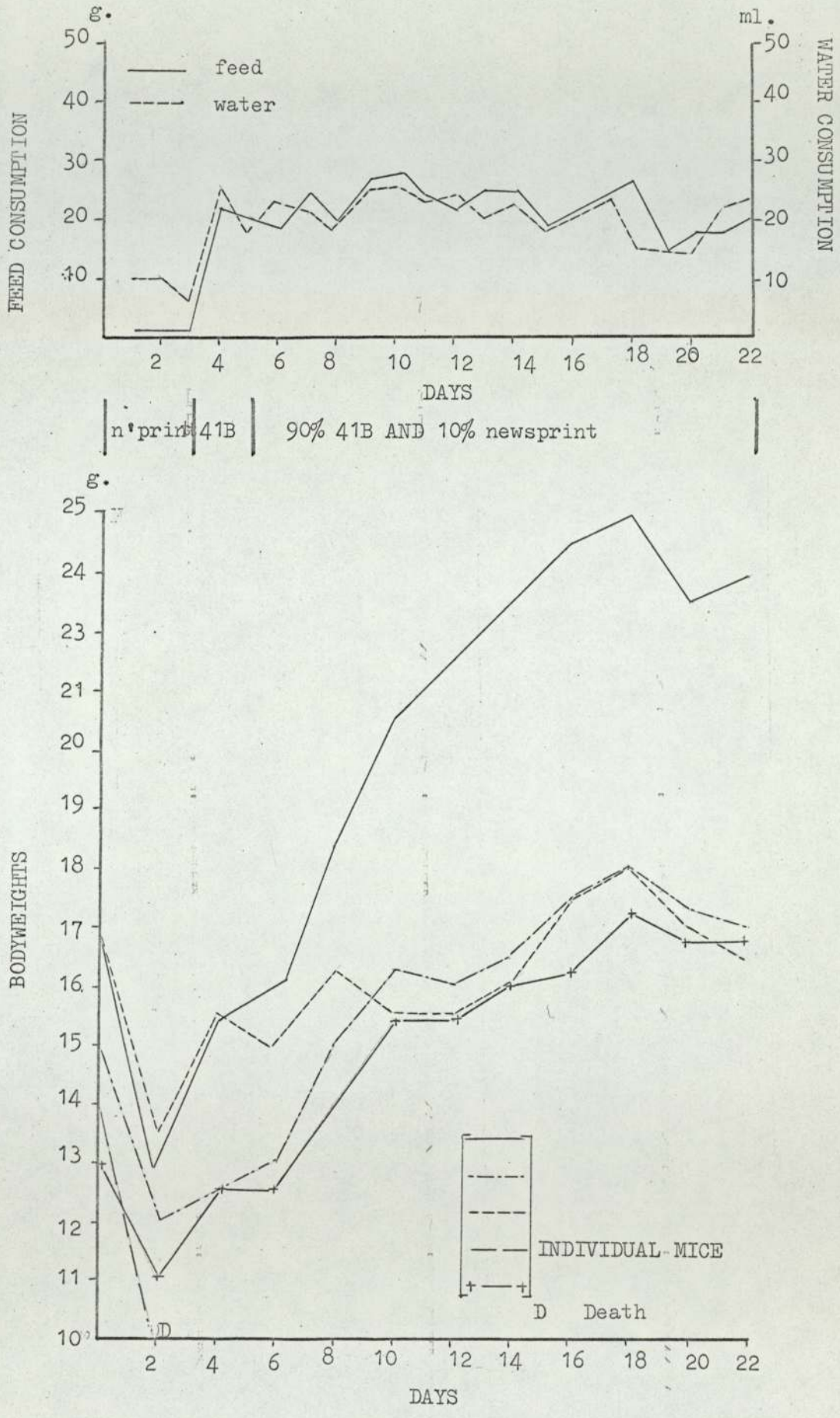


maximum amount consumed in any one day being less than 2g. During the feeding trials it became clear from the results, that if the remaining mice in the group were fed solely on the product of the solid substrate fermentations, then they would die. Thus on day 4 the mice were fed the normal diet of 41B and in a very short time (between days 4 and 6) they began to increase in weight and daily food consumption increased from zero to 15g. At the beginning of day 6 the mice were fed a diet consisting of 90 percent 41B and 10 percent of the product of the solid substrate fermentations and despite an initial drop in the weight of food consumed, between days 6 and 7, the 4 remaining mice continued to increase in weight until the termination of the feeding trials. After day 7 the weight of food consumed, each day, increased from 3g to 24g by day 9 and it remained approximately constant at this level until day 19 after which time it decreased to 19g. However, on the termination of the feeding trials the rate of food consumption had again started to increase. When the mice were placed on a diet of 90 percent 41B and 10 percent of the product of the solid substrate fermentations the volume of water consumed each day increased and then remained approximately constant at 20 ml until the termination of the feeding trials. During the early stages of the feeding trials the behaviour of the mice in this group had become very different from those of the control group, the mice were almost completely inactive remaining huddled together in the centre of the cage and suffering from periodic attacks of shivering. However, after being

fed the normal diet of 41B and then a second experimental diet of 90 percent 41B and 10 percent of upgraded newsprint their behaviour gradually became comparable with that of the control group. With the second experimental diet there was no detectable evidence of any rejection of the feedstuff by the mice.

Figure 7.5 shows the body weights and the weight of food and volume of water consumed each day by the group of mice fed initially on the newsprint. The initial weights of the mice varied from 13 - 17 g but after 2 days this variation had fallen to 10.5 - 13.5 g showing that all of the mice had lost weight. During these first 2 days one of the mice died. Only a minimal amount of the food (newsprint) provided had been consumed during the first 2 days and it became obvious that the remaining mice would also die unless their diet was changed. At the beginning of day 3 the mice were fed the normal diet of 41B and this quickly led to increases in body weights and the consumption of food and water. At the beginning of day 5 the diet was changed to 90 percent 41B and 10 percent newsprint, and the gains in body weight shown by the mice on the diet of 41B were continued until day 18, after which time small losses were again recorded. This second decline in the body weights of the mice continued until day 20, after which time one of the mice started to gain weight and the weight of another remained constant. When the 4 remaining mice were being fed the second experimental diet, one of them

FIGURE 7.5 BODYWEIGHTS OF THE MICE FED ON A DIET OF NEWSPRINT AND THE WEIGHTS OF FEED AND VOLUME OF WATER CONSUMED.



increased weight at a much faster rate than the others and on the termination of the feeding trials it weighed 23 g compared with an average weight of 16.75 g for the remaining 3 mice. The greater weight of this one mouse was probably due to the order of dominance in the mice; this could also account for the presence of the mice which showed very small gains in weight in other groups although fed an adequate diet. After day 4 the weight of food consumed each day varied from 15 - 28 g but remained essentially constant between these limits for the remainder of the feeding trials. During the same period of time the consumption of water also remained approximately constant fluctuating between 16 and 26 ml. In the first 3 days the mice in this group were almost completely inactive displaying the symptoms of shivering previously described for the mice on other diets. In the later stages of the feeding trials, when the mice were fed with 90 percent 41B and 10 percent newsprint their behaviour was comparable with the mice in the control group but there was again evidence of rejection of the newsprint in the diet by the mice.

Discussion

The feeding trials have shown that the product obtained from the solid substrate fermentation of newsprint by Sporotrichum thermophile var 2 is not acutely toxic. This is demonstrated by the fact that at neither the 10 percent

nor the 50 percent level of concentration of the product in the diet, were any ill effects suffered by the mice, even after prolonged consumption of the product.

When the product of the solid substrate fermentations was used in the diet at a concentration of 50 percent, the mice apart from an initial loss in weight suffered no ill effects and 4 out of the 5 in this group showed increases in body weight during the feeding trials. The fifth member of this group remained at a constant weight after the first 4 days and it appeared that this mouse did not fail to grow because of the diet but because of the domination exerted by the other mice in the group which were often aggressive towards it. There was some evidence that the mice rejected part of the product (i.e. the upgraded newsprint) when it was fed at this high level of 50 percent of the diet.

The gains in body weight of the mice fed on the diet of 50 percent upgraded newsprint and 50 percent 41B were not nearly as large as those of the control group fed exclusively on 41B. This type of result was expected because the upgraded newsprint was never meant to be a 'complete' feedstuff and mice are not able to completely digest this type of cellulose rich diet. The nutritional value of the upgraded newsprint can readily be seen by comparing the growth of the mice fed 50 percent upgraded newsprint plus 50 percent 41B with the mice fed on 50 percent plain newsprint plus 50 percent 41B. In the latter case, after 14 days 3 of the

5 mice had died and the other 2 mice had shown substantial losses in body weight and also the rejection of the newsprint in the feed pellets by the mice was particularly marked.

The feeding trials have also shown that the upgraded newsprint can not support the growth of the mice on its own and that the mice can not derive any nutritional benefit from the newsprint on its own. When the diets of these two groups of mice were changed and they were fed 90 percent 41B plus 10 percent upgraded newsprint and 90 percent 41B and 10 percent ordinary newsprint substantial gains in the body weights of the mice were observed for both groups. The first group of mice, fed 90 percent 41B plus 10 percent upgraded newsprint were placed on this diet at the beginning of day 6 and the second group of mice fed on 90 percent 41B plus 10 percent ordinary newsprint were placed on this diet at the beginning of day 5. Prior to this both groups of mice had been fed for two days on the normal diet of 41B. In a 16 day period after the two groups of mice had been placed on these diets (for the group 1 mice which were fed the diet containing 10 percent upgraded newsprint this period was between days 6 and 22 and for the group 2 mice which were fed the diet containing 10 percent ordinary newsprint this period was between days 5 and 21) the first group of mice appeared, from Figure 7.4, to increase in weight at a uniform rate but with the second group of mice the rate of increase in bodyweights noticeably declined after day 16 and shortly after this decreases in the body weights

of the mice were recorded. The differences in the changes of body weights, between the two groups of mice would indicate that the mice of group 1 were deriving some nutritional benefit from the upgraded newsprint which was not available to the mice of group 2 fed on the diet supplemented with ordinary newsprint.

A 't' test to detect the level of significance of the difference between the changes in body weight of the group 1 and group 2 mice described above produced the following results:-

Group 1

Gains in bodyweight (x_1)	$(x_1)^2$
8.00 g	64.00
5.25 g	27.56
7.00 g	49.00
10.75 g	115.54
$\leqslant x_1 = 26$	$\leqslant x_1^2 = 256.10$
$n_1 = 4$	
$\bar{x}_1 = 6.5$	
$(\leqslant x_1)^2 = 6.76$	
$\leqslant (x-\bar{x}_1)^2 = 87.1$	

Group 2

Gains in bodyweight (x_2)	$(x_2)^2$
4.25	18.06
4.50	20.25
1.75	3.06
7.00	49.00

$\sum x_2 = 17.5$ $\quad \sum x_2^2 = 90.37$

$n_2 = 4$

$\bar{x}_2 = 4.375$

$(\sum x_2) = 306.25$

$(x-\bar{x}_2)^2 = 13.81$

The general formula for the calculation of 't' is:-

$$t = \frac{\text{deviation between means to be compared}}{\text{standard deviation of the difference of the means}}$$

and

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where

$$s = \sqrt{\frac{\sum (x - \bar{x}_1)^2 + \sum (x - \bar{x}_2)^2}{n_1 + n_2 - 2}}$$

From the general formulae it can be calculated that:-

$$s = \sqrt{16.82}$$

and from this value of 's' it can be calculated that:-

$$t = 2.06$$

Therefore with a value of t = 2.06 and with 6 degrees of freedom a value of $P > 0.05$ but < 0.1 is obtained.

The results of the 't' test indicate that the value of 'P' lies between 0.05 and 0.1, which means that the differences observed in the gains in body weight between the mice in group 1 and group 2 were 90 - 95 percent certain to have been caused by the difference in the diets with only a 5 - 10 percent probability of the difference occurring by chance alone. However, with most biological research the level of significance taken is usually at least 95 percent ie $P < 0.05$ (Bishop, 1971). The inconclusive nature of the result obtained from the 't' test in this case was almost certainly due to the very small sample size of 5 mice in each group.

The 't' test has, however, indicated that it is probable that the difference in gains in body weights between the two groups of mice which were fed 90 percent 41B plus 10 percent upgraded newsprint and 90 percent 41B plus 10 percent ordinary newsprint, was due to the 10 percent of upgraded newsprint in the diet of one of the groups of mice. Thus it can be concluded that the newsprint was upgraded by the process of solid substrate fermentation with Sporotrichum thermophile var 2 to a feedstuff which was at least palatable (acceptable), digestible and to some extent nutritious and not acutely toxic.

CHAPTER 8

Chapter 8

Discussion and Conclusions

The micro-organisms which are thought to be principally responsible for causing the biodegradation of cellulose in composts, the thermophilic fungi and actinomycetes, were found to be indigenous to the samples of domestic refuse examined. Secondly many species of the thermophilic and mesophilic fungi isolated from the samples of domestic refuse taken from household dustbins, refuse wagons and the composting plant were also isolated from the experimental windrow.

The three isolation techniques employed to study the changes occurring in the microbial populations of the experimental windrow, during composting, revealed that the thermophilic actinomycetes became the dominant microbial population during the thermophilic phase of composting. Four species of thermophilic actinomycetes were isolated of which Thermomonospora curvata was the most prevalent, being frequently isolated by all three techniques. Stutzenberger (1971) had previously shown by comparing the 'C_x' cellulase enzyme produced in refuse composts with that produced by Thermomonospora curvata, that this actinomycete could probably be considered as a major cellulose decomposing organism in the composting process of municipal refuse. The results obtained from the experimental windrow lend

support to Stutzenburger's (1971) hypothesis.

The most frequently occurring cellulolytic thermophilic fungus isolated during the thermophilic phase of the experimental windrow was Chaetomium thermophile which was also consistently isolated by all three techniques. Chang (1967), Fergus (1969), Malik (1970) and Tansey (1971) have all reported upon the extremely cellulolytic nature of Chaetomium thermophile.

The fact that both Thermomonospora curvata and Chaetomium thermophile were isolated by the screened substrate tube (Eggins and Lloyd, 1968) indicates that both organisms were actively growing in the windrow and therefore it should be expected that both Thermomonospora curvata and Chaetomium thermophile are major cellulose decomposers in the composting process for municipal refuse and that cellulose degradation, the rate limiting step in the composting of refuse could be enhanced by encouraging the development of these organisms, especially the more numerous thermophilic actinomycetes.

Although the thermophilic actinomycetes were much more numerous in the experimental windrow than the thermophilic fungi they were not considered suitable micro-organisms for use in the development of a process for the microbial upgrading of waste cellulose because thermophilic actinomycetes are known to be pathogenic, causing farmers' lung disease (Cross, Maciver and Lacey, 1968). Research by

Waksman, Cordon and Umbreit (1939) and Chang (1967) had also demonstrated that the thermophilic fungi Humicola insolens and Chaetomium thermophile, respectively, could degrade cellulosic materials at a comparable rate to the total microbial population and much faster than thermophilic actinomycetes in pure culture.

In the screening programme designed to isolate cellulolytic fungi suitable for the microbial upgrading of newsprint thirteen species of thermophilic fungi were isolated. A comparison of the linear growth rates of thermophilic and mesophilic fungi, isolated in the screening programme and also of those fungi selected on the basis of reports in the literature, indicated that generally the thermophilic fungi had much faster growth rates on cellulose and newsprint agars than the mesophilic fungi. Tansey (1971) has shown, previously, that the cellulolytic rates of several of the thermophilic fungi were 2 - 3 times those of the mesophilic species.

In a secondary screening programme for the eight fastest growing thermophilic fungi, involving the use of shake-flask cultures with suspensions of ball-milled newsprint as substrate, Chaetomium thermophile var. dissitum and Sporotrichum thermophile var 2 were found to achieve the production of the highest protein contents with reference to the amount of substrate utilised. From the shake-flask cultures it was also found the use of urea as a nitrogen

source was highly favourable for promoting the synthesis of fungal protein and rapid degradation of the newsprint, this agrees with the findings of Chahal and Gray (1969) and Jones and Irvine (1972).

With the submerged fermentations, of the newsprint by both Chaetomium thermophile var. dissitum and Sporotrichum thermophile var 2 there was an almost complete utilisation of the available substrate. The generation times recorded for Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2, in this system of fermentation, were 5.7 hours and 1.6 hours respectively. These times are very short, especially for fungi, the shortest generation times previously recorded for fungi being in the order of 4 - 12 hours (Litchfield, 1968). Sporotrichum thermophile var 2 exhibited a growth rate comparable to those of some bacteria and yeasts and is remarkable when the nature of the substrate, containing 30 percent lignin, is taken into consideration. Spicer (1971) reported that fermentations designed to utilise, cellulose, hemicellulose and lignin substrates, could be very specialised of long duration and even then they may well not be economically feasible. Previously these criticisms have been justified because attempts to ferment cellulosic substrates have always required expensive pre-treatment of the substrate to make it amenable to microbial attack. The most common method of pre-treatment has nearly always involved acid hydrolysis and recently in the U.S.A. proposals have been made for the production of yeast from the cellulose fraction

of refuse by first hydrolysing the cellulose to glucose (Meller, 1968). The economics of such a system have, however, been described as highly unfavourable.

More recently Rogers, Coleman, Spino and Purcell (1972) reported that because of the large quantities of cellulose available, in refuse, for microbial upgrading, a process without the need for any pre-treatment would save time and money. However, after comparing the growth of Aspergillus fumigatus on untreated cellulose and samples of cellulose variously treated by alkali-oxidation, high temperature hydrolysis, nitrite photochemical treatment and electron irradication Rogers et al (1972) concluded that without an effective pre-treatment process recycling cellulose can not become an economically feasible reality. This conclusion was reached because of the poor growth rates obtained by Aspergillus fumigatus on the untreated cellulose. In the present investigation, with the submerged fermentations, both Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2 were able to obtain almost a complete breakdown and utilisation of the newsprint; Chaetomium thermophile var dissitum achieving protein production of 19.7 percent and utilisation of 97.6 percent of the substrate after 20 hours and Sporotrichum thermophile var 2 achieving protein production of 19.2 percent and 90 percent utilisation of the substrate within 11 hours. These results clearly indicate that it is, practically, possible to ferment cellulose substrates, such as waste newsprint, without the

need for any form of pre-treatment other than simple mechanical grinding.

The degree of utilisation of the newsprint which contained, approximately, 30 percent of lignin indicated that both Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2 must have degraded substantial proportions of the lignin as well as utilising the cellulose and hemicellulose fractions. The yeild factors (weight of mycelium formed divided by weight of substrate used) for Chaetomium thermophile var dissitum and Sporotrichum thermophile var 2 were 0.61 and 0.56 respectively. These yield factors are extremely encouraging as those reported for other fungi are usually in the region of 0.48 - 0.50 (Spicer, 1971).

Thus the system of submerged fermentation, which presented an opportunity to study the growth of the thermophilic fungi on a cellulose substrate under optimum conditions, has demonstrated that at least two species of the thermophilic fungi can very rapidly degrade cellulose, even when that cellulose is associated with lignin, without the need for expensive and complicated pre-treatment processes.

In the system of solid substrate fermentation of the newsprint it was only possible to obtain a good degree of growth with Sporotrichum thermophile var 2. Repeated attempts to culture other thermophilic fungi, in this

system were unfortunately not successful. It was subsequently shown that this inhibition of the other fungi was almost certainly caused by the high osmotic pressure of the concentrated nutrient solution used. With Sporotrichum thermophile var 2 a product was obtained, after 6 days growth, with a protein content of 6.5 percent, which was shown in the preliminary feeding and toxicity trials, with mice as the test animals, to be nutritious and not acutely toxic. It is realised that a 'scale-up' in production would be desirable so that feeding trials with either herbivores or ruminants could be carried out to more accurately assess the nutritional qualities of the newsprint; such feeding trials were outside the scope of the present investigation.

During the development of the technique of solid substrate fermentation it was found that the system of inoculation and manipulation of the environmental conditions was more than adequate to suppress the growth of contaminants such as the heavily sporing Aspergillus fumigatus, even after large numbers of its spores had been deliberately introduced onto the substrate. In a system of fermentation where it is not intended to use the more usual methods of sterilising the substrate and media it is very important to establish a dominant fermentation by preventing microbial encroachment with possible contamination by pathogenic or parasitic micro-organisms.

The two systems of fermentation developed during the present investigation are widely different in concept. The system of submerged fermentation used in the present investigation differs from most other types of submerged

fermentation in consisting of 4 phases which are; two solid phases, the cellulose suspension and the growing micro-organism; an aqueous phase, the fermentation medium and a gaseous phase required for aeration. Most systems of submerged fermentation contain only one solid phase, the micro-organism, the substrate usually being soluble. All of the ingredients essential for the growth of a micro-organism in this system are present in the aqueous phase with the exception of oxygen and since oxygen must cross gas-liquid interfaces it can be the component limiting cell growth. The rapid and extensive growth of two species of thermophilic fungi on the suspensions of ball-milled news-print indicates that, for the first time, the fermentation of cellulose using a method of submerged fermentation could be competitive with the fermentation of other, more amenable, carbohydrates such as molasses and starch. However, all fermentations of this nature suffer from the same draw-backs, in particular that of processing costs. A flow diagram of the steps which would be involved in the fermentation of cellulose by this technique is presented in Figure 8.1.

Submerged fermentations require sophisticated apparatus to provide optimum conditions of growth for micro-organisms including expensive sterilising and product processing equipment and a large labour force for their operation. Understandably these fermentation techniques can only be operated economically on a large scale and are unsuitable for small scale production and so cannot take advantage of the localised production of small quantities

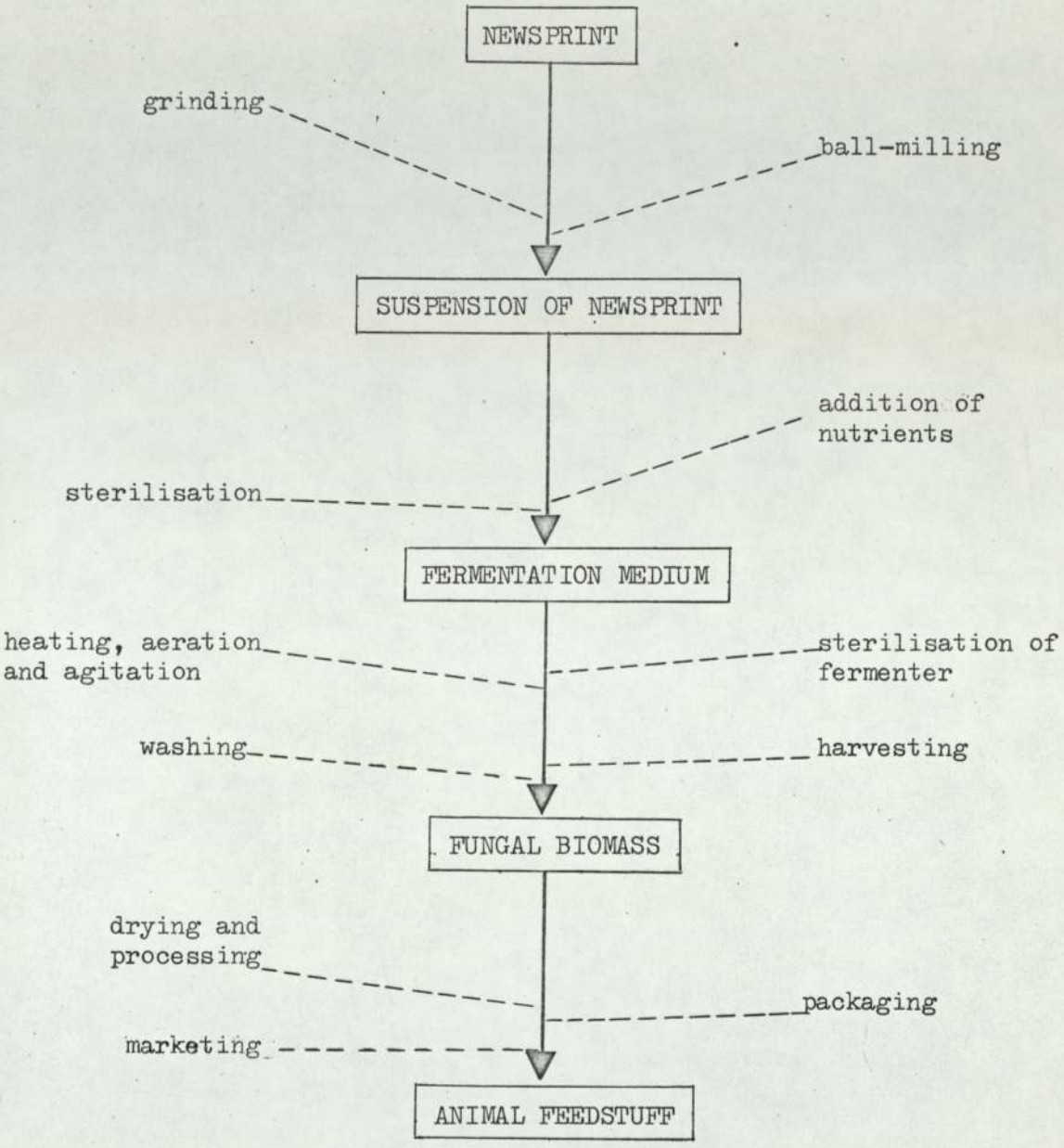


FIGURE 8.1 STAGES IN THE SUBMERGED FERMENTATION OF SUSPENSIONS NEWSPRINT.

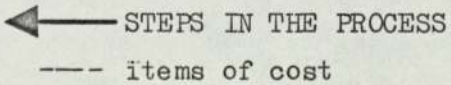


Table 8.1 Items of economic importance for the production of microbial protein by a standard process of submerged fermentation.

(A) CAPITAL INVESTMENT

1. Plant and Machinery
2. Land
3. Buildings (Laboratories and Offices)

(B) PROCESSING COSTS

1. Raw material
   - (a) ease of collection to a central site
   - (b) availability to a given site
   - (c) bulk handling properties
   - (d) seasonal fluctuations in availability
2. Sterility requirements
   - (a) Microbial encroachment
3. Fermentation
   - (a) residence time in reactor (doubling time)
   - (b) cell concentrations attainable
   - (c) operating temperature (cooling water)
   - (d) oxygen requirements
   - (e) power requirements for mass transfer
   - (f) heats of reaction
   - (g) cell yields per pound of substrate consumed
   - (h) foaming tendency

Table 8.1 (continued)

4. Cell harvesting techniques
5. Washing and purification techniques for removal of
   (a) substrate residues
   (b) raw material impurities
   (c) nucleic acids
   (d) metabolic by products
6. Product value
   (a) percentage protein
   (b) limiting amino acid
   (c) digestibility

(C) LABOUR COSTS

1. Scientific staff
   (a) Microbiologists
   (b) Chemists
2. Managerial staff
3. Process operators
4. Maintenance engineers
5. Instrument engineers.

of substrates which occurs with the production of cellulosic wastes. The items of cost for such techniques are listed in Table 8.1 which was compiled from information given by Callihan and Dunlap (1969) and Spicer (1971).

The process of solid substrate fermentation developed from the traditional food fermentation processes for the production of tempeh, ragi, etc., is much simpler than the technique of submerged fermentation and is ideal for small scale, local upgrading of cellulose wastes. The steps necessary for upgrading cellulose wastes by this process together with the items of cost are outlined in Figure 8.2. It is envisaged that the process of solid substrate fermentation, after further development could be carried out by unskilled labour, requiring only a simple grinding machine to reduce the substrate to a condition suitable for fermentation, the addition of a nutrient solution, inoculation with the desired fungus (which could be achieved by seeding with the product of the previous fermentation once the process has been initiated), and fermentation in a growth chamber similiar to those used in the mushroom industry.

The simplicity of solid substrate fermentation, assumes greater significance than merely being a desirable alternative for wastepaper disposal because waste cellulose is readily available everywhere. In rural areas cellulose is available as corn cobs, rice hulls, wheat straw, bagasse, etc. In urban areas solid wastes contain large portions of newspapers, books, rags, towels, wood, etc. Clearly a process of solid substrate fermentation developed for the

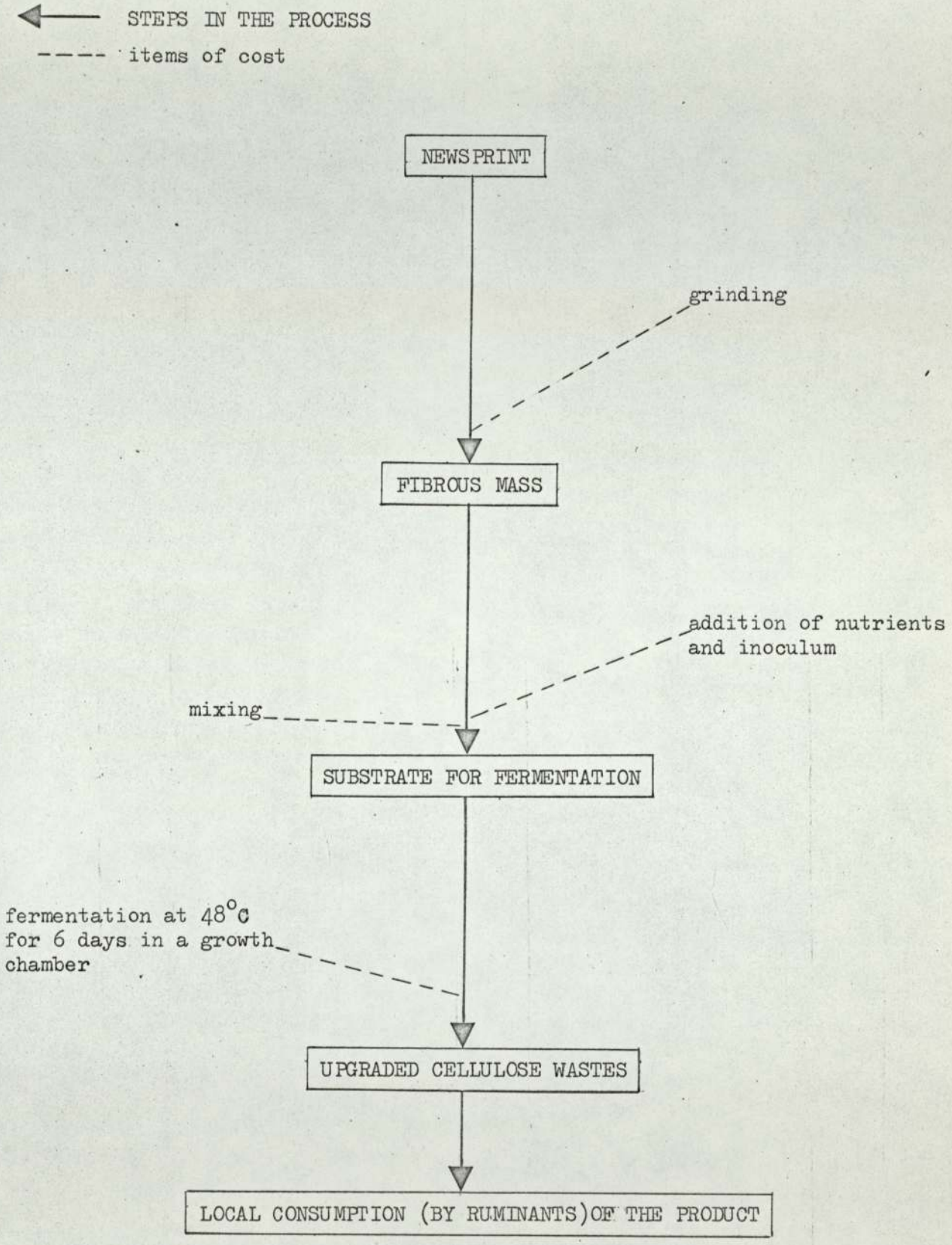


FIGURE 8.2 STAGES IN THE SOLID SUBSTRATE FERMENTATION OF NEWSPRINT

utilisation of these locally produced wastes could make a significant contribution to the production of animal feedstuffs. If, instead, a centralised plant were built to utilise such wastes in a system of submerged fermentation, the costs of transporting the raw materials to the plant and the product back to the farm might be prohibitive.

It must be emphasised that the processes described above are only at the laboratory scale and at the present time it is not possible to produce a detailed breakdown of the processing costs. At this stage cost can only be considered on the basis of the items in each process described above. Reviewing protein production by microfungi, Spicer (1971) suggested that although it was not possible to give a general answer to the question of cost preliminary cost estimates could be made in terms of elements in the process.

Industrial concerns producing protein rich microbial foods, such as yeast from hydrocarbons, have so far concentrated their marketing efforts towards the animal feed market. This has been a deliberate choice and not one that was dictated by any shortcomings in the products (Shacklady, 1970). If animals in one country find the material acceptable and do well on it, then it is highly probable that essentially similiar results could be obtained generally. Whilst as far as animals are concerned it is possible to think in terms of a universally acceptable product, the same cannot be said of man. Although local and regional tastes in food are being investigated and attempts made to produce feeds aesthetically pleasing

and economically acceptable, the deficiency in protein rich feedstuffs is worsening.

Feeding man via the animal is less efficient than feeding him directly, but improving the nutritional status of man by improving the production of livestock is a more sure and rapid way than trying to induce him to consume something to which he is unaccustomed.

In practice the yeast produced from such processes as hydrocarbon fermentation will, in the developed countries, be used in pig and poultry feeds (Shacklady, 1970). The reason for this decision is primarily an economic not a nutritional one because ruminants, such as cattle and sheep, are capable of utilising low quality proteins and even simple nitrogenous compounds, such as urea, quite effectively an attribute denied to non-ruminants. Shacklady (1970) considers that the poorer quality proteins and urea will be cheaper than such materials as fishmeal, soya and single cell proteins and equally effective for ruminants. Consequently it is doubtful if yeasts or other high quality proteins will be competitive with low grade proteins and nitrogenous substances.

The upgraded newsprint produced from the solid substrate fermentations of the present investigation contains undegraded cellulose, some protein and almost certainly some vitamins present in the fungal mycelium. This indicates that the upgraded newsprint should be an excellent feedstuff for ruminants, satisfying both their energy and protein requirements. The fungal protein

content of the upgraded newsprint should produce better growth results in cattle than a corresponding amount of nitrogenous material of low biological value, such as urea, since it has been reported by Spicer (1971) that biosynthesised protein is more efficient as a feedstuff.

In the introduction to this thesis, the increasing cellulose content of municipal refuse of many cities in the U.K. and the U.S.A. was mentioned as were the present major methods of refuse disposal, tipping, incineration and composting. It was shown that, at the present time, no wholely acceptable method of disposal for these wastes existed and one of the major reasons for this was the contamination of wastes with other materials present in the refuse. It was suggested that these difficulties could be overcome by organising separate collecting systems for the major components of town waste, which would have the effect of creating valuable resources of raw materials readily available for recycling processes. The present investigation has shown that it is feasible by using highly cellulolytic micro-organisms, species of thermophilic fungi, to upgrade waste paper, specifically newsprint, and produce an animal feedstuff which is both nutritious and non-toxic.

The research which demonstrated that cellulosic materials, such as newspaper and other grades of waste papers, are acceptable animal feedstuffs has been fully discussed in this thesis. It has recently been found that a number of benefits are obtained by feeding cattle rations of waste paper over a prolonged period of time.

Meinhardt and Kolb (1971) reported that cattle fed on wastepaper, produced carcasses exhibiting a lower percentage of fat resulting in a higher percentage of prime and total retail cuts and also gained weight 5-20 percent faster than those cattle fed with normal rations. The findings of Meinhardt and Kolb (1971) indicate the potential for a product, such as the upgraded newsprint, which incorporates fungal protein and vitamins into a raw material of proven nutritional value.

The purpose of feeding upgraded waste paper to cattle is twofold, first the reduction of the burden of solid wastes and secondly the production of high class animal protein to alleviate the world protein deficiency. Meinhardt and Kolb (1971) estimated that in the U.S.A. 3.5 billion tons of solid wastes are generated each year of which 2 billion tons consists of agricultural crop and animal wastes and 295 million tons of cellulose and other organic materials, this represents a total of 2.3 billion tons, the remainder being largely mineral wastes. From this information Meinhardt and Kolb (1971) calculated that of the 2.3 billion tons of organic wastes 0.8 billion would be sufficient to feed the U.S.A.'s present 112 million cattle population, with the proviso that it was given some processing, adequate distribution and necessary ration supplements; a system of solid substrate fermentation could make a significant contribution towards supplementing this type of ration. Three times the present cattle population of the U.S.A. could consume two thirds (i.e. 2.3 billion tons) of all the solid wastes generated in that country.

Even the most selective utilisation of available organic wastes would be more than sufficient to feed all such animals releasing all or most of the cereal grain presently used for such purposes for human consumption. In the U.K. it has been estimated (Fulbrook et al, 1973) that, on average, about 4½ million tons of straw are produced per annum. While a considerable proportion is used for bedding much of the straw is burnt in the field, although it would make an ideal substrate for upgrading to a feedstuff for ruminants.

Recent estimates of the world's population and food supplies, suggest that in the near future an increasing deficiency of the raw materials currently used in concentrate feeds in intensive or semi-intensive systems of animal production is likely to develop (Meller, 1968; Burt, 1972). The use of high protein feedstuffs is also estimated to increase faster than that of the concentrates (Meller, 1968). The deficit described above is likely because of the probability that human population will increase more rapidly than food crop production, giving rise to an increased demand for animal products and decreasing surplus foods to be used in their production (Burt, 1972). Spicer (1971) expressed the opinion that in the future there would be a market for biosynthesised proteins because the demand for protein is increasing and a considerable amount of material that until recently was used for animal feeding is now going for human food. Thus the predictions for worsening shortage of animal feedstuffs mean it is likely that a market for processes and products, such as that developed in the present investigation, will become established.

In conclusion it can be stated that species of the thermophilic fungi, by virtue of their enhanced rate of metabolism and cellulolytic activity, may, in the future have an important role as industrial micro-organisms; particularly in the field of biodegradation of cellulose wastes. In the past the thermophilic fungi have virtually been ignored as industrial micro-organisms and to the best of the author's knowledge there are not any publications available which mention the deliberate selection of thermophilic fungi for inclusion in a currently operative industrial process. The exclusion of thermophilic fungi from industrial processes is surprising, when in the fermentation industry problems have so often been encountered with cooling the fermenters to allow the growth of mesophilic or thermotolerant strains (Meller, 1968; Mateles, 1968). Recent investigations by Crisan (1969) indicated that the proteins of thermophilic fungi are similiar to those of mesophilic fungi and that they are not peculiarly adapted to achieve thermostability, indicating that they are almost certainly as valuable, commercially, as those of mesophilic fungi. Finally, it should be made clear that in spite of the natural abundance of cellulose a very insignificant portion of the estimated 190 billion metric tons of available cellulose has been exploited for commercial purposes (Ghose, 1969). The reason for this state of affairs may be attributed to the sluggish hydrolytic breakdown of cellulose achieved by the cellulase enzymes of most micro-organisms. Therefore it is all the more surprising that a group of micro-organisms, the thermophilic fungi, which in the past have frequently been associated with the specialised ecological niches (composts),

where it is known that cellulose degradation occurs at an enhanced rate, should for so long have been almost totally ignored.

APPENDICES

APPENDIX 1

Eggins and Pugh (1962) Cellulose Agar

Potassium dihydrogen orthophosphate	1.0g
Ammonium sulphate	0.5g
Potassium chloride	0.5g
Yeast extract	0.5g
L. asparagine	0.5g
Magnesium sulphate	0.2g
Calcium chloride	0.1g
Oxoid agar No. 3	20.0g
Ball-milled cellulose suspension (4.0%)	250ml
Rose Bengal	0.066g/litre
Distilled water	to 1 litre

For the glucose-starch agar the cellulose suspension was substituted with 5g glucose and 5g starch.

APPENDIX II

Tables 1-12 from Chapter 2 showing the percentage frequency of occurrence of those fungi isolated from samples of refuse and bin scrapings taken from household dustbins.

Table 1, Part A

Percentage frequency of occurrence of fungi isolated from samples of refuse from household dustbins on cellulose agar by Warcup's technique at 50°C.

FUNGI	Bins from residential area										Bins from council estate									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	30	85	60		10	60	10		85	25	15	25	15	15	5	5	25	15	5	20
Cephalosporium sp.													5			35			40	
Chaetomium thermophile	50			35	20		25			55	10	20		5		20	25		20	
Humicola grisea	10																			
Humicola insolens		65							70		5		10					10		
Humicola lanuginosa	80									80	10		30			10	20		10	
Malbranchea pulchella	10			20				15		15										
Mucor pusillus												5								
Sporotrichum thermophile																				20
Talaromyces duponti		75							75				65				15			65
Torula thermophila	80	90		20					85	80		20								
Actinomycetes	40	15	15		30	25	30	35	10	50	5	55		5		65	50	20		20

Table 1, Part B — Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on glucose-starch agar at 50°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	45	85	95	45	60	90	55	40	80	40	80	45	75	80	10	95	40	65	90	80
Cephalosporium sp.													10			60			55	
Chaetomium thermophile	35			25	45		40			10	25	5		20		20	10		15	
Humicola grisea	15																			
Humicola insolens		15							10		15		10	20		20				
Humicola lanuginosa	80									75	25		25	20			5			
Malbranchea pulchella	20									20										
Mucor pusillus																				
Sporotrichum thermophile																				
Talaromyces duponti	40				20		25		35				25				5			
Torula thermophila	55	10		10							60						5			
Actinomycetes	45				20		25	5		40	15		20			5	10		10	

Table 2, Part A — Percentage frequency of occurrence of fungi isolated from samples of bin scrapings, from household dustbins, on cellulose agar by Warcup's technique at 50°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	15		20	50	15	5			15	45	80	65	5	80		80	5	25	85	70
Cephalosporium sp.																				
Chaetomium thermophile	20		15	45	15				20	40										
Humicola grisea																				
Humicola insolens	10									70										
Humicola lanuginosa				70																
Malbranchea pulchella		5		60				5		55										
Mucor pusillus																				
Talaromyces duponti																				40
Thermoascus aurantiacus				15						15										
Torula thermophila				15						10										
Actinomycetes				10					15		25		45					40	30	

Table 2, Part B — Percentage frequency of occurrence of fungi isolated from samples of bin scrapings, from household dustbins, on glucose-starch agar by Warcup's technique at 50°C.

	Bins from residential area										Bins from coucil estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	5		5		10	10		15		5	95	80	55	45		40	10	45	100	85
Cephalosporium sp.																20				
Chaetomium thermophile	10			40						40										
Humicola grisea																				
Humicola insolens	5									5										
Humicola lanuginosa				60						55										
Malbranchea pulchella		10		10																
Mucor pusillus		5						5												
Talaromyces duponti																				
Thermoascus aurantiacus																				
Torula thermophila				25						20										
Actinomycetes			40							35			5					5		

Table 3, Part A

Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on cellulose agar by Warcup's technique at 45°C.

	Bins from a residential area										Bins from a council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	20	75	60	100	80	60	80	100	75	15	75	100	95	70	100	100	100	100	100	95
Cephalosporium sp.		10							5				80			60	15		65	80
Chaetomium thermophile	25			35	20		20			30						5			5	
Humicola grisea																				
Humicola insolens											15		5	15						
Humicola lanuginosa												20	20			20	25		20	
Malbranchea pulchella				25				20												
Mucor pusillus												40	10				45			
*Penicillium funiculosum			40			35														
Talaromyces duponti			5						5											
Torula thermophila	85			20						80										
Acintomycetes	70	40	20	80	75	15	85	70	40		25	20		35			15		5	

* Denotes mesophile

Table 3, Part B

Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on glucose-starch agar by Warcup's technique at 45°C.

	Bins from a residential area										Bins from a council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	60	90	50	100	85	55	80	100	90	65	85	100	100	80	100	100	100	100	95	100
Cephalosporium sp.		5							5				10			40	90		40	5
Chaetomium thermophile	15			30	5		5			10						15			15	
Humicola grisea	20																			
Humicola insolens											5									
Humicola lanuginosa													5				5			
Malbranchea pulchella																				
Mucor pusillus												100	5				100			
*Penicillium funiculosum																				
Talaromyces duponti		5																		
Torula thermophila	60									65										
Actinomycetes	50			80	95		90			15	30			35		40			40	

* Denotes mesophile

Table 4, Part A

Percentage frequency of occurrence of fungi isolated from samples of scrapings, from household dustbins, on cellulose agar by Warcup's technique at 45°C.

	Bins from residential area										Bins from a council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	75	10	95	80	75	10	5	70	95	90	30	50	25	30	5	100		20	20	55
Cephalosporium sp.									10											
Chaetomium thermophile	10			20	5					15										
Humicola insolens	5				10															
Humicola lanuginosa																20				
Malbranchea pulchella	20		90				20		85											
Mucor pusillus	5	5																		
Actinomycetes	5		5		5				5		15		75		20	80	25		15	

Table 4, Part B — Percentage frequency of occurrence of fungi isolated from samples of scrapings, from household dustbins, on glucose-starch agar by Warcup's technique at 45°C.

	Bins from residential area										Bins from a council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	80	20	80	85	85		50	100	85	85	70	65	25	100	40	30	35	100	60	70
Cephalosporium sp.			20						30											
Chaetomium thermophile																				
Humicola insolens																				
Humicola lanuginosa																				
Malbranchea pulchella		5		25						20										
Mucor pusillus	20	25		5	20			5		10										
Actinomycetes											10		20		10	25	10		5	

Table 5, Part A — Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on cellulose agar by Warcup's technique at 40°C.

	Bins from a residential area										Bins from a council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	30	70	65	100	50	65	40	100	65	30	80	95	75	85	100	80	95	100	85	75
Aspergillus fumigatus (O.V.)																				
Aspergillus niger																	5			
Cephalosporium sp.												20	15			60	25		65	20
Chaetomium thermophile	5			40				35		5										
*Coprinus sp.	5																			
*Eurotium sp.												10				15				
Humicola insolens										50		10	45							15
Humicola lanuginosa													25							
Mucor pusillus					20		15					75	15				80			20
*Penicillium funiculosum			45			60														
Talaromyces duponti		5																		
Torula thermophila	20			25				20		100	40			35						
Actinomycetes	75	80	80	20		75		25	75	70	30			35						

* Denotes mesophile

Table 5, Part B

Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on glucose-starch agar by Warcup's technique at 40°C.

	Bins from a residential area										Bins from a council estate									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	75	90	45	100	55	45	55	100	85	75	85	100	100	90	100	75	100	100	70	100
Aspergillus fumigatus (O.V.)				35				40												
Aspergillus niger																	10			
Cephalosporium sp.													45			60	100		60	40
Chaetomium thermophile	10			35				40		10										
*Coprinus sp.																				
*Eurotium sp.																5				
Humicola insolens												10		5						
Humicola lanuginosa													15							
Mucor pusillus					45		45					100	5				95			5
*Penicillium funiculosum			20			20														
Talaromyces duponti		35							30											
Torula thermophila	35									25										
Actinomycetes	25	20						15						20			50		40	

*Denotes thermophile

Table 6, Part A

Percentage frequency of occurrence of fungi isolated from samples of scrapings, from household dustbins, on cellulose agar by Warcup's technique at 50°C.

	Bins from a residential area										Bins from a council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	50	5		80	55	15	10	5	95	90	60	55	70	40	45	40	45	20	60	75
Cephalosporium sp.	85	10		75	90	5	5	20	85	85	50	85	100	95	35	100	35	100	55	85
Chaetomium thermophile	25				20															
Malbranchea pulchella				30						25										
Mucor pusillus							10	5												
Actinomycetes									5		20			70		40			15	

Table 6, Part B

Percentage frequency of occurrence of fungi isolated from samples of scrapings, from household dustbins, on glucose-starch agar by Warcup's technique at 40°C.

	Bins from a residential area										Bins from a council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	60	10		90	50	30	20	15	100	100	80	75	80	65	50	40	55	35	55	100
Cephalosporium sp.	80			80	75		10	30	80	85	65	100	100	80	30	100	40	95	45	60
Chaetomium thermophile																				
Malbranchea pulchella				15						10										
Mucor pusillus				35			10	20		40										
Actinomycetes									20					15	20	20			20	

Table 7, Part A

Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on cellulose agar by Warcup's technique at 35°C.

FUNGI	Bins from residential area										Bins from council estate									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	75	65	60	60	40	60	40	65	60	80	95	100	100	95	100	100	100	100	100	100
Aspergillus fumigatus (O.V.)												20				60			65	
*Aspergillus niger	20								30	25		70					70			
*Aureobasidium pullulans						10		15												
Cephalosporium sp.		25			80		75		20							40			45	
Malbranchea pulchella																	5			
*Monilia brunnea																				
Mucor pusillus		45		15	10		15	20	40			100	15				100			10
*Paecilomyces varioti																				
Penicillium funiculosum			70	40		75		45				95				20	95		20	
Torula thermophia	60									55										

* Denotes mesophile

Table 7, Part B

Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on glucose-starch agar by Warcup's technique at 35°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	100	10	60	85	40	65	40	80	10	100	95	100	100	100	100	100	100	100	100	100
Aspergillus fumigatus (O.V.)												55							5	
*Aspergillus niger									75			10								
*Aureobasidium pullulans					10		5													
Cephalosporium sp.					60		55									20	40		15	
Malbranchea pulchella												40								
*Monilia brunnea			30																	
Mucor pusillus	70	100			40		45		95	65	100	45					100			40
*Paecilomyces varioti			15			20														
*Penicillium funiculosum	5	85	35	95		40		100	90	5	25			30	50			45	5	
Torula thermophila	15									55										

* Denotes mesophile

Table 8, Part A

Percentage frequency of occurrence of fungi isolated from samples of scrapings, from household dustbins, on cellulose agar by Warcup's technique at 35°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	60	5	45	50	55	15	15	5	50	45	15	100	100	100	100	100	100	100	15	100
*Aspergillus niger														10						
Cephalosporium sp.	10		50						50											
Malbranchea pulchella				10						5										
Mucor pusillus								5				10								5
*Paecilomyces varioti																				
*Penicillium funiculosum										25										

*Denotes mesophile

Table 8, Part B

Percentage frequency of occurrence of fungi isolated from samples of scrapings, from household dustbins, on glucose-starch agar by Warcup's technique at 35°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus fumigatus	90	10	60	20	90	10	5	20	70	25	80	100	100	100	100	100	100	100	80	100
*Aspergillus niger														15						
Cephalosporium sp.	50		55		45				60											
Malbranchea pulchella																				
Mucor pusillus																				
*Paecilomyces varioti			15						20											
*Penicillium funiculosum			20						25											

*Denotes mesophile

Table 9, Part A

Percentage frequency of occurrence of fungi isolated from samples of refuse, froun household dustbins, on cellulose agar by Warcup's technique at 30°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alternaria sp.							15			30										
Arthrobotrys sp.																				
Aspergillus fumigatus			50		5	50	5				70	70		75	90			95		
Aspergillus niger																	15			
Aspergillus sp.												20								
Cephalosporium sp.	100	95	20	65	95	15		60	95	100	5									20
Cerastomella sp.																				20
Chaetomium globosum										20										
Eurotium vermiculatum																15			10	
Eurotium sp.																				
Fusarium sp.	75	100	20		65	30	70	60	100	75	45	85		40	25	100	85		100	
Gliocladium roseum	100	5	20	100		25		100	5	100		95	100	65	95	75	100	95	80	100
Gliomastix sp.											75									
Humicola grisea							10			80		30		40		10	30			

Cont'd/.....

Table 9, Part A Cont'd

FUNGI	Bins from residential area										Bins from council estate									
	1	2	3	4	5	6			9	10	11	12	13	14	15	16	17	18	19	20
Monilia brunnea																				10
Mucor globosus	100	100	25	85		20	50	80	100	100	100	95	25	100	100	100	100	95	100	20
Paecilomyces varioti																		20		55
Penicillium funiculosum	100	100	100	45	85	100	85	45	100	100	100	65	100	100	55	25	70	55	30	100
Penicillium sp. 2		25		10	20		20						5		35					
Periconia sp.							15													
Rhizopus nigricans				75															25	
Sporotrichum pruinosum		15							5											
Stachybotrys atra	60									65										
Stysanus sp.											40	40	100	35	20		40	15		100
Torula thermophila +											5									
Trichoderma viride	75			60				65	80			85		15	10	75			15	80
Ulocladium sp.													25		20			30		
Verticillium lateritium					85		85													
Zygorhyncus moelleri																				

+ Denotes thermophile

Table 9, Part B — Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on glucose-starch agar by Warcup's technique at 30°C.

FUNGI	Bins from residential area										Bins from council estate									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alternaria sp.							25			15										
Arthrobotrys sp.	20																			
Aspergillus fumigatus		15	40			40			15		70	100		75		90				80
Aspergillus niger									40			100					95	100		
Aspergillus sp.														40						
Cephalosporium sp.	85	100	20	5	100	20		5	100	85	50			45						
Cerastomella sp.																				
Chaetomium globosum																				
Eurotium vermiculatum																		5		
Eurotium sp.																				
Fusarium sp.		5			100		100	10		5	35	20		40		20	25		15	
Gliocladium roseum	30			10		10		5		30		10	20	5	80	15	15	85	15	15
Gliomastix sp.																				
Humicola grisea							5			70	5									

Cont'd/....

Table 9, Part B Cont'd

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Monila brunnea																				
Mucor globosus	45	100	40	100		40	60	100	100	50	70	100	60	65	70	20	100	65	20	65
Paecilomyces varioti					20															5
Penicillium funiculosum	95	100	100	100	85	100	90	100	100	95	80	100	100	80	100	95	100	100	100	100
Penicillium sp.2		25			25	5		5			25		80	20	30	100		80		75
Periconia sp.							5													
Rhizopus nigricans				100				100								100			100	
Sporotrichum pruinosum																				
Stachybotrys atra																				
Stysanus sp.																				
Torula thermophila +																				
Trichoderma viride	20			100				95		25			25	40	5	10	70		10	20
Ulocladium sp.																				
Verticillium lateritcum					45		40													
Zygorhyncus moelleri																		5		

+ Denotes thermophile

Table 10, Part A

Percentage frequency of occurrence of fungi isolated from samples of scrapings, from household dustbins, on cellulose agar by Warcup's technique at 30°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alternaria sp.		25						20	5								50			
Aspergillus fumigatus			15	15	20	5	5			20								55	5	
Aspergillus niger	5		25											45		40				
Aspergillus sp.			5												75		80			
Aureobasidium pullulans		35		25	15			40												
Cephalosporium sp.	45	30	100	50	40		100	25	100	55	30	50					15	15	30	45
Cerastomella sp.												10								
Chaetomium globosum	5		25						20											
Fusarium sp.	45	20		60	40			50	15	40		25			60		60			20
Eurotium sp.																				

Cont'd/...

Table 10, Part A Cont'd

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Eurotium vermiculatum																				
Gliocladium roseum	45				40													60		
Humicola grisea	55				50													40		
Monilia brunnea		25	35					20			75				55		60	60	80	40
Mucor globosus	70		20	90	70				20	90	100	65						60	100	60
Paecilomyces varioti									5											
Penicillium funiculosum	70	90	95	40	65	65	60	85	100	40	45	80	65	100	100	100	100	60	50	80
Penicillium sp.2		25	20			5		20			20			65	40	60	40		25	
Rhizopus nigricans										15								20		
Sporotrichum pruinosum	30																			
Trichoderma viride	40			50	35				5	45	100	90							100	80
Ulocladium sp.		40						35												

Table 10, Part B

Percentage frequency of occurrence of fungi isolated from samples scrapings, from household dustbins, on cellulose agar by Warcup's technique at 30°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alternaria sp.		5						40	5								10			
Aspergillus fumigatus			15	35	10	10	10			40			50		35			50		
Aspergillus niger	20		25											60		65				
Aspergillus sp.		5													20		20			
Aureobasidium pullulans		5			5															
Cephalosporium sp.	100	30	50	75	95		100		55	70		10		35				40		
Cerastomella sp.												5								
Chaetomium globosum			25						5											
Fusarium sp.	15	5		85	20			20		90					20		15			
Eurotium sp.												10								

Cont'd/...

Table 10, Part B Cont'd

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Eurotium vermiculatum										20										
Gliocladium roseum	5				20								10					5		
Humicola grisea	5												5							
Monilia brunnea		25	35																	20
Mucor globosus	90		55	100	90				60	100	100	95	100					100	100	100
Paecilomyces varioti									10											
Penicillium funiculosum	70	90	100	30	65	95	90	70	100	25	55	100	100	100	80	100	100	100	55	100
Penicillium sp.2		5	55			30		20						100	80	100	75		60	
Rhizopus nigricans	70	10			65			5	50											
Sporotrichum pruinosum	5																			
Trichoderma viride	10			30						25	100	10							100	
Ulocladium sp.		30						25												

Table 11, Part A

Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on cellulose agar by Warcup's technique at 25°C.

FUNGI	Bins from residential area										Bins from council estate									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alternaria sp.	40				15		20	20		45										
Aspergillus fumigatus	5	20	90		45		40		15											
Aspergillus niger															15					
Aspergillus sp.															25					
Cephalosporium sp.	55	65		55	75				60	55	15	60		20	20		65			
Chaetomium globosum	25							20		25										
Fusarium sp.	80	90	45		100	50	100	80	95	85	100			100	85		85	80	100	
Geotrichum candidum	20									15										
Gliocladium roseum	100	45	20	100		25		100	40	100	100	100	100	100	85		100	85	80	100
Gliomastix sp.											100			100						
Graphium sp.		20							20											
Humicola grisea	100																			
Monilia brunnea																				

Cont'd/....

Table 11, Part A Cont'd

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Mucor globosus	20	55	20	80	40	30	45	80	100	100	100		55	100	95	60	95	100	30	50
Paecilomyces varioti			40			35														
Penicillium funiculosum		100	100	50	100	100	100		100	80	50	100	30	5	75	100	100	80	45	30
Penicillium sp.2			60			60							5							
Periconia sp.			55			55	50													
Rhizopus nigricans				100				100											45	
Sporotrichum pruinosum		40							35											
Stachybotrys atra	60									65										
Stysanus sp.											45		100	40	10		45			100
Trichoderma viride	15	15		60				60	10	20		100	100		20	100	100	20	100	100
Ulocladium sp.																	30			
Verticillium lateritium					20		25													
Verticillium sp.								20												
Zygorhyncus moelleri																				

Table 11, Part B — Percentage frequency of occurrence of fungi isolated from samples of refuse, from household dustbins, on glucose-starch agar by Warcup's technique at 25°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alternaria sp.	25				65		30			20										
Aspergillus fumigatus	15				55		20													
Aspergillus niger																	95			
Aspergillus sp.																				
Cephalosporium sp.	20	40		10	75				35	25	25	20		20			25			
Chaetomium globosum																				
Fusarium sp.	100	5			100		100		10	100	40			35			25		25	
Geotrichum candidum	20								25	100										
Gliocladium roseum	30		20	15		15		20		25		100	95	100	100		100	100	20	100
Gliomastix sp.											10									
Graphium sp.																				
Humicola grisea							10			95										
Monila brunnea											25			20						

Cont'd/...

Table 11, Part B Cont'd

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Mucor globosus	100	100	20	100		35	15	100	100	95	100	100	75	100	100	25	100	100	60	75
Paecilomyces varioti						25														
Penicillium funiculosum	85	100	100	100	100	100	100	100	100	100	100	100	15	100	100	40	100	100	95	
Periconia sp.			40		55	40	55													
Rhizopus nigricans				100				100											100	
Sporotrichum pruinosum																				
Stachybotrys atra										5										
Stysanus sp.											5		25							
Trichoderma viride	70	5		75				80		75		100	100		95	100	100	100	100	100
Ulocladium sp.																				
Verticillium lateritium							30													
Verticillium sp.																				
Zygorhyncus moelleri	10																			
Penicillium sp.2			20			15				55				5	50	5				

Table 12, Part A

Percentage frequency of occurrence of fungi isolated from samples of scrapings, from household dustbins, on cellulose agar by Warcup's technique at 25°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alternaria sp.		25	10						10						60		65			
Aspergillus fumigatus			15			15	15		10							100		60		
Aspergillus niger																				
Aspergillus sp.		15						10							20		20			
Aureobasidium pullulans		45	5	20						15										
Cephalosporium sp.	45	30	100	60	40		100	100				100	40		20		20	40	75	100
Cerastomella sp.										10	80	10								
Chaetomium globosum	65				60															
Eurotium sp.																				
Eurotium vermiculatum																				
Fusarium sp.	80	15	20	95	75			20	25	95		15			80		65			20
Geotrichum candidum										25										

Cont'd/...

Table 12, Part A Cont'd

FUNGI	Bins from residential area										Bins from council estate									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gliocladium roseum	70				65								100					100		
Gliomastix sp.																				
Humicola grisea	100				100															
Monilia brunnea		20	45								100	70	55		65		5	60	95	80
Mucor globosus	70			75	65					80	100	50	100					100	100	40
Paecilomyces varioti																				
Penicillium funiculosum	90	100	100	85	85	100	100	95	100	85	50	80	65	100	65	100	100	60	50	80
Penicillium sp.2		15				15		15			20			100		100				
Rhizopus nigricans	80	10	45		75			10	45	30										
Sporotrichum pruinosum	100				100															
Stysanus sp.							5													
Trichoderma viride	10		10	5	10				15		100	25							100	20
Ulocladium sp.		40											30					30		

Table 12, Part B

Percentage frequency of occurrence of fungi isolated from samples of scrapings, from household dustbins, on cellulose agar by Warcup's technique at 25°C.

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Alternaria sp.					20				5								10			
Aspergillus fumigatus					30	45	45								45			45		
Aspergillus niger	20			20										65		60				
Aspergillus sp.																	5			
Aureobasidium pullulans		5	5	45						60								20		
Cephalosporium sp.				65																
Cerastomella sp.										15										
Chaetomium globosum	10																			
Eurotium sp.																				15
Eurotium vermiculatum										5										
Fusarium sp.	30			10	25			5	10		5			65		65				
Geotrichum candidum										25										

Cont'd/...

Table 12, Part B Cont'd

	Bins from residential area										Bins from council estate									
FUNGI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gliocladium roseum	10												10							
Gliomastix sp.											20									
Humicola grisea	20										20									
Monilia brunnea				5							5	15								20
Mucor globosus	100			100	100					100	100	100	100				5	100	100	100
Paecilomyces varioti			5							5										
Penicillium funiculosum	70	100		100	70	100	100	100	5	100	80	100	100	100	100	100	100	100	85	100
Penicillium sp.			75			10					20			100	40	100	45		15	
Rhizopus nigricans	95	50	65		90			50	65	45										
Sporotrichum pruinosum	50																			
Stysanus sp.																				
Trichoderma viride	55		5	20	45				5		100	20							95	5
Ulocladium sp.		30																		

APPENDIX III

A. H. ALLEN & PARTNERS

PARTNERS:
HUGH CHILDS, B.SC., (MANC.) F.R.I.C.
A. O. JONES, M.A., (OXON.) F.R.I.C.
E. P. UNDERWOOD, B.SC., (LOND.) F.R.I.C.

ANALYTICAL AND CONSULTING CHEMISTS

PUBLIC AND AGRICULTURAL ANALYSTS

COUNTY OF NOTTINGHAM.
CITIES OF SHEFFIELD, YORK & LINCOLN
COUNTY BOROUGHS OF BARNSLEY, DONCASTER, GRIMSBY & ROTHERHAM
BOROUGHS OF CHESTERFIELD & SCUNTHORPE

TELEPHONE NO. 21687

Public Analysts Laboratory,
67, Surrey Street,
Sheffield
S1 2LH

Report No.1291/68

REPORT on a sample of Compost received from Chesterfield Rural District Council, per Mr. Murray, Council Offices, Saltergate, Chesterfield, on August 20th, 1968.

The sample showed on analysis:-

Moisture	29·82	per cent
Nitrogen	0·67	" "
Phosphate (P_2O_5)	0·60	" "
Potash (K_2O)	0·06	" "
Carbonaceous Matter	20·4	" "

The compost is fairly normal in composition and contains the usual traces of nitrogen, phosphate and potash. The carbonaceous matter represents the organic content of the sample.

September 7th, 1968.

FOR A. H. ALLEN & PARTNERS

E. P. Underwood

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