THE DECOMPOSITION OF AGRICULTURAL CELLULOSIC WASTES BY *COPRINUS CINEREUS*

by

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Being a thesis submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy

University of Aston in Birmingham

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SUMMARY

This study was part of a larger research programme designed to obtain a method of improving the feed value of waste straw to ruminants, using a selected microorganism. The work reported here was carried out to assess the potential of Coprinus cinereus (Schaeff ex Fr.) S.F. Gray sensu Konr. for this purpose, to upscale the most promising treatments and to investigate the natural colonisation of straw by the fungus.

The breakdown of sterile barley straw was investigated over a range of pHs maintained with ammonia and hydrochloric acid solutions. Hemicellulose and cellulose were found to be attacked, with hemicellulose decomposed preferentially. At optimum growth conditions, in vitro digestibility of the straw was increased to the level of good quality hay, although this declined after peaking. Digestibility increase was correlated with utilisation of hemicellulose and cellulose in the short term.

Larger scale biodegradation trials using unsterile straw showed that a consistently upgraded product could be obtained using an inoculum of C. cinereus spores in 1% w/w ammonia solution with loose straw in polythene sacks. The low protein content of the upgraded material indicated that it was unsuitable as a complete ruminant diet.

A technique was developed to study the activity of C. cinereus and other fungi in cereal field soil. It was shown that C. cinereus is an active component of the fungus flora of barley field soil, indicating that the propagules of C. cinereus found in harvested straw arise from the soil.

The feasibility of linking a straw biodegradation process to an animal slurry treatment system, which serves as an ammonia source was investigated. The economic aspects of such a scheme were discussed.

KEY WORDS

Coprinus cinereus : straw : biodegradation : ammonia : feedstuff

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GENERAL INTRODUCTION

1.1 AGRICULTURAL CELLULOSIC WASTE

1.1.1 The size of the problem

The two main waste products of agriculture which cause problems by excessive build-up are livestock manures and cereal straw. Both are the result of modern intensive specialisation which has overloaded the decay capacity of the carbon cycle. For many centuries straw was valued as a useful by-product of cereal production, largely for feeding or bedding draught animals. However, the development of the internal combustion engine rapidly caused animal power to become obsolete in many countries and demand for straw became drastically reduced. At about the same time, synthetic fertilisers were manufactured as a compact, standardised substitute for farmyard manure, and after the Second World War they became the principal means of maintaining soil fertility in the Western World.

Since then the combination of chemical herbicides, plentiful "artificial" fertilisers and varieties of cereals bred for the new circumstances has enabled cereal growers over much of the world to dispense with mixed farming rotations and concentrate on mechanised cereal growing, usually accompanied by a reduction in livestock husbandry, which has tended to move elsewhere.

In the U.K. this has led to the present situation where the drier eastern counties support the majority of cereal production, while the stock farming is found mainly in the wetter and hillier western and northern counties, thus separating the supply and traditional demand for straw (Fulbrook et. al. 1973).

Large increases in the price of oil in the early 1970's and the concomitant increase in fertiliser and animal feed prices encouraged interest in the profitable use of waste products. The problem of waste straw became the subject of a number of reports detailing potential disposal outlets (ACAH, 1973; McLean, 1973; N.F.U., 1973). However, none were able to make recommendations for any fundamental or widespread changes in the practice of straw disposal.

Farmers seldom weigh the straw from their crops, however grain yields are generally known with some accuracy and it has been observed that, under certain conditions, there is good correlation between straw and grain yields (Donald and Hamblin, 1976; Gallagher and Biscoe, 1978). In the U.K. it is reasonable to calculate the straw yield, as normally harvested, from official grain yield estimates and the best available estimates of straw to grain ratios (Staniforth, 1979). Straw production calculated in this way from 1973 onwards is shown in Table 1.1.

TABLE 1.1

STRAW PRODUCTION IN THE U.K. FROM 1973 - 1979

YEAR	WHEAT		BARLEY		OATS		TOTAL STRAW YIELD IN TONNES (MILLION)
	total grain yield in tonnes (Million)	straw yield in tonnes (Million)	total grain yield in tonnes (Million)	straw yield in tonnes (Million)	total grain yield in tonnes (Million)	straw yield in tonnes (Million)	
1973	4.90	3.67	9.00	5.40	1.08	0.86	9.93
1974	6.13	4.60	9.12	5.47	0.95	0.76	10.83
1975	4.49	3.37	8.51	5.11	0.79	0.63	9.11
1976	4.74	3.55	7.66	4.60	0.76	0.61	8.76
1977	5.27	3.96	10.73	6.44	0.80	0.64	11.04
1978	6.60	4.95	9.84	5.90	0.71	0.57	11.42
1979	7.14	5.36	9.56	5.74	0.54	0.43	11.53
Straw: Grain ratio	0.75*		0.60*		0.80+		

*Hughes, 1977

+Staniforth, 1979

Straw yield depends on a number of factors including species, variety, height of cut, seasonal factors, soil types, fertiliser treatments, cutting time, denseness of plant and the degree of lodging. Thus the above figures are only a general estimate of straw production but give a coherent indication of the amount that could be harvested annually.

It can be seen that straw production varies from year to year, but the amount produced is tending to increase. Farm requirements for straw will also vary but a general increase in straw production will probably generate further excess. The NFU report (1973) estimated 3.57 million tons of straw to be surplus to farm requirements, of which 3.42 million tons were burnt. In 1976 there were 3.30 million tons for ex-farm use and 5.20 millions tons in 1977. In these years 2.19 and 4.23 million tons respectively were burnt (Farmers Weekly, April 7th 1978). From the above figures it appears the present situation is that about 50% of the straw produced in the U.K. is unused and most of it is burnt in the field.

1.1.2 Use and disposal of straw

Hughes (1979) reported the fate of straw produced in 1977 for the cereal dominant areas of England and Wales, and the results are summarised in Table 1.2.

TABLE 1.2

STRAW DISPOSAL IN ENGLAND AND WALES 1977

(% OF AREA CROPPED)

	Baled	Cultivated or ploughed in	Burnt
Wheat	34	4	62
Barley	73	trace	27

The main on farm uses of straw are as bedding for livestock and as a fibrous feedstuff. Wheat straw with its relatively low nutrient content is generally used for bedding, while barley and oat straws are usually reserved as components of feed rations. With the decline of livestock production in arable areas, as the table shows, wheat straw causes the chief surplus with 62% of its production burnt in 1977.

Straw is a difficult material to use economically away from its site of production. It is a high bulk, low density commodity which is expensive to transport. It is variable in quality and seasonal in nature, being produced at the farmers' time of greatest workload. These problems are of great significance when considering use of straw away from the field. Some developments have been made in producing denser bales for easier, more economic transportation, and several commercial high density balers have been produced, but they are, as yet, uneconomic to operate under U.K. conditions (Klinner and Chaplin, 1977; Bull, 1978). These difficulties have led to very limited use of straw outside agriculture. The main outlets are paper manufacture, building boards, boiler heating and packaging. The scale of these and other current uses has been reviewed by Staniforth (1979).

Apart from burning, the farmer has very few alternatives for disposal of waste straw. One possibility is to plough

it in after chopping, but successful use of this technique depends on adequate replacement of nitrogen immobilised temporarily by the decomposing straw (Uhlen, 1973).

The other possibilities involve reduced cultivation and direct drilling, which enable the next crop to be planted only a short time after harvest of the previous one. The field is not ploughed and crop residues remain, thus saving in time and labour, particularly in a wet autumn, enabling maximum sowing of the higher yielding winter cereal varieties. Straw from the previous crop has been shown to cause serious problems under these circumstances. Cochran et. al. (1977) showed that phytotoxins are produced from decomposing wheat and barley straw when in soil contact. This is of considerably greater significance with minimal tillage than in conventional systems of cultivation. Ellis and Lynch (1977) suggested that the short interval between successive crops gave little opportunity for leaching of toxic substances, mainly organic acids, and that germinating cereal seeds were very likely to be in close contact with decomposing straw, and hence exposed to high concentrations of toxic substances. Also, when straw is present on the soil surface there can be mechanical difficulties in sowing by direct drilling or reduced cultivation.

The nature of these problems dictates that removal of the straw is the simplest solution; with burning the most convenient, except in wet weather. The difficulties

involved in direct use of straw in cultivation systems is reflected by the small amount of straw dealt with in this manner.

In the U.K., straw and stubble burning is frequently regarded as a beneficial operation. A field can be cheaply and quickly cleared in preparation for the next crop, and the risk of nitrogen immobilisation and toxic chemical production from decomposing straw is avoided. Trials at experimental husbandry farms have shown that the sequential yields of winter cereals drilled direct or with minimal cultivation can benefit considerably from straw and stubble burning (Ellis and Lynch, 1977). There is some evidence that burning can reduce volunteer cereal and weed seeds, especially Alopecurus myosuroides (black grass) and Avena spp. (wild oats) (Ellis, 1979). Edwards and Lofty, (1978) showed that burning may decrease insect pests; although soil invertebrates important in maintaining soil fertility may be reduced in the long term.

The field burning of straw has its disadvantages, particularly in areas such as eastern England, where it is an integral part of post harvest operations. It is difficult to achieve an effective burn in wet weather, whilst dry, windy conditions may cause fires to get out of control and cause damage to hedgerows, neighbouring woods and property. To reduce these attendant problems of pollution

and damage the farmers'union in the U.K. have introduced a revised code of practice for field burning (N.F.U., 1978). It has been shown that properly conducted straw burning operations cause very little peripheral damage (Wood, 1977).

Recently, much work has been done to develop outlets for waste straw, notably in the field of pulp production. Dean (1978) described a process with the potential to produce a range of products, working on the basis of a small pulp mill. By varying chemical concentrations and cooking time the products can include animal feed, corrugated packing material, bleached pulp, hardboard, sizing agents and soil stabilisers. The process is under development at the pilot stage.

Other possibilities include simple combustion for direct local use (Rexen, 1976), methane production and pyrolysis (McCann and Saddler, 1976), and production of chemicals by acid hydrolysis (Sachetto, 1977). Machinery has been developed to plant straw in rows, to prevent wind erosion and crop removal in some susceptible areas (Staniforth, 1979) whilst it is possible to briquette straw, in much the same way as peat, for low grade fuel production (Hansford, 1975).

These trends in non-feedstuffs research are encouraging but, as can be seen from the figures, the amount of waste straw burnt has not been reduced.

1.1.3 Whole crop harvesting

As mentioned previously, most barley straw and oat straw is used as animal feedstuff while wheat straw is usually regarded as waste. Some developments have been made in harvesting the whole cereal crop for processing either on farm or at a central site (Wilton, 1976; Vahlberg, 1978). The method is suitable for all cereals and it is possible to obtain three separate fractions: grain, coarse straw and a feedstuff mixture of broken grain, chaff or awns, leaves, fine straw and weeds. The coarse straw is burnt in the grain drier furnace and should provide sufficient heat to dry the crop (Wilton, 1976). Some progress has been made in commercial application of this process in Sweden but equipment installation and transport costs of the high bulk harvest remain prohibitively high.

1.2 UPGRADING STRAW

Non agricultural uses of straw are often uneconomic because of high transportation costs. In view of this fact alone it would seem prudent to consider maximum use of straw on the farm. It is relatively free from pesticides having been subjected to growing conditions necessary for food production. It is a potentially useful source of energy for ruminants, containing roughly half the fixed energy of the cereal crop (Heslop-Harrison, 1975), but the availability of this clean, carbohydrate energy is low.

The nature of the relationship between the cellulose, hemicellulose and lignin components of straw which imparts such a low energy availability will be discussed in a later section.

Chemical, physical and biological methods have been employed in attempts to upgrade straw, by either increasing protein content or improving energy availability. In the main, methods have centred on improving straw digestibility and palatability to increase intake. A brief review of current research follows.

1.2.1 Chemical treatment

It has been known for nearly a century that the alkali treatment of straw can increase its value as a ruminant feedstuff. The earliest methods of treatment involved the heating or pressure cooking of straws in dilute sodium hydroxide solutions, followed by washing with clean water to remove the alkali (Kellner and Kohler, 1900). Kellner and Kohler (1900) observed that rye straw hydrolysed by their process had a digestibility of about 88%.

In a later version Beckmann (1921) replaced pressure cooking with a simpler cold water steep to reduce costs, although the quality of the finished product was lower, with a digestibility of about 70%. The major disadvantages of the process were a high requirement for labour, the

need to preserve the product by drying or ensiling and the use of large quantities of wash water to remove the alkali. Washing caused loss of much of the solubilised material and produced large quantities of polluted waste water. This process is still used in Norway although it is known that the effluent discharged from straw treatment plants into rivers is highly injurious to fish populations (Snekvik, et al., 1976)

In the Beckmann process a dilute solution of 1 - 2% sodium hydroxide is used. Wilson and Pigden (1964) and Donefer et al. (1969) indicated that it was possible to improve straw by spraying it with more concentrated solutions, followed by neutralisation of excess alkali with an organic acid.

Further research has been carried out in a number of laboratories to streamline the treatment and remove the need to wash out or neutralise excess alkali (Singh and Jackson, 1971; Carmona and Greenhalgh, 1972; Rexen et al., 1976) The process has been developed to the point where straw treatment may now be carried out on farm or at a central factory. A number of machines have been developed to treat straw in the field (Farmers Weekly, July 1st 1977; Wilkinson, 1977) while a large British animal feed firm has several process plants operating in this country (Anon, 1977).

Opinions on the economic viability and usefulness of such straw treatments to the farmer are conflicting and the present position is unclear (Taylor et al., 1977; Parry, 1978; Greenhalgh, 1980).

The use of other alkalis for improving the digestibility of straw has been investigated. These include KOH, $Ca(OH)_2$, NH_4OH and tetramethylammonium hydroxide but none has proved to be as effective as NaOH (Guggolz et al., 1971; Hartley and Jones, 1978, Solaiman et al., 1978). Ammonia treatment of straw has been investigated in Russia by Laguta (1961) and Bondarev and Gurtikh,(1962) and in Norway by Arnason and Mo (1977). Although digestibility results are lower than those using sodium hydroxide, ammonia has several advantages in straw treatment. No special processing of the straw is required, and available nitrogen is incorporated into the straw (Ørskov, 1980). Also the problem of copious urine production caused by ingestion of large quantities of sodium ions is removed (Hovell, 1980 personal communication). Chandra and Jackson (1971) found that sodium hypochlorite was as effective as sodium hydroxide in increasing straw digestibility but residual chlorine apparently inhibited rumen microbial activity.

1.2.2 Physical treatment

Donefer (1977) summarised recent attempts to increase

straw digestibility by pressure cooking, but concluded that such treatments were uneconomic and impractical. Various types of mechanical treatment have been investigated including chopping, milling and grinding but evidence of improved feed value is inconclusive. Wilton (1975) reported two feeding trials in which economically optimum levels of ground straw in ruminant diets were as high as 30% and as low as 10%.

1.2.3. Biological treatment

Most methods of biologically upgrading cellulosic wastes have involved utilisation of the carbohydrate content as an energy source to produce microbial protein. Several workers have used bacteria for this purpose. Han (1975) working on rice straw, used the cellulolytic bacterium Cellulomonas sp. in symbiosis with Alicagenes sp.; the latter utilising the cellobiose produced by the former. Ramasamy and Verachtert (1979) used a Pseudomonas sp. to ferment wheat straw up to the level of 38% protein.

A wide range of fungi have also been investigated, usually in axenic culture but occasionally paired with another microorganism. Kristensen (1978) investigated single cell protein production from barley straw using the yeast Candida utilis and the bacterium Cellulomonas sp. Peitersen (1975), in the fermentation of barley straw with Trichoderma viride, found that the rate of protein and

cellulase production increased when T. viride was grown together with Candida utilis.Han et al. (1976) grew Aureobasidium pullulans on an acid hydrolysate of rye grass straw to produce single cell protein whilst the fungus Sporotrichum pulverulentum has been used to produce a protein enriched product from the waste fibres of a newsprint mill (Eriksson and Larsson, 1975). The problem with all of these processes is that they require the use of complex fermentation technology, usually involving substrate pretreatment such as grinding, hydrolysis and sterilisation, and continuous flow or batch fermentation systems. This approach has not proven economically practical (Dunlap, 1975).

The use of relatively simple techniques for increasing protein production has received little attention. Heltay and Petofi (1965) composted rice straw with mineral nutrients, bacteria and various waste materials to produce a substance with 15% protein content. The compost was pasteurised, inoculated with Agaricus bisporus and stored in an air conditioned chamber for 2 - 3 weeks. The result was termed "mycofutter" and consisted of up to 20% protein. It could be ground up to produce livestock meal, but the process does not appear to have been taken up widely.

Enzymatic hydrolysis of waste cellulose by extracellular cellulases has been investigated by a number of workers

but most processes have proven uneconomic (Dunlap, 1975; Andren, et al., 1976).

The use of microorganisms to increase the digestibility of cellulosic waste materials has received scant attention until recently; although the natural conversion of wood by fungi into fodder called "Palo podrido" has been known and exploited in Chile since at least 1929 (Kuhlwein, 1963). Hartley et al. (1974) investigated the use of white rot fungi to improve the feed value of barley straw and wood wastes. The fungi were grown on ground straw and also on beech, oak and poplar sawdusts. After fermentation, some samples were treated with sodium hydroxide solution. The digestibility of straw after fungal and alkali treatment ranged from 70% to 82% depending on the fungus and alkali treatment used, compared to 46% for the untreated control. Most of this increase was reported to be due to the action of alkali with only a small contribution made by the fungus.

Zadrazil (1977) investigated the growth of four basidiomycetes on wheat straw and found Stropharia rugosoannulata increased straw digestibility from 40% to 60% and 70% when incubated at 22°C and 30°C, although the incubation time was very long, 120 days in this case. Latham (1979) investigated the activity of 134 isolates of white rot fungi on barley straw and found only three improved straw digestibility, notably Schizophyllum commune which gave 11% increase above the control at 30°C

after 4 weeks incubation.

The small number of investigations into increasing cellulosic waste digestibility by fungal activity reflect the economic and technical difficulties of upscaling such processes, including equipment installation and running costs, substrate availability and extent of pretreatments, sterility requirements, incubation time and quality of final product.

1.3 THE FEEDING VALUE OF STRAW TO RUMINANTS

The straws most often encountered in Britain are those of wheat, barley and oats. The nutritional value of these straws varies considerably, depending on variety, method of harvesting, seasonal factors and also on their biochemical composition and structure (Table 1.3).

The digestibility of straw, particularly barley straw, varies between varieties and this could represent significant differences in the energy contribution to diets, yet few workers report the variety of straw used in their feeding trials (Palmer, 1976). Where the straw is carefully selected and of a type that is relatively digestible, even when fed to full appetite it cannot provide enough energy, protein, minerals or vitamins to maintain dairy cows, beef cattle or sheep (ADAS, 1977). The low digestibility limits both its intake and feed potential and it must be

TABLE 1.3

COMPOSITION AND NUTRITIVE VALUE OF STRAW

(DRY MATTER BASIS)

	Winter Wheat	Spring Wheat	Winter Barley	Spring Barley	Winter Oats	Spring Oats
Crude protein %	2.4	3.4	3.7	3.8	2.2	3.4
Ether extracts %	1.5	1.5	1.6	2.1	1.7	2.2
Crude fibre %	42.6	41.7	48.8	39.4	40.2	39.4
N-free extracts %	47.3	46.3	39.2	49.3	50.1	49.3
Total ash %	6.2	7.1	6.6	5.3	5.7	5.7
Gross energy $MJkg^{-1}$	17.7	17.6	17.8	18.0	17.8	18.0
Metabolisable energy $MJkg^{-1}$	5.7	5.6	5.8	7.3	6.8	6.7
D value %	39	39	39	49	46	46

(M.A.F.F., 1979)

adequately supplemented by the remainder of the diet. Clearly, there is considerable scope for a straw product of improved digestibility level for ruminants.

1.4 THE RUMINANT DIGESTIVE SYSTEM

The ruminant digestive system differs markedly from monogastric animals and its basic functioning must be appreciated before considering the production of a ruminant feedstuff. The digestive system of the ruminant consists of four main parts, the rumen, the reticulum, the omasum and the abomasum. Ruminants depend on the activity of microorganisms for the conversion of plant polysaccharides to utilisable substances. With the exception of starches, these polysaccharides are attacked little, if at all, by the animals' own gastric and intestinal secretions. Microorganisms in the gut are relied upon to provide the hydrolytic enzymes to break down cellulose, hemicellulose and pectins.

The rumen and reticulum form a continuous sac which is the site of main microbial activity, the favourable conditions in "the rumen" permitting the growth of many species of bacteria and protozoa. There is a practically continuous flow of saliva to the rumen which forms a 'basal salts' medium for the microorganisms, and added to this at varying intervals is the feed. Cellulose, hemicellulose and the other dietary components are converted to volatile

fatty acids by the rumen microorganisms. These volatile fatty acids are the major energy source for the animal, while the bacterial protein so formed is absorbed in the lower gut, thus contributing to the protein requirement of the animal (Hungate, 1966). Bacterial species capable of degrading cellulose in the rumen are few. These include Ruminococcus flavefaciens, Bacteroides succinogenes and Ruminococcus albus. There are a total of about 10^7 cellulolytic bacteria per millilitre in the rumen and these are thought to be responsible for the breakdown of the cellulose component in feeds (Summers, 1978).

The retention time of the fermenting material and suspended microorganisms in the rumen is about one day although large, fibrous material may remain for several days. These fibres are gradually reduced in size by both microbial action and by regurgitation and chewing (rumination) until they are small enough to leave the rumen.

There are many factors affecting the utilisation of straw in the rumen. Digestibility is lowered by the physical and chemical links between the major structural polymers as well as the presence of waxes and silica (Van Soest and Jones, 1968). Another factor for consideration is the low nitrogen content of straw which is normally not enough to support microbial protein synthesis on a sufficient scale. Additional nitrogen, often in the form of urea, has to be given to animals on straw based diets. The carbohydrate and nitrogen requirements of the ruminant and its

microflora are closely interrelated and this must be taken into account when considering a ruminant feedstuff. Proteins are the most common nitrogenous materials in forages and these are usually directly digested and assimilated by the rumen microflora. Some rumen micro-organisms ferment protein to provide energy and ammonia (Hungate, 1966). This ammonia may be re-assimilated into amino acids but this is only possible if carbohydrates are available. The relative importance of proteins as energy sources or as components for cell synthesis depends on the carbohydrate:nitrogen ratio in the rumen.

The fact that nitrogenous constituents of feed undergo microbial conversions in this way before they are assimilated by the host has led to the practice of feeding ruminants simple forms of nitrogen, particularly with high carbohydrate containing feeds such as straw (Loosli and McDonald, 1968). The feeding of good quality proteins would be largely wasted as the ruminant would eventually receive its standard, symbiont protein; regardless of its diet. It has been shown that the rumen microorganisms convert urea to protein of sufficiently high quality to support efficient production (Loosli and McDonald, 1968), although it is important to feed it at low levels or poisoning will result (Frøslie, 1977).

1.5 THE STRUCTURE AND CHEMISTRY OF STRAW

Straw is the aerial part of the cereal plant which remains after the grain has been removed at harvest. This consists mainly of stems and some leaf material. At harvest the straw consists of dry cell walls with an outer cuticle layer and a middle lamella between the cells. These walls consist of primary cell walls formed during growth. Certain cells, particularly epidermal, fibre and xylem cells have a secondary cell wall laid down after growth has ceased. This is composed of three layers, the S1 layer (thin outer), S2 (thick middle), and S3 (thin inner). The main constituents of straw are shown in Table 1.4.

1.5.1 Cellulose

Cellulose is the most abundant compound found in straw, forming 40 - 45% of its dry weight (Owen 1976). It is a polymer of D-glucose joined through β 1-4glycoside linkages. The structural formula is conventionally represented in Figure 1. Cellulose is highly susceptible to microbial attack, being degraded by an enzyme complex collectively known as cellulases. The exact nature and mode of action of this enzyme complex is still not fully understood. Reese et al. (1950) postulated that a non-hydrolytic enzyme C_1 "activates" the cellulose enabling hydrolysis by two enzymes C_x to take place, with subsequent

TABLE 1.4

CHEMICAL COMPOSITION OF STRAW

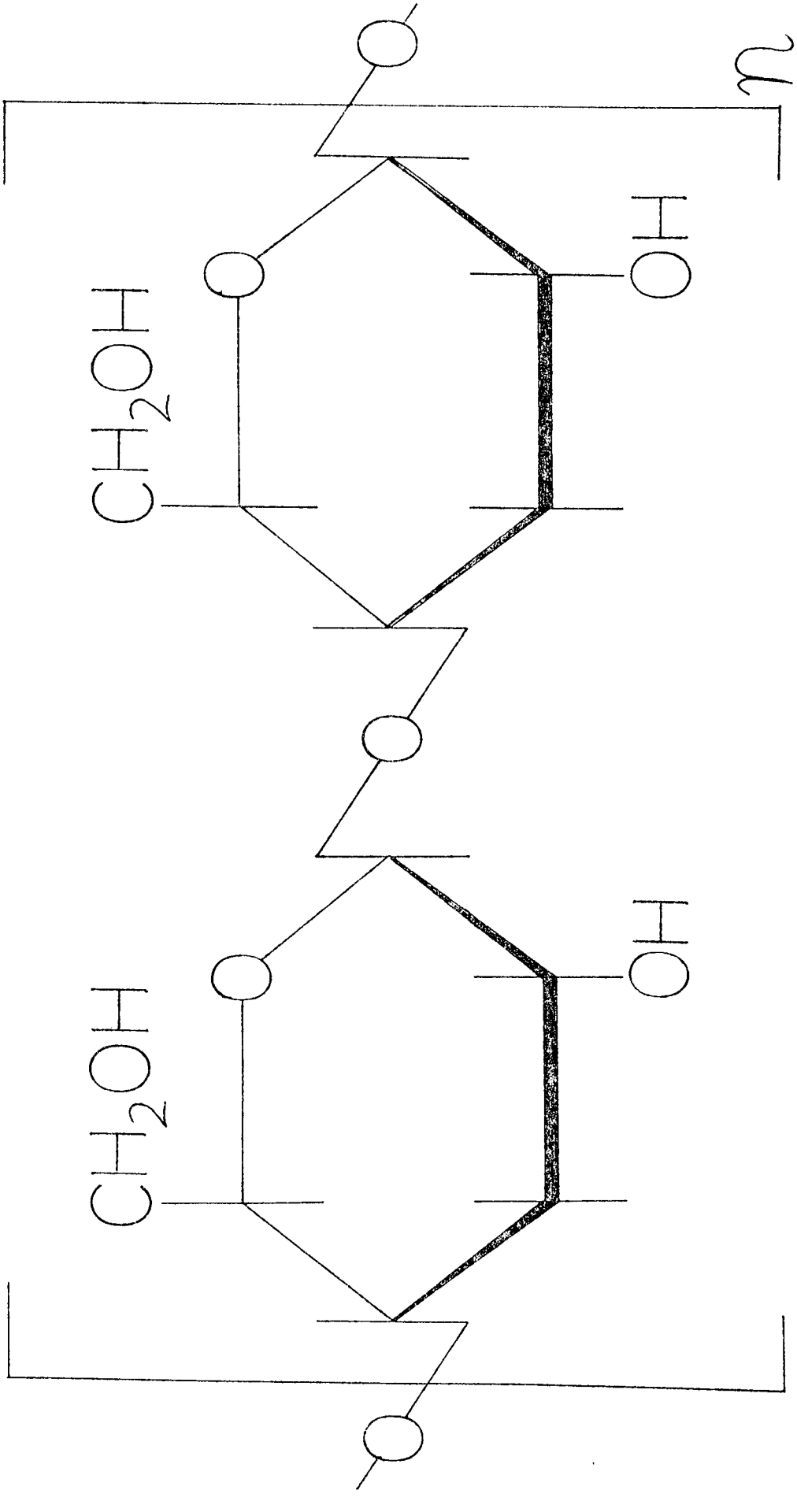
(% DRY MATTER)

Straw	Cell contents	Cell walls	Hemi-cellulose	Cellulose	Lignin	Ash
Wheat	18.6	81.4	29.2	40.3	8.7	6.9
Barley	19.6	80.4	30.3	43.2	6.9	6.1
Oats	16.6	83.4	28.2	44.6	9.3	4.9

(Owen, 1976)

FIGURE 1.1

THE STRUCTURE OF THE CELLULOSE MOLECULE



hydrolysis of reaction products by cellobiase to glucose. Wood (1975) showed that the cellulase complex contains the following components, listed in order of occurrence of their activity on cellulose:-

1. C_1 is an enzyme whose action is unspecified. It is required for the hydrolysis of native cellulose by β 1-4 glucanases.

2. β 1-4 glucanases ($=C_x$) are the hydrolytic enzymes and are of two types:-

   a. exoβ 1-4 glucanase acts at the non-reducing end of the cellulose chain, and large reaction products of endo β 1-4 glucanase, to produce single glucose units.

   b. endo β 1-4 glucanase acts randomly at the internal glycoside bonds producing shortened molecules.

3. β glycosidases are highly active on all the β dimers of glucose but are often descriptively referred to as cellobiase.

Some fungi, for example Trichoderma viride and Aspergillus fumigatus are known to possess all the above components but are unable to effect significant breakdown of cellulose combined with other polymers in a natural

substrate. It has been postulated that an additional factor, absent in such fungi, is required to initiate attack under these circumstances.

1.5.2 Hemicelluloses

Hemicelluloses are metabolised by a wide range of microorganisms and constitute about 30% of the dry weight of straw (Owen, 1976). The hemicelluloses of straw are mainly pentosans which yield pentose sugars on hydrolysis with fungal enzymes, the most common product being D-xylose. These polymers consist of a polyxylose backbone with L-arabinose and uronic acid side chains, although during botanical ageing the percentage of side chains on xylans decreases markedly (Morrison, 1974) hence straw hemicelluloses are found to have a relatively low arabinose content.

1.5.3 Lignin

Lignin comprises about 6-8% of the dry weight of straw (Owen, 1976). It is a three dimensional branched polymer formed by the oxidative polymerisation of three substituted cinnamyl alcohols: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Kirk, 1971). Lignin is relatively resistant to microbial attack with the ability to effect its degradation being confined largely to certain basidiomycete fungi. The main exoenzymes

produced by fungi capable of degrading lignin are laccase and peroxidase, and are active in the process of demethoxylation of vanillic acid and lignin (Trojanowski, 1969). The demethoxylation process constitutes an important step in the primary process of lignin breakdown allowing loosening and possibly eventual cleavage of bonds joining the phenyl propane subunits in lignin (Trojanowski et al., 1967).

1.5.4 The relationship between the basic cell wall polymers of straw and its resistance to microbial attack in the rumen.

The linear polymeric molecules of cellulose are aligned together to form fibrils with a diameter of about 35Å, and these aggregate to form microfibrils with a diameter of about 250Å (Talmadge et al., 1973). Within the microfibrils are crystalline regions called micelles, interrupted at regular intervals by amorphous regions. The microfibrils form the fairly open framework of the cell wall changing direction in each layer of the wall adding strength in all planes. In addition each microfibril is stressed as the cell extends and then locked by an amorphous hemicellulose matrix into its stressed position.

In the later stages of cellular differentiation, lignin is encrusted into certain cell types, mainly in the middle

lamella and primary cell wall. It locks very effectively onto the surfaces of other wall materials imparting strength and rigidity where it is required. Lignin and hemicellulose appear to be covalently bound to one another forming a macromolecular matrix in which the cellulose fibrils are embedded, but there is no evidence that cellulose is bound to any cell wall component (Morrison, 1979). In this way a structure highly resistant to the digestion processes in the rumen is formed. The enzymatic activity of the rumen microorganisms may be hindered by this structural arrangement in several ways. The lignin may act as a physical barrier around the cellulose preventing cellulase attack, it may inhibit enzyme activity by its chemical structure due to the presence of phenolic groups, or it may chemically mask the hemicellulose present so that rumen hemicellulases do not recognise their substrate (Morrison, 1979).

Other components found in the cell wall are pectins, some glycoproteins and silica. The presence of the latter may be of importance in reducing the susceptibility of the cell wall to rumen microbial attack (Van Soest and Jones, 1968) together with the effects of interaction of the cell wall polymers.

1.6 THE BIODEGRADATION OF STRAW

The terms biodeterioration and biodegradation may

be seen as two biological processes which are identical in principle but distinguished by their interpretation in human terms. Conventionally, biodeterioration refers to damage to materials, constructions and processes of relatively high value whereas biodegradation refers to enhancement of materials only. Eggins and Oxley (1980) concluded that a precise definition of biodegradation was impossible. They confined the term to those instances where the original material is of relatively low or negative value and there is an element of disposal in the process. If there is a valuable product, this is a bonus.

The biological upgrading of wastes has frequently been investigated using organisms whose characteristics are well known but occupy an ecological niche unrelated to the substrate under investigation. This usually means that the waste material has to undergo some form of expensive pretreatment including sterilisation before the organism can produce the desired result. This tends to increase the energy input of the process such that the whole project becomes uneconomic to operate on an applied scale.

An organism which occurs naturally in a waste material and has an active role in the decomposition of that waste is more likely to be a candidate for use in its biodegradation. Such an organism may be ecologically and physiologically adapted for efficient utilisation of the proposed substrate and could therefore be at a distinct

advantage over non indigenous organisms. Provided the organism can be encouraged to grow under conditions which can be easily and cheaply controlled and reproduced, the biodegradation of the waste becomes a feasible proposition.

By providing suitable selective conditions it is possible to encourage the dominance of selected organisms in non-sterile substrates. Beer and wine production and the process of cheese maturation are examples of this. It was decided to look at the upgrading of waste straw from this angle and to investigate techniques which would rely on low energy input and could be carried out on the site of straw production. The overall aim of the process was to encourage the growth of an indigenous organism from straw so that it became the dominant colonist. The product of this controlled compost would have increased digestibility to ruminants and also increased protein content, although the latter is of lesser importance.

Earlier workers (Eggins and Seal, personal communications) have isolated Coprinus cinereus (Schaeff ex Fr) S.F. Gray sensu Konr. fairly consistently from composting pig slurry and straw mixtures, and Chang and Hudson (1967) isolated C. cinereus from nitrogen amended straw composts after peak heating. The pH of these composts was alkaline which was probably due to ammonia production by microbial breakdown of urea or urine in the composts. Other work carried

out in this laboratory has suggested that C. cinereus has physiological characteristics which may be useful in a straw biodegradation process (McShane, 1976; Penn, 1977; and Kelley, 1979). The starting point of this project was to investigate the effect of C. cinereus growth on straw amended with ammonia.

There were two main reasons for choosing ammonia as the selective agent in this work. Urea had previously been used to successfully promote C. cinereus growth, probably due to the release of ammonia, but ammonia solution appeared to be much more selective. The second factor which was considered was the possibility of linking this upgrading process to an animal slurry treatment technique which was under investigation (Seal, personal communication). This involved separating the slurry into solid and liquid fractions using a straw bale filter. The liquid was treated with lime to coagulate suspended solids together with phosphates and to raise the pH. The pH increase due to lime addition enabled ammonia to be released physically from solution. The supernatant was passed down a specially constructed trickling tower whilst a counter current of air removed ammonia from the top of the tower. This ammonia could be redissolved in water to form a convenient concentrated ammonia source for use in the straw upgrading process. The ammonia would be used to adjust the environmental conditions to select for the growth of an indigenous

fungus, C. cinereus, in the waste straw.

1.7 C. CINEREUS, THE ORGANISM AND ITS CONFUSED NOMENCLATURE

C. cinereus was selected for use in the proposed technique. This organism is a mesophilic basidiomycete often associated with manure piles, compost heaps and other masses of self heating material. The genus Coprinus has been of interest to physiologists and taxonomists for over a century. Unfortunately the value of the early literature for present day use is limited because the nomenclature of the organisms studied has been applied in a very confusing manner. This is still true today. Although many Coprinus species have been studied, by far the most use has been made of 'C. lagopus' and its allies, yet it is the nomenclature of this group which has become the most confused. In investigating this confusion Pinto-Lopes and Almeida (1970) revealed the wide variety of specific names that have been used to designate particular species, and conversely, the large number of different species which have been described with the same specific epithet.

Coprinus cinereus (Schaeff ex Fr.) S. F. Gray sensu Konr has been studied as C. lagopus (Anderson, 1959; Casselton, 1966; Cowan and Lewis, 1966; Darbyshire, 1972; Day, 1963;

Day and Anderson, 1961; Lewis, 1961; Madelin, 1956; Milne, 1975; Moore, 1967; and Morgan, 1966), as C. stercorarius (Brunswick, 1924; Mounce, 1921 and 1922), as C. macrorhizus (Buller, 1924; Dickson, 1936), as C. fimetarius (Fries, 1955) and as C. fimetarius var. cinereus (Buller, 1931). The major confusion has arisen between the identities of C. lagopus and C. cinereus, very similar species which arise on soil and litter and straw and dung respectively. Many workers have used C. cinereus as a tool in genetic experiments because of its ease of culture in the laboratory. However most of these have named their fungus C. lagopus so that the confusion has been perpetuated.

The isolates used in this piece of work have all been positively identified as C. cinereus. The initial isolate was identified by R. Watling at the Royal Botanic Gardens, Edinburgh and subsequent isolates were identified by crossing monokaryons with those of a known C. cinereus culture (Kelley, 1979). C. cinereus is the only Coprinus species which will grow well at 30 - 35^{o}C and also produces highly characteristic microsclerotia (Kemp, 1975).

1.8 OBJECTIVES OF THE RESEARCH PROGRAMME

The objectives of the project as a whole are discussed in a previous section. The scope of the work detailed in this thesis may be listed as follows:-

1. To investigate the activity of C. cinereus in the breakdown of the components of straw, maintained at a range of pHs with ammonia, to give greater understanding and eventual control of the biodegradation process.

2. To investigate changes in digestibility and protein content of the above straw, to upscale the most promising treatments and to attempt to produce a consistently upgraded product on the larger scale.

3. To investigate the activity of C. cinereus and other fungi on straw in the field throughout the year. This involves the devising of a technique for the isolation of actively growing fungi from soil. It will be used to investigate the origin of the natural inoculum of C. cinereus which is encouraged to grow during the straw biodegradation process.

4. To investigate ammonia utilisation by C. cinereus and the possible on farm production of ammonia for use in straw biodegradation.

C H A P T E R T W O

THE DECOMPOSITION OF STERILE BARLEY STRAW BY *COPRINUS CINEREUS*

2.1 INTRODUCTION

Much of the carbohydrate in highly lignified plant material is unavailable for breakdown by the rumen microflora, due mainly to the interaction of the polymers composing the cell wall. Mature straw, unlike hay, is not grown primarily as a feedstuff. It is harvested with all its strengthening tissues deposited and has a low digestibility, with much of the potentially available carbohydrate shielded by this interaction. The problem has been discussed in greater depth in Chapter 1, and this indicates that fungal activity on these polymers will have great bearing on increasing the feed value of straw to ruminants.

Kelley (1979) showed the capacity of C. cinereus for rapid growth on ammonia supplemented straw, which gave it a selective advantage over other potential colonists. This indicated that C. cinereus might be a suitable organism for use in a straw upgrading process. A study by Chang-Ho and Yee (1977) on the activity of C. cinereus on rice straw was designed largely to assess its competitive abilities on commercial mushroom beds of Volvariella volvacea. The activity of C. cinereus on certain components of this rice straw, particularly hemicellulose and cellulose, suggested that a study of the activity of the fungus on ammonia supplemented straw was necessary. It was thought that a greater understanding of the breakdown of ammonia

supplemented straw could eventually allow controlled decomposition to occur, resulting in an upgraded product with enhanced feed value.

The experiments described in this chapter were designed to assess the activity of C. cinereus on the components of ammoniated barley straw, maintained at a range of pHs. From this information it was thought that changes in nutritional value of the material, which are presented in the next chapter, could be linked to the breakdown of some of these straw components by the fungus. Also, of equal importance, was the comparison of activity of several isolates of C. cinereus from different localities to determine strain differences, if any, for future work.

2.2 MATERIALS

2.2.1 The straw

In Chapter 1 it has been shown that wheat straw constitutes the majority of straw that is surplus to farmers' requirements, and most of it is burnt in the field. The availability of harvested wheat straw is therefore limited. Barley straw is fed to livestock as a low grade feed and is stored in quantity for this purpose. An improvement in nutritional value of barley straw would be of great benefit to the farmer and the principles of upgrading it could

almost certainly be applied to wheat straw, as it has been shown to have a very similar microflora (Kelley, 1979). It was also decided to concentrate on barley straw for two further reasons. The technique used to estimate straw digestibility, described in Chapter 3, was limited to material above 35% digestibility, which excluded most varieties of wheat straw. Also, the barley straw obtained was a known variety obtained from a single field which lent a further degree of uniformity to the project.

Spring barley straws may range from 36% to 49% digestibility (Palmer, 1976), thus the variety "Hassan" a Spring malting barley was used for consistency. This particular straw batch was obtained in Autumn 1976 from one field in Lower Harlestone, Northamptonshire (706,650. 1:50,000, Ordnance Survey Sheet 152). This field was also the site of investigations described in Chapter 5.

2.2.2 The inoculum

It is important to note that C. cinereus, in common with many other fungi exhibits physiological strain variation. This has been demonstrated by Fries (1955), Darbyshire (1972), Milne (1975), McShane (1976), and Kelley (1979). Kelley (1979) used isolates of C. cinereus from geographically separate areas to assess their comparative cellulose and lignin decomposing activities on artifical media. These isolates were shown to have significant differences in

their growth rates, cellulolytic abilities and responses to the 'Bavendamm' test. It was thought that these differences in activity could be of significance in the production of a uniform feedstuff from straw. Thus, to investigate this, and to lend uniformity to the group project, the isolates listed in Table 2.1 were used.

In order to accommodate possible upscaling of some of the treatments, a spore suspension was used to give dispersed growth through the straw. Recent unpublished work done at the Biodeterioration Centre has shown that the spores of C. cinereus have an average of 58% germination after 24 hours incubation at 30°C and pH 6.8, making them very suitable as a uniform inoculum for this study.

2.2.3 Methods

All four isolates of the fungus grew and produced numerous sporocarps in pure culture on potato maltose agar, recommended by Kemp and Watling (1977). The technique for the culturing of sporocarps, harvesting of spores and the production of spore suspensions is detailed in Appendix Ia.

Barley straw was chopped to roughly 2cm lengths, placed in weighed 100ml conical flasks in about 5 gram lots and oven dried to constant weight at 85°C (Trinder and Hall, 1972),

TABLE 2.1

ORIGIN OF C. CINEREUS ISOLATES

Isolate No.	Area
As2	Shrewley, Warwickshire
As3	Harlestone, Northamptonshire
As4	Sarn Bach, Gwynedd
As6	Spalding, Lincolnshire

to determine initial dry weight. 5ml of distilled water were added to each flask which was then topped with aluminium foil. Each filled flask was steamed for twenty minutes on three successive days to sterilise. After preliminary trials it was decided to use an inoculum of 2ml of 5×10^4 spores/ml suspension, standardised using a haemocytometer. This concentration of spores gave a thorough coverage of the straw and could be easily reproduced. Each flask was inoculated with a spore suspension of one of the four isolates to give five replicates of each treatment, to be analysed at five day intervals over 15 days. Uninoculated sterile controls were also removed at each sampling day to provide base lines for the subsequent analyses. All flasks were incubated at $30^{\circ}C \pm 1$.

The pH range 5 to 10 was investigated using 5ml volumes of dilute membrane filter sterilised ammonium hydroxide or hydrochloric acid to produce the required pH of the straw. Each inoculated flask was carefully shaken to ensure even dispersal of the spores and acid or alkali. It was found that daily pH monitoring of the flasks was necessary as buffer solutions were not used. These would have tended to alter the nutrient regime within the flasks. To adjust the pH to their initial values, single samples (blanks) of each treatment were sacrificed daily and "titrated" with acid or alkali to adjust the pH to the required level. Each replicate was then aseptically

amended with the same amount of solution as the sacrifice. Earlier trials with this technique showed there could be differences of $\pm$ 0.25 pH units within replicates so the investigation was conducted between the limits of whole pH units, i.e. between 5 and 6, 6 and 7, 7 and 8, 8 and 9, and 9 and 10.

The pH of the blanks was taken with a glass electrode and Pye model 78 pH meter. 5ml of distilled water was added to the flask, thoroughly shaken into the straw and allowed to run off for 30 minutes. The pH of the excess liquid was then taken.

Loss in dry weight was determined gravimetrically. Each sample was then chopped in a Moulinex coffee grinder for two minutes and finally hammer milled in a Culatti micro-hammer mill to pass a 0.8mm. mesh sieve. The powder thus obtained was analysed for various biochemical fractions, the value for each fraction being calculated back as a percentage of the original dry weight before fungal attack. The following analyses were carried out:-

Ethanol-benzene soluble fraction (containing sugars, glucosides, essential oils, colouring matter and resinous substances) was determined by contiuous extraction of the straw in a Soxhlet thimble for 12 hours with 33 volumes of 95% ethanol and 67 volumes of benzene.

Pepsin-diastase soluble fraction (containing protein, starch and glycogen) was determined by the enzymatic hydrolysis of the ethanol-benzene extracted straw. This method is explained in Appendix Ib.

Hemicellulose was determined by extracting the pepsin-diastase extracted straw with 24% potassium hydroxide for 4 hours at 25^{o}C (Chang, 1967).

Cellulose was determined by proximate analysis by hydrolysis of the alkali extracted straw with 72% sulphuric acid according to the method of Ellis, et al., (1946). This method is explained in Appendix Ic.

The residue following this acid hydrolysis was taken to be lignin.

2.3 RESULTS

After 72 hours incubation, growth of the spore inoculum was visible to the unaided eye. At this point, daily HCl amendment of the flasks in the neutral to acid pH ranges became necessary to maintain pH within the set limits. Flasks in the alkaline range maintained their initial pHs until day 10 when all the flasks in the neutral to alkaline ranges required daily amendment with ammonia. Flasks in the neutral to acid ranges continued to require

amendment with HCl until the experiment terminated.

The results of the analyses were subjected to an analysis of variance, recorded in Appendix II which showed there were no significant differences ($p = 0.2$) between isolates in their activity on barley straw. The values obtained for each of the isolates were treated as replicates and are presented in Figures 2.1 to 2.5 and Tables 2.2 and 2.3. Without further statistical analysis being necessary it is obvious that the activity of C. cinereus varies with pH.

Ethanol-benzene soluble fractions did not vary much during the experiment, tending to remain at 5.7% of the original straw dry weight. Pepsin-diastase soluble fractions increased as the pH rose, with all fraction values except those in the most alkaline pH range peaking at the end of the experiment. The highest fraction values were obtained within the 8 to 9 pH range. Total weight losses could be accounted for by decreases in the hemicellulose and cellulose fractions. Both hemicellulose and cellulose decomposition was maximum at pH 8 to 9, with least decomposition occurring at the lowest pH range. Lignin decomposition could not be demonstrated, with values remaining similar to those of untreated straw.

2.4 DISCUSSION

From the results obtained it can be seen that growth

TABLE 2.2

ETHANOL-BENZENE SOLUBLE FRACTIONS OF BARLEY STRAW AFTER GROWTH OF C. CINEREUS

pH range	% ORIGINAL DRY WEIGHT			
	DAY 0	DAY 5	DAY 10	DAY 15
5 to 6	5.70 ± .25	5.69 ± .14	5.71 ± .08	5.78 ± .04
6 to 7	5.70 ± .25	5.62 ± .17	5.73 ± .07	5.79 ± .08
7 to 8	5.70 ± .25	5.75 ± .06	5.68 ± .09	5.85 ± .02
8 to 9	5.70 ± .25	5.58 ± .03	5.73 ± .03	5.80 ± .02
9 to 10	5.70 ± .25	5.65 ± .13	5.68 ± .08	5.84 ± .03

FIGURE 2.1

CHANGES IN DRY WEIGHT OF BARLEY STRAW AFTER GROWTH OF C. CINEREUS OVER A RANGE OF PHs

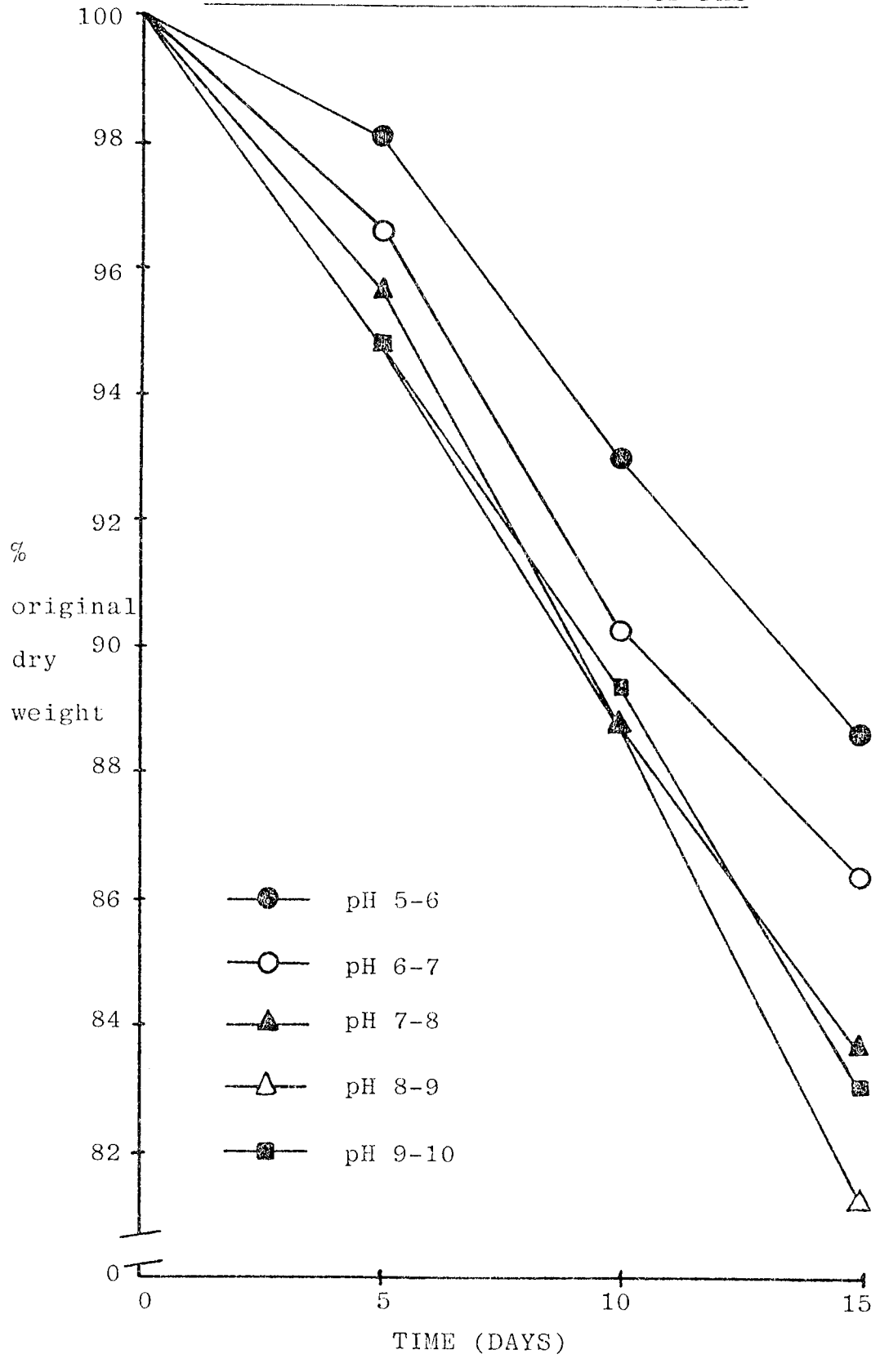


FIGURE 2.2

CHANGES IN PEPSIN-DIASTASE SOLUBLE FRACTION OF BARLEY STRAW AFTER GROWTH OF C. CINEREUS

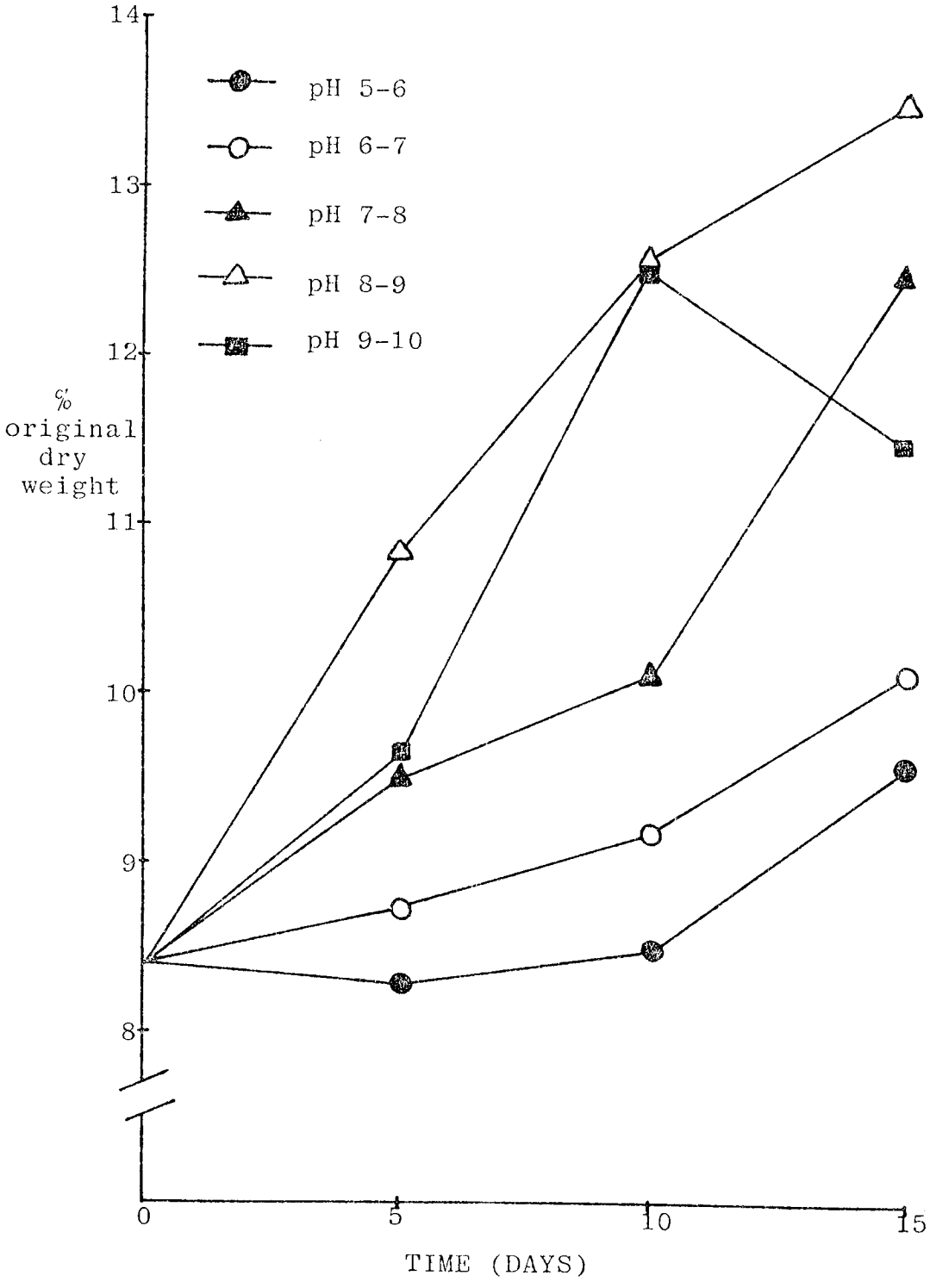


FIGURE 2.3

CHANGES IN HEMICELLULOSE CONTENT OF BARLEY STRAW AFTER GROWTH OF C. CINEREUS

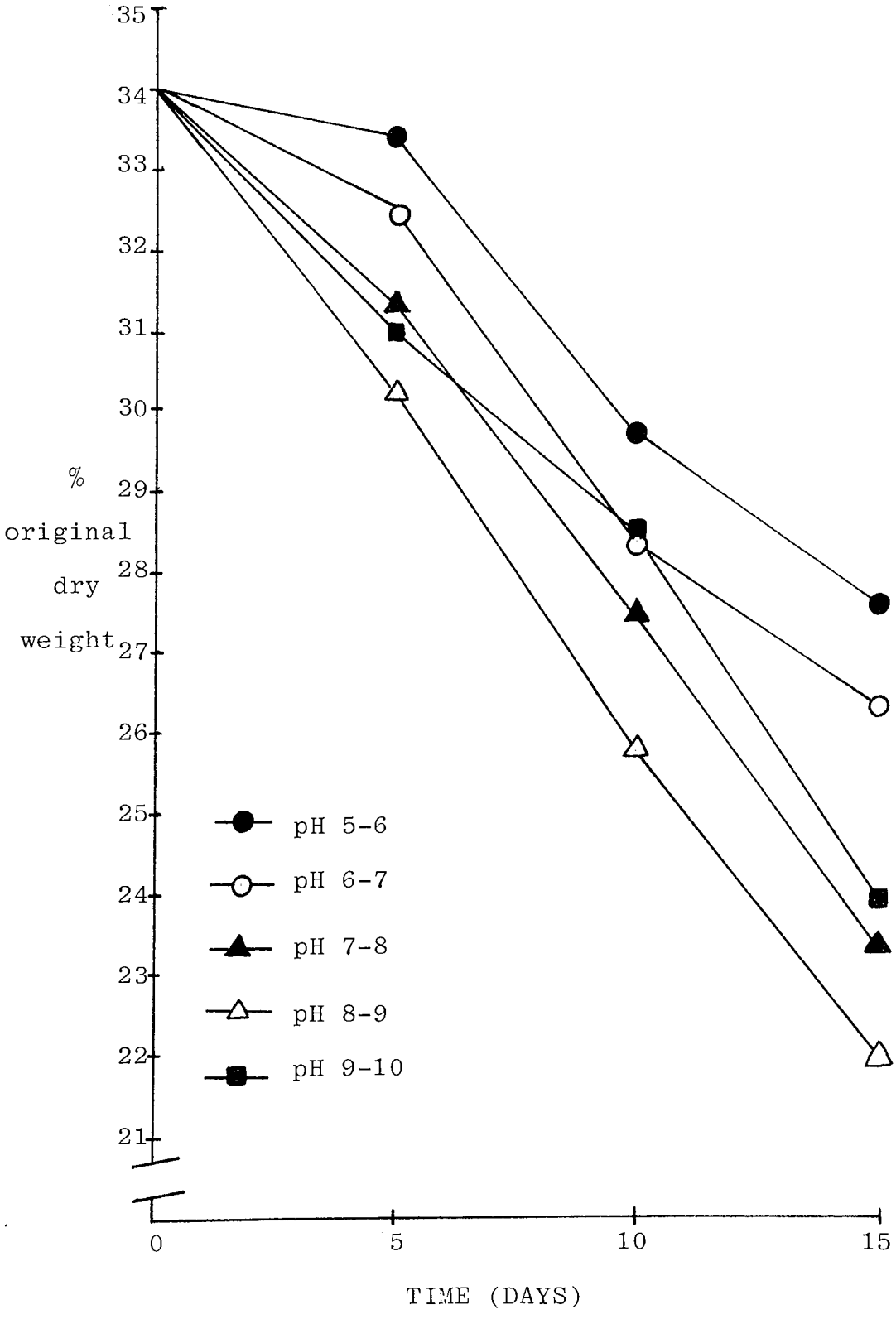


FIGURE 2.4

CHANGES IN CELLULOSE CONTENT OF BARLEY STRAW AFTER GROWTH OF C. CINEREUS

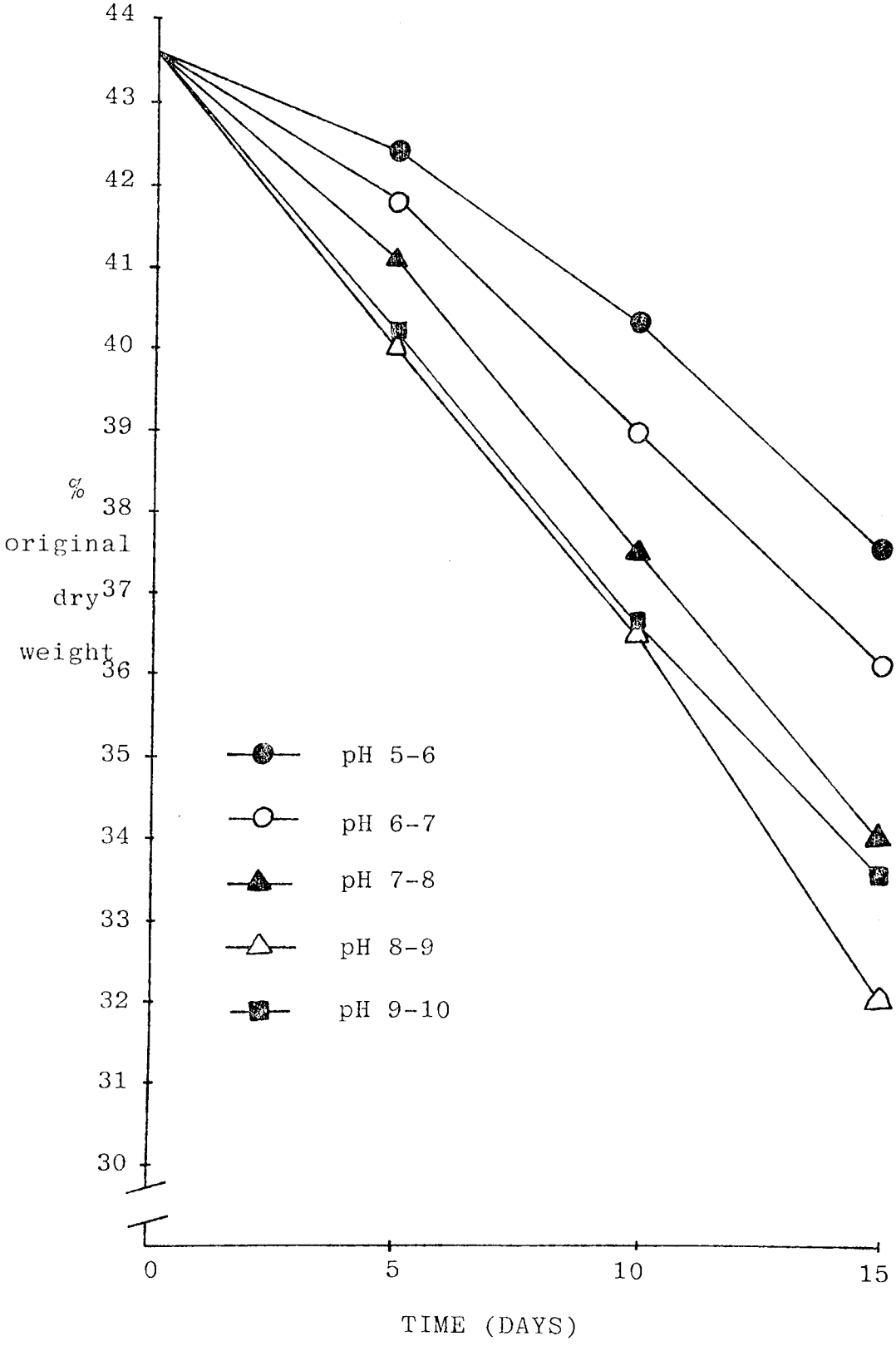


FIGURE 2.5

HEMICELLULOSE AND CELLULOSE CONTENT OF BARLEY STRAW AFTER GROWTH OF C. CINEREUS

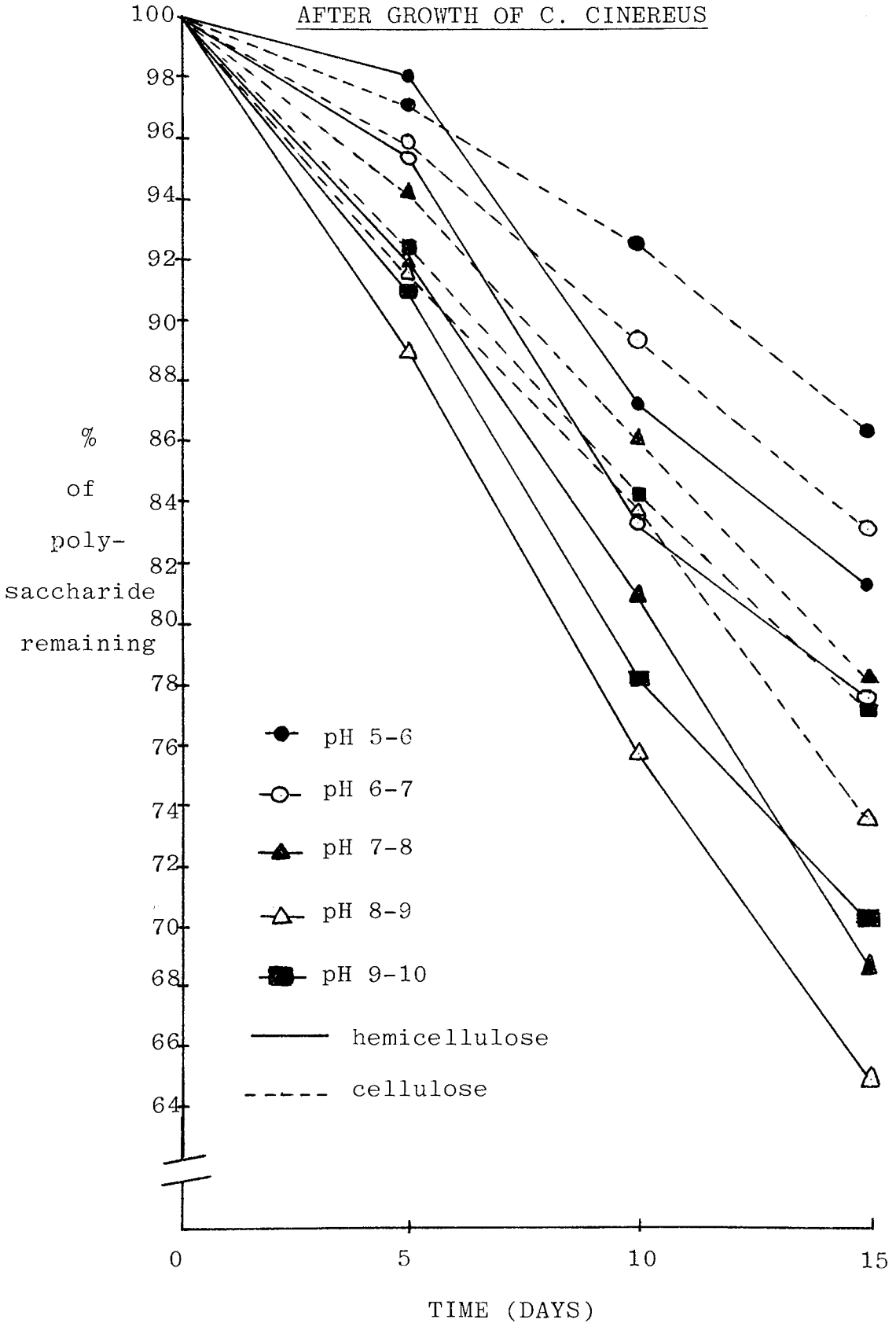


TABLE 2.3

LIGNIN CONTENT OF BARLEY STRAW AFTER GROWTH OF C. CINEREUS

pH range	% ORIGINAL DRY WEIGHT			
	DAY 0	DAY 5	DAY 10	DAY 15
5 to 6	8.17 ± .20	8.16 ± .06	8.17 ± .20	7.93 ± .08
6 to 7	8.17 ± .20	7.87 ± .12	7.96 ± .14	7.93 ± .16
7 to 8	8.17 ± .20	7.90 ± .21	7.82 ± .14	7.94 ± .15
8 to 9	8.17 ± .20	7.95 ± .29	8.01 ± .03	7.96 ± .25
9 to 10	8.17 ± .20	8.18 ± .18	8.05 ± .18	8.01 ± .32

of C. cinereus is linked with pH. Figure 2.1 indicates C. cinereus grew best on straw amended with ammonia to give a pH value of 8 to 9. This is in agreement with the work of Milne (1975) and McShane (1976) who found C. cinereus to have a growth optimum between pH 7.9 and 8.3. C. cinereus is part of a succession of fungi found on dungs (Webster, 1970) thus a tolerance to the alkaline conditions frequently found in these habitats may be a prerequisite for the effective colonisation of dungs by coprophilic species.

In Table 2.2 the static values of the ethanol-benzene soluble fractions possibly reflect a rapid turnover of the products of cellulolysis to storage products. Chang-Ho and Yee (1977) reported similar results from growth of C. cinereus on rice straw, with no change in ethanol soluble materials after 21 days incubation at 30°C. Rapid conversion of the products of cellulolysis is supported by the relatively low levels of reducing sugars found in C. cinereus, compared to other fungi from wheat straw composts, by Hedger and Hudson (1974), despite it being strongly cellulolytic. The results of the pepsin-diastase soluble fractions in Figure 2.2 further support the idea of rapid conversion of sugars to storage products. These fractions contain protein, starch and glycogen and probably represent accumulation of storage products and the conversion of ammonia to fungal protein. The maximum values obtained were in the 8 to 9 pH range which is the optimum pH range

for growth of C. cinereus in this experiment. C. cinereus has been shown to utilise ammonium nitrogen by several workers (Voderberg, 1948; Fries, 1955; Madelin, 1956; Milne, 1975; and Kelley, 1979), although the actual role of ammonia in promoting its growth is not fully understood. This will be discussed in greater depth in a later chapter.

Decomposition of hemicellulose, shown in Figure 2.3, was most rapid at the optimum pH range for growth. Its breakdown rate at pH 8 to 9 was almost linear and showed the affinity of the fungus for this particular component. Chang (1967) found that xylan was utilised to a greater extent than cellulose by C. cinereus when these polysaccharides were supplied as sole carbon sources. The results shown in Figure 2.4 show very similar trends in cellulose decomposition with greatest activity at pH 8 to 9. A number of workers have studied the cellulolytic abilities of C. cinereus (Rege, 1927; Fries, 1955; Chang, 1967; Chang-Ho and Yee, 1977; Jodice, 1971; Penn, 1977 and Kelley, 1979) and some have come to opposing conclusions,finding that C. cinereus is not cellulolytic (Rege, 1927) or that it shows "strong cellulolytic activity" (Jodice, 1971). Most researchers, however, have obtained results somewhere between those two extremes, and find that C. cinereus is cellulolytic though not as active as such organisms as Chaetomium thermophile, Aspergillus fumigatus and Trichoderma viride (Chang, 1967; Penn, 1977).

Hemicellulose and cellulose are closely bound together as components of the plant cell wall. Figures 2.3 and 2.4 show that the rates of decomposition by weight of these two components were very similar. This indicates that hemicellulose is preferentially decomposed by C. cinereus as it is less abundant in straw than cellulose (Owen, 1976). Figure 2.5 shows that the amount of hemicellulose decomposed compared to cellulose was greatest at the optimum pH range for growth of the fungus. This indicates that, despite being cellulolytic, the fungus has a definite affinity for hemicellulose in barley straw. Highley (1976) showed that the decomposition of cellulose in coniferous wood by brown rot fungi depended on the prior removal of hemicellulose, while Lyr (1960) found that the hemicellulasic activities of several wood destroying fungi reached a maximum rapidly and before cellulase activity reached a maximum. This could explain the apparent affinity of C. cinereus for hemicellulose, in that the organism has no alternative but to decompose hemicellulose first, by virtue of the organisation of the polymers of the cell wall. Decomposition of the encrusting hemicellulose would then enable the crystalline cores of cellulose microfibrils to be digested.

Table 2.3 shows that no lignin decomposition could be demonstrated during the course of the experiment. Many workers have reported C.cinereus to give positive results in the "Bavendamm" test (Fries, 1955; Jodice, 1971; Penn,

1977; and Kelley, 1979) and those using lignin containing substrates have found a slow but definite loss of lignin over a long period. Those using shorter periods have reported little, if any, loss of lignin (Chang, 1967; Chang-Ho and Yee, 1977). The short period of this experiment may have prevented the detection of lignin decomposition by weight loss, although this does not necessarily mean there had been no attack on straw lignin by C. cinereus over this period. Lignin has been shown to be covalently bound to hemicellulose (Morrison, 1979) and it is possible that some modification of the lignin structure had taken place during hemicellulose decomposition by C. cinereus.

The changes in pH of the sets of straw treatments indicate a certain self buffering capacity by the organism. This is probably due to the production of ammonia by C. cinereus (Moore, personal communication) and is discussed in depth in a later chapter.

2.5 CHAPTER CONCLUSIONS

The activity of C. cinereus on barley straw amended with ammonia indicates that it is physiologically adapted for colonisation of alkaline substrates. This is reflected in its natural occurrence on dungs. Its ability to grow rapidly through the straw and to efficiently utilise the straw polysaccharides show an opportunistic potential in

artificial circumstances such as straw-dung mixtures and composts, which are frequently alkaline due to urea or urine decomposition.

The rapid growth of the fungus on the straw at pH 8 to 9, the resultant increase in protein and glycogen and the selective decomposition of hemicellulose indicate that C. cinereus may be of great importance in producing a material of enhanced digestibility in the short term. The next chapter will describe the nutritional evaluation of barley straw as a ruminant feedstuff after growth of C. cinereus.

C H A P T E R T H R E E

A STUDY OF THE VALUE OF BARLEY STRAW AS A RUMINANT FEEDSTUFF AFTER GROWTH OF *COPRINUS CINEREUS*

3.1 INTRODUCTION

3.1.1 General

The results of the investigations in the previous chapter indicated that the growth of C. cinereus on barley straw may enhance its value as a ruminant feedstuff. The object of the work in this chapter was to interpret these results, and subsequent nutritional analyses of the treated straw with this aim in view. It was hoped that this would result in greater understanding of the controllable conditions that give the most desirable product. The feasibility of upscaling the most promising treatment may also be indicated by this investigation.

3.1.2 Digestibility and its estimation

The digestibility of a feedstuff is simply a measurement of the amount of material solubilised by the digestive activities of the animal. Direct measurement of this value can often be difficult and time consuming to perform. One in vivo method used by the Agricultural Development and Advisory Service involves the measurement of feed intake compared to excretory products. This gives an accurate assessment of digestibility and is used to provide standard values for feedstuffs (M.A.F.F., 1979). However, this procedure is very time consuming and is restricted to

establishments equipped to use ruminants as test animals. A quicker method involves introduction of a forage sample in a nylon bag directly into the rumen via a fistula (Mehrez and Ørskov, 1977). After one day the nylon bag is removed and weight loss, and hence digestibility, is determined. Unfortunately this technique also requires highly specialised laboratory facilities and is inconvenient for evaluating a large number of samples.

Many laboratories rely on an _in vitro_ fermentation of the forage using rumen liquor (Tilley and Terry, 1963). However, this method again requires the close proximity of laboratory animals and can only be carried out at establishments with these facilities. Further, rumen liquor is considered difficult to maintain at a standard activity (Guggolz et al., 1971; Kellner and Kirchgessner, 1976) as fibre digestion capabilities are conditioned by the type of feed the donor animals have been receiving.

An alternative to rumen liquor for digestion is an enzyme solution as proposed by Jarrige et al. (1970) and Jones and Hayward (1973). Guggolz et al. (1971) used successive incubations with commercial cellulase and protease preparations for digestibility estimations of 29 forages. The correlation between the amount of material solubilised by enzyme treatment and the _in vivo_ digestibility of the forages was 0.90. McQueen and Van Soest (1975) used

commercial preparations of cellulase, hemicellulase and pepsin in a method that correlated at 0.92 with the *in vitro* digestibility of 18 forages. Clark and Beard (1977) used cellulase and pepsin for digestibility estimations of 23 forages and found significant correlation with dry matter digestibility *in vivo* ($r = 0.84$).

It would be desirable to have such enzymatic determinations as a single operation with low protein content cellulosic materials ($< 2\%$) such as most straws it is possible that a proteolytic stage may be omitted to obtain an overall digestibility determination. Rexen (1977) investigated the potential of this approach for alkali treated straw and found that his technique provided a simple and fast estimate of digestibility *in vitro* ($r = 0.91$).

Where no facilities for *in vivo* or rumen liquor digestibility determinations exist, the simplest method for low protein content cellulosic materials appears to be that of Rexen (1977). This technique was slightly modified and used to predict the digestibility of straw samples throughout this investigation.

3.2 MATERIALS AND METHODS

The straw samples for digestibility determination were those produced by the method described in the previous

chapter. The technique described by Rexen (1977) involved the use of straw ground to pass a 0.8mm. screen. The milled straw was oven dried at 85°C (Trinder and Hall, 1972) and a 1g sample placed in a universal bottle with metal cap and rubber washer.

Distilled water was added to 15ml followed by 5ml of citrate buffer solution and 0.75g of cellulase powder. The bottle was sealed and placed in a 40°C water bath and shaken mechanically for 24 hours.

The contents of the universal bottle were transferred to a centrifuge tube and centrifuged for 10 minutes at 3,000 rev/min. The supernatant was discarded and all residual particles were washed from the universal bottle to the centrifuge tube with approximately 30ml of distilled water. The sample was resuspended, centrifuged again and washed into a previously weighed glass filter crucible (pore size 15 - 40μ). The residue was washed with water, oven dried at 85°C and the amount solubilised determined by weight difference.

Rexen (1977) investigated the activity of five commercial brands of cellulase, interpreting the results in terms of the percentage of the activity of one of them. Onozuka SS produced by Yakult Biochemicals Company Limited, Japan, was taken as the standard and BDH cellulase produced by British

Drug Houses Limited from Trichoderma viride gave the highest activity. Due to easy availability, BDH cellulase was used throughout these investigations.

The enzyme preparation was standardised by the method of Mandels and Weber (1969). The percentage of material solubilised was converted to an Onozuka SS value and included in Rexen's regression equation:-

$$y = 33.24 + 0.83x$$

where

y = organic matter digestibility *in vitro*

and

x = Onozuka SS soluble organic matter

Reagents

Cellulase: British Drug Houses Ltd., Batch No. 6564220

Citrate Buffer: 1.0M, 210.14g citric acid ($1H_2O$) and 88.0g sodium hydroxide in 1 litre, pH adjusted to 4.8.

The same batch number of cellulase was used throughout this investigation as it was thought there may have been some differences in activity between batches. This was later confirmed by Baker (1980, personal communication).

This technique did not detect increases in protein content due to the presence of fungal tissue, thus the

results obtained were probably lower than the actual digestibility of the material, although the degradability of the fungal tissue in the rumen was not determined. To avoid including an unknown factor in the digestibility results the values of digestibility and crude protein content were considered separately.

Crude protein was determined by the micro Kjeldahl method which is described in Appendix Id.

Samples for these analyses were chosen randomly from the sets of five replicates from the four isolates of C. cinereus. Two samples were chosen per isolate and mean values for each treatment were taken giving eight replicates in all for each datum point.

It has been shown in Chapter 1 that ammonia can be used to increase straw digestibility and protein content chemically. This is usually achieved with concentrations in excess of 3.5% anhydrous ammonia per kg dry straw (Arnason and Mo, 1977). To evaluate the contribution of the added ammonia during the growth of C. cinereus on straw,the "blank" samples (without C. cinereus) were analysed. These were single samples per isolate giving four replicates for each datum point.

3.3 RESULTS

The results are presented in Figures 3.1 and 3.2 and Tables 3.1 and 3.2. Kjeldahl protein or crude protein values increased throughout the period of the experiment. The maximum value of 7.42% was obtained after 15 days at pH 8 to 9. Table 3.1 shows that only 0.2 percentage units of crude protein were due to chemically bound ammonia in the straw.

Digestibility increased over the whole pH range up to day 10. The highest values occurred in the alkaline pH ranges with the maximum value of 58.5% obtained at pH 8 to 9. Table 3.2 shows that less than one percentage unit increase in digestibility was due to the chemical activity of ammonia on the straw. All digestibility values dropped to around the original level or below at day 15. The greatest decreases in digestibility occurred in the alkaline pH ranges.

3.4 DISCUSSION

From the results obtained it can again be seen that growth of C. cinereus on barley straw is linked with pH. Figure 3.1, in common with the results from Chapter 2, shows C. cinereus grew best within the pH values of 8 to 9. It has been shown by several workers that C. cinereus is

FIGURE 3.1

KJELDAHL PROTEIN CONTENT OF BARLEY STRAW AFTER GROWTH OF C. CINEREUS

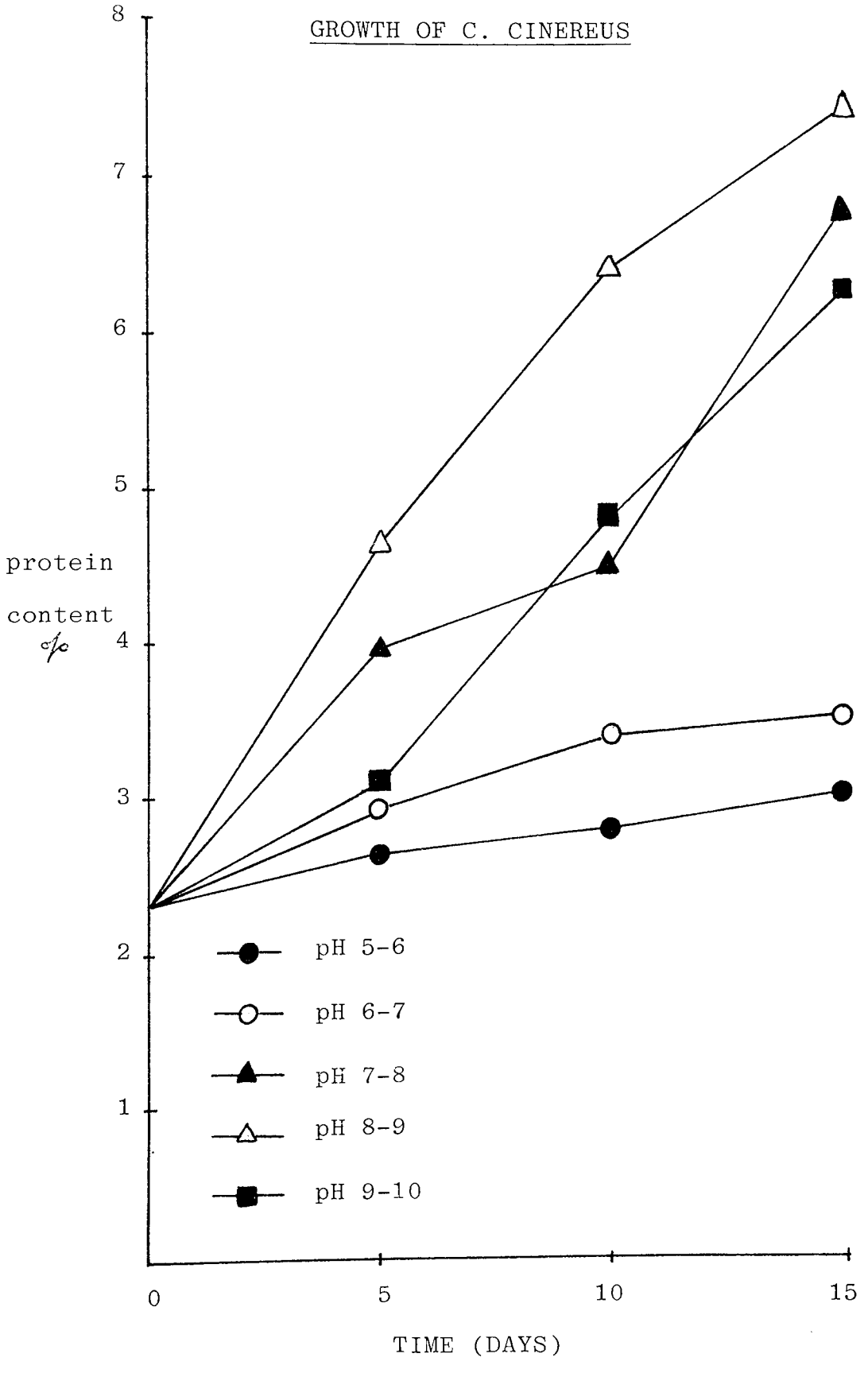


TABLE 3.1

CONTRIBUTION OF AMMONIA TO CRUDE PROTEIN CONTENT

pH range	CRUDE PROTEIN CONTENT %			
	DAY 0	DAY 5	DAY 10	DAY 15
5 to 6	2.33 ± .06	2.33 ± .11	2.28 ± .07	2.37 ± .12
6 to 7	2.34 ± .12	2.33 ± .10	2.28 ± .08	2.36 ± .12
7 to 8	2.33 ± .11	2.34 ± .10	2.32 ± .12	2.44 ± .05
8 to 9	2.32 ± .11	2.34 ± .10	2.35 ± .12	2.49 ± .07
9 to 10	2.33 ± .11	2.28 ± .05	2.37 ± .13	2.50 ± .12

FIGURE 3.2

DIGESTIBILITY OF BARLEY STRAW AFTER GROWTH OF C. CINEREUS

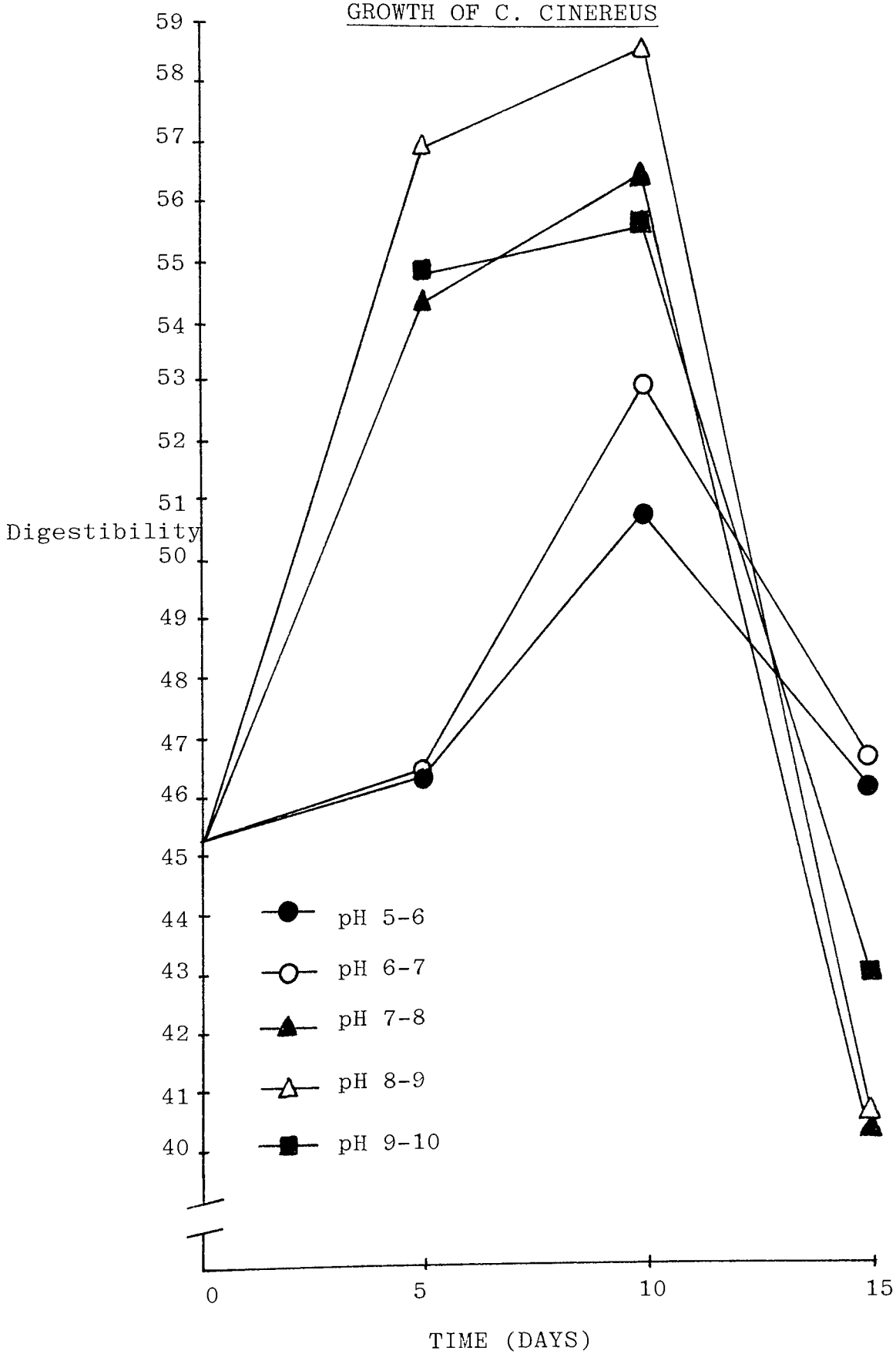


TABLE 3.2

CONTRIBUTION OF AMMONIA TO STRAW DIGESTIBILITY

pH range	DIGESTIBILITY %			
	DAY 0	DAY 5	DAY 10	DAY 15
5 to 6	45.3 ± .1	45.3 ± .1	45.4 ± .1	45.3 ± .2
6 to 7	45.4 ± .1	45.4 ± .1	45.3 ± .1	45.3 ± .2
7 to 8	45.2 ± .1	45.4 ± .1	45.5 ± .1	45.5 ± .1
8 to 9	45.4 ± .1	45.6 ± .3	45.9 ± .4	45.9 ± .2
9 to 10	45.4 ± .1	45.7 ± .3	45.9 ± .3	46.2 ± .2

capable of utilising ammonia as a sole source of nitrogen (Darbyshire, 1974; Penn, 1977 and Kelley, 1979). Increase in crude protein content largely indicates the production of fungal mycelium in this experiment, reflecting the ability of the organism to synthesise amino acids from ammonia. It is likely that the energy required for amino acid synthesis from ammonia is provided by the breakdown products of straw carbohydrates in this case.

The maximum crude protein content obtained was 7.42%. The ARC (1965) recommended a minimum crude protein content of 12% of the dry matter of the feedstuff to sustain the microbial activity of the rumen. This figure is not, however, a true indication of the digestible protein when this material is fed to a ruminant. The amount of protein required depends on its degradability in the rumen. Much less is required of a protein of low degradability (provided it is still well digested in the small intestine) than one of high degradability. The minimum crude protein concentration is 8.6% of the dry matter when all the crude protein is available for microbial metabolism. The microbial protein yield is calculated to be adequate for maintenance. As the degradability decreases, the crude protein requirement increases such that at a degradability of 70% in the rumen the required crude protein is 12.3% of the dry matter to supply ruminal needs (Miller, 1978). The remaining 30% is absorbed by the small intestine and contributes to milk production.

The crude protein content of barley straw after growth of C. cinereus is too low to maintain the microbial activity of the rumen, even if the degradability of the fungal protein is high. If the material was fed simply as a protein source, then supplementation would be required in the form of non-protein nitrogen or protein depending on the degradability of the fungal material. From Table 3.1 the small amount of ammonia chemically bound to the straw would contribute a little non-protein nitrogen to the ruminant diet.

The maximum digestibility value obtained was 58.5%. This figure is equivalent to the digestibility of good quality grass hay (M.A.F.F., 1979), thus the feeding potential of the initial straw has beenraised to that of a material commonly used as a maintenance feed for ruminants. From Table 3.2 the contribution of ammonia to this figure is 0.8% which indicates that the increases in digestibility over the whole range investigated were due largely to fungal activity. The decreases in digestibility following the peaks probably reflect extensive utilisation of the carbohydrate fractions by the fungus. It has been shown in Chapter 2 that a steady decrease in hemicellulose and cellulose occurs during the growth of C. cinereus on straw.

Lignification has been considered to be one of the primary barriers to ruminant microbial digestion of cellulosic materials. The digestibility of these materials such as

straw can be increased by physical, chemical or biological methods. Usually these methods involve modification of lignin or its removal to increase the availability of the carbohydrate fraction to the ruminant digestive system. Polčin and Bezúch (1977) showed that grinding of spruce wood increased the susceptibility of cell walls to enzyme hydrolysis by breaking the shielding lignin layer. Kamstra et al. (1980) investigated the delignification of cellulosic wastes by peroxyactetic acid treatments and found that although almost complete delignification could be achieved, 60% delignification was sufficient for preparation of ruminant feeds.

The biological decomposition of lignified cellulosic materials to increase digestibility has received little attention to date although some work has shown promise. Latham (1979) obtained an 11% increase in dry matter digestibility using a Schizophyllum commune isolate capable of degrading extracted lignin (Indulin A). Although it possesses an active polyphenol oxidase system (Kelley, 1979), the results presented in the previous chapter indicate that C. cinereus did not actually decrease the amount of lignin in barley straw. It is likely, therefore, that the activity of C. cinereus on another straw component, or on lignin to effect some undetected modification of its shielding properties, or both of these activities caused the digestibility of the straw to be increased.

Waite et al. (1964) showed by analysis of increasingly mature grasses that decreases in digestibility with maturity were probably linked with increasing amounts of lignin and hemicellulose. It has been shown that lignin and hemicellulose are covalently bound in the plant cell wall (Morrison, 1979), and that the components of birch wood cell walls are arranged in an organised fashion around the cellulose fibre axis (Kerr and Goring, 1977). It is likely that the polymers of the straw cell wall are arranged in a similarly ordered fashion. Disruption of this order could lead to the increased penetration of diffusing materials such as cellulolytic enzymes, as shown by Tarkow and Feist (1969) when hardwoods were treated with 1 % sodium hydroxide.

It follows that removal of any of the major cell wall components would effectively disrupt the order of the remaining cell wall polymers. This could lead to increased availability of the remaining carbohydrate, thus it was attempted to reveal links between hemicellulose and cellulose utilisation and straw digestibility. Since digestibility values only increased up to the tenth day, only these results were used in the scatter diagrams (Figures 3.3 and 3.4) relating percentage of hemicellulose and cellulose utilised against digestibility. The correlation coefficients were calculated and found to be very similar. The values obtained were found to be significant at a

FIGURE 3.3

CORRELATION BETWEEN HEMICELLULOSE UTILISED BY C. CINEREUS AND DIGESTIBILITY OF BARLEY STRAW OVER 10 DAYS

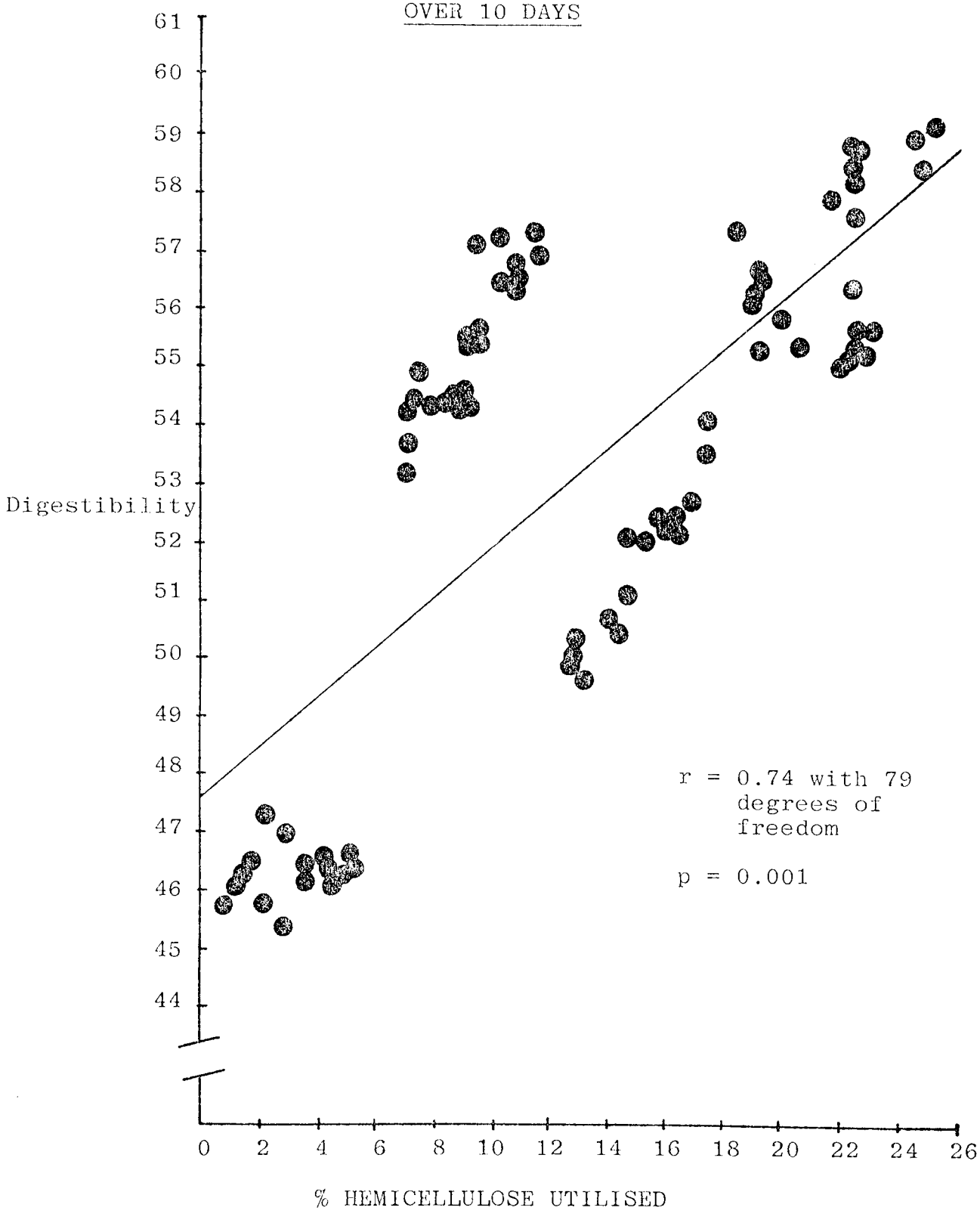
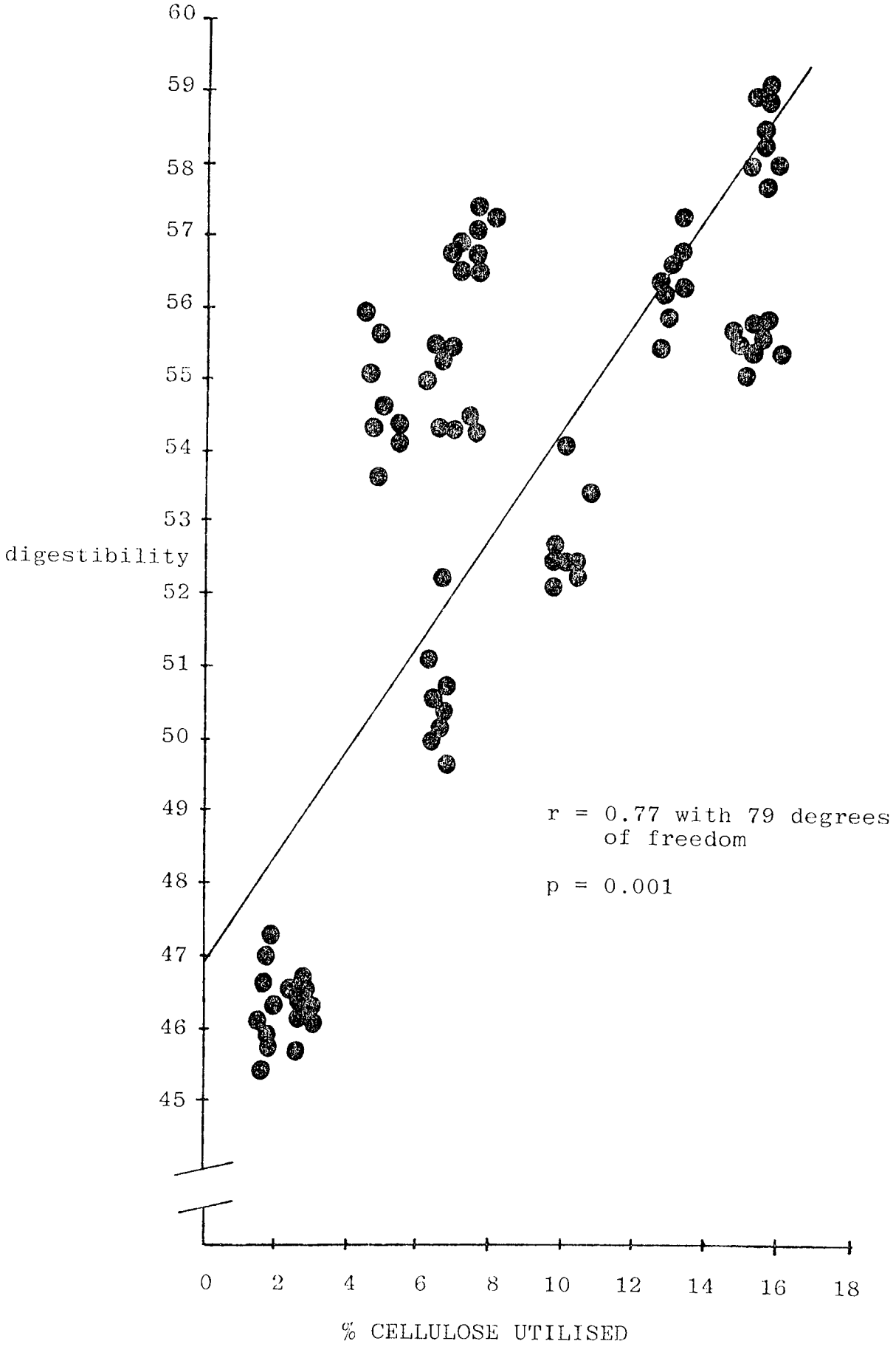


FIGURE 3.4

CORRELATION BETWEEN CELLULOSE UTILISED BY C. CINEREUS AND DIGESTIBILITY OF BARLEY STRAW OVER 10 DAYS



probability level of $p = 0.001$. Thus the percentage of hemicellulose and cellulose utilised by C. cinereus and the resulting digestibility of straw were positively related.

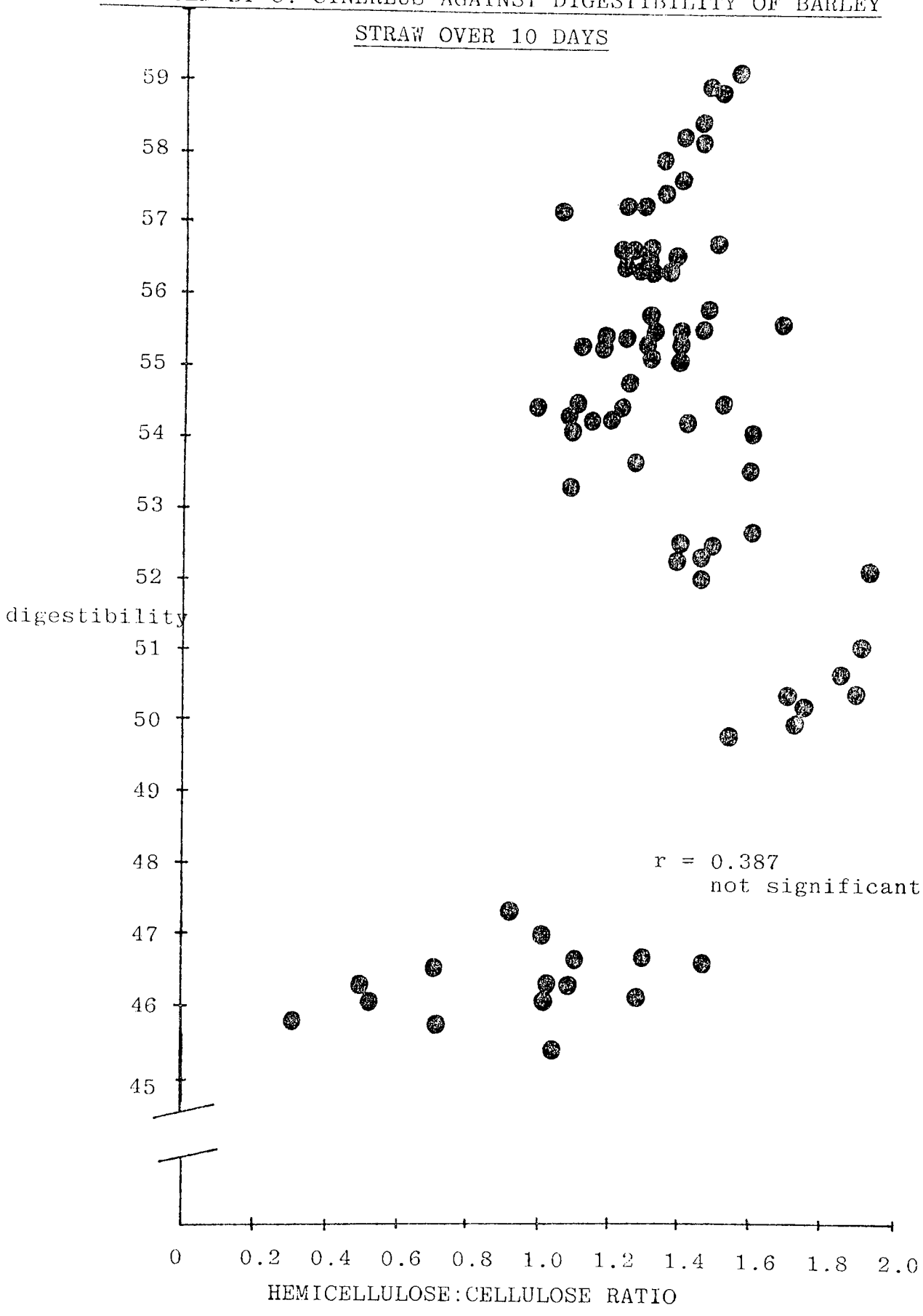
Waksman (1931) showed that some microorganisms will attack hemicelluloses more readily during the early stages of decomposition of a plant material, and attack the cellulose later on. C. cinereus shows a definite affinity towards hemicellulose although it has been shown in the previous chapter to simultaneously degrade cellulose but at a slower rate. This may be a result of the organisation of the cell wall polymers as discussed earlier. The hemicellulose components are probably the first to be encountered and solubilised by the fungal hyphae ramifying the straw.

It was attempted to demonstrate the suspected link between hemicellulose utilisation and straw digestibility by a scatter diagram (Figure 3.5) relating the ratio of hemicellulose to cellulose utilised against digestibility. The correlation coefficient was found to be not significant. This indicates that although hemicellulose utilisation is positively related to the straw digestibility, another factor must also be considered.

It has been mentioned previously that C. cinereus possesses the ability to slowly degrade lignin, but this

FIGURE 3.5

CORRELATION BETWEEN RATIO OF HEMICELLULOSE AND CELLULOSE UTILISED BY C. CINEREUS AGAINST DIGESTIBILITY OF BARLEY STRAW OVER 10 DAYS



was not apparent in this investigation. However, a comparison of hemicellulose content and digestibility values between day 10 and day 15 from selected pH ranges shows that fungal activity other than hemicellulose decomposition contributed part of the digestibility increases. Maximum digestibility values of 58.5% were obtained after 10 days at pH 8 to 9, with hemicellulose content at 25.8%. After 15 days at pH 6 to 7 the digestibility values were 45.9% with hemicellulose content at 26.4%. This indicates that some activity occurred at alkaline pH only which contributes towards increase in digestibility. It is most likely that this effect is brought about by fungal activity on lignin, but it is not detectable as a decrease in lignin content.

The fact that all digestibility values fell to around the level of the original straw or below after day 10 indicates rapid utilisation of the carbohydrates that were previously available for ruminant digestion. This is of great importance when considering the upscaling of such a process for feed production as the material has only a short period of actual increased feed value. Up to the tenth day, barley straw has been increased in nutritional value to levels equivalent to good quality grass hay in both digestibility and protein content, however the decreases in digestibility afterwards could be a severe drawback to practical application of the laboratory scale experiment.

3.5 CHAPTER CONCLUSIONS

The increases in protein content and digestibility of barley straw after growth of C. cinereus at pH 8 to 9 indicate that it is possible to produce a material of increased feed value to ruminants. It has also been shown that digestibility increases are positively related to hemicellulose and cellulose utilisation in the short term by the fungus. It is also thought that modification of lignin structure by C. cinereus contributed to this digestibility increase.

The major disadvantage demonstrated so far is that the upgraded straw rapidly decreased in digestibility after reaching its peak on the tenth day of incubation. This decrease was probably due to extensive carbohydrate utilisation by C. cinereus.

The next chapter will describe the upscaling of the most promising treatments and the nutritional evaluation of the materials produced.

C H A P T E R F O U R

INVESTIGATION OF THE CAPACITY OF *COPRINUS CINEREUS* TO UPGRADE BARLEY STRAW ON THE LARGE SCALE

4.1 THE USE OF BALED STRAW TO ENCOURAGE THE GROWTH OF C. CINEREUS

4.1.1 Introduction

The results obtained in the previous chapter indicated that a product equivalent in feed value to good quality hay could be obtained from sterile barley straw after growth of C. cinereus.

Seal and Kelley (1980) showed that ammonia solutions can produce highly selective conditions for the isolation and growth of C. cinereus from barley straw, while the growth of other microorganisms is suppressed. Kelley (1979) investigated the mechanism of selection of C. cinereus in ammoniated barley straw and concluded that competitive advantage was conferred by a tolerance to high ammonia levels rather than through the success of ammonia as a nitrogen source. The inhibition of competitive organisms in ammoniated straw enabled C. cinereus to grow at the same rate on unsterile straw as on sterile straw.

This implies that an upgrading process using unsterile barley straw treated with ammonia solution to encourage growth of C. cinereus is feasible provided suitable conditions can be maintained. Upscaling the previous work would highlight some of the practical problems to be encountered and could also indicate the value of the method used in terms of

product quality. The next step was taken with ease of operation and economy in mind, for use of the method in farm-type conditions.

4.1.2 Materials and methods

An ammonia solution of suitable concentration to create the conditions for maximum growth of C. cinereus in barley straw was required. Kelley (1979) investigated growth of C. cinereus over a range of ammonia concentrations and found levels between 0.5% w/w and 2.0% w/w to produce optimum growth of C. cinereus with maximum suppression of other species.

A rapid method of introducing 1% w/w ammonia into the straw was devised. It was found that a half bale, soaked in water and drained, retained approximately its own weight of water. Use of a 1% w/w ammonia solution for spraying or soaking thus incorporated a concentration of ammonia into the straw which was within the limits most suitable for growth of C. cinereus.

C. cinereus has been demonstrated to be part of the indigenous microflora of barley straw (Penn, 1977) and has been induced to grow in such straw by the addition of certain ammonia concentrations (Kelley, 1979). The requirement for a supplement to this natural inoculum had not been studied,

so this was investigated as part of the large scale trials.

The following experiments were conducted in an unlit cellar of variable temperature. The temperature fluctuated between 27°C and 31°C at that time of year, which reflected the probable level of control to be encountered in an on farm application of the composting process. These temperatures were still within the favourable limits for growth of the fungus (McShane, 1976).

Straw bales were divided in half and six straw-baited screened substrate isolation tubes (as described in Chapter 5) were placed in both the centre and outer 5cm of each half bale. The bale was re-tied using polypropylene bailer twine.

Two methods of introducing the inoculum of C. cinereus were investigated, and uninoculated controls were included to indicate the value of the naturally present C. cinereus propagules as the sole inoculum.

1. A half bale was soaked thoroughly in a 1% w/w ammonia solution contained in a 100 litre capacity glassfibre tank, and allowed to drain. A spore suspension of 1×10^5 spores/ml was injected in 10 x 10ml doses into the centre of the bale. A further 50ml of suspension was sprinkled over the bale surface. The bale was wrapped in a black polythene sheet and incubated at 29°C $\pm$ 2.

2. A spore suspension of 1 x 10^6 spores/ml was prepared and 100ml added to approximately 30 litres of 1% w/w ammonia solution in a glassfibre tank. A half bale was soaked in this suspension, drained, wrapped in a black polythene sheet and incubated at 29^{o}C $\pm$ 2.

Two replicates of each method including controls were set up, and samples for digestibility determination and pH monitoring were removed on days 0, 5, 10 and 15. Duplicate samples were taken from the centre and periphery of each bale. Temperatures of the bale centre and at 5cm depth were taken on sampling days using a Comark portable thermometer. On sampling days following the initial setting up, two screened substrate tubes were removed from both the centre and outer series of tubes. The straw baits were removed and plated out onto malt extract agar. The plates were incubated at 30^{o}C and the fungi isolated were sub-cultured and identified.

4.1.3 Results

These are shown in Tables 4.1 and 4.2. From Table 4.1 it can be seen that a marked variation in straw digestibility occurred between the bale centre and periphery in all cases. Digestibility increased up to day 10 on the periphery of the bales followed by a decline. The bale centres all showed a general decline in digestibility to levels below 35% by the end of the experiment.

TABLE 4.1

DIGESTIBILITY, TEMPERATURE AND pH OF AMMONIATED BALED STRAW DURING COMPOSTING

	DAY	DIGESTIBILITY (%)		TEMPERATURE (^{o}C)		pH	
		Bale periphery	Bale centre	Bale periphery	Bale centre	Bale periphery	Bale centre
A	0	45.5	45.5	28.5	28.5	9.35	9.35
	5	47.8	41.6	32.5	38.0	9.00	9.05
	10	55.8	37.3	33.0	49.5	9.00	8.25
	15	42.7	35	35.0	55.5	8.75	8.00
B	0	45.5	45.4	28.5	28.5	9.35	9.35
	5	47.4	41.7	31.0	38.5	9.15	8.90
	10	56.1	36.2	33.0	51.0	9.00	8.15
	15	43.2	35	34.5	57.0	8.85	7.95
C	0	45.4	45.5	28.5	28.5	9.35	9.35
	5	46.0	41.2	30.5	38.0	9.05	8.75
	10	49.4	37.2	31.5	49.0	8.90	8.10
	15	46.7	35	33.0	55.5	8.70	7.90

A = injected

B = soaked

C = uninoculated

TABLE 4.2.

FUNGI ISOLATED BY THE SCREENED SUBSTRATE METHOD FROM AMMONIATED BALED STRAW COMPOSTS

METHOD OF INOCULATION	SAMPLING DAY	LOCATION OF ISOLATE	
		BALE CENTRE	BALE PERIPHERY
INJECTED	5	C. cinereus A. fumigatus G. roseum	C. cinereus
	10	A. fumigatus C. thermophile	C. cinereus
	15	A. fumigatus C. thermophile	C. cinereus
SOAKED	5	C. cinereus A. fumigatus G. roseum	C. cinereus
	10	A. fumigatus	C. cinereus
	15	A. fumigatus C. thermophile	C. cinereus
UNINOCULATED	5	A. fumigatus G. roseum	C. cinereus G. roseum
	10	A. fumigatus C. thermophile	C. cinereus G. roseum
	15	A. fumigatus C. thermophile	C. cinereus G. roseum

Temperature rose in the centre of all the bales to over 55°C by day 15 while the peripheral temperatures rose to around 34°C. Initial pHs were 9.35. These dropped by the end of the experiment to between 8.7 and 8.85 at the bale peripheries to between 7.90 and 8.00 in the bale centres.

C. cinereus was consistently isolated from the peripheries of the bales but did not occur in the bale centres after day 5. Aspergillus fumigatus was consistently isolated from the bale centres.

4.1.4 Discussion

The digestibility values obtained at the periphery of the bales indicated that a product of increased feed value could be obtained from growth of C. cinereus on unsterile ammoniated barley straw. The low digestibility values obtained from the bale centres indicated that conditions within the bales were not conducive to the growth of C. cinereus.

The temperature increases for all the bales were similar and reached 57°C in the centre of the bales inoculated by soaking. Chang and Hudson (1967) reported temperatures of 70°C from the centre of a wheat straw compost after five days. The comparatively lower temperatures of the bales can probably be accounted for by the smaller amount of straw involved, allowing more rapid heat dissipation.

pH gradually decreased, with the most marked falls occurring in the bale centres. This may have been due to the dissipation of ammonia by the increased core temperature or by its utilisation as a nitrogen source by the contaminant microorganisms. The slower pH drop recorded at the bale peripheries may have been due to ammonia movement from the centre to the exterior or by ammonia production by C. cinereus. C. cinereus has been shown to produce ammonia as a regulator between sclerotium and sporophore production (Moore and Jirjis, 1976).

The fungi isolated by the screened substrate technique indicated that the bale centres had been colonised by a small range of thermophilic or thermotolerant species. These organisms have been frequently reported from straw composts (Eastwood, 1952; Chang and Hudson, 1967) along with a wide range of other fungi. It is likely that the fungi isolated in this investigation were the few species of the usual composting succession capable of withstanding the high initial pH. Kelley (1979) also isolated Aspergillus fumigatus and Gliocladium roseum from ammoniated straw and concluded that their characteristics of tolerance to a wide range of conditions, fast growth rates, high spore production and antagonistic properties enabled them to compete in this situation.

4.1.5 Conclusions

Straw bales inoculated with spores of C. cinereus did not provide suitable conditions for the exclusive growth of C. cinereus. An upgraded product was recorded on the periphery of the bales but the bale centres became colonised with contaminant microorganisms that reduced the straw digestibility to levels below that of the original straw.

The presence of A. fumigatus, a potential human and livestock pathogen was disturbing, and it will be necessary to modify methods of growing C. cinereus on straw to prevent the growth of A. fumigatus and other contaminant species.

4.2 THE USE OF LOOSE STRAW TO ENCOURAGE THE GROWTH OF C. CINEREUS

4.2.1 Introduction

The results from the previous section indicated that C. cinereus did not grow adequately enough through tied bales of straw to produce an upgraded material. It was thought, however, that the fungus could possibly colonise loose straw, since all previous experiments had been successful with this approach on the bench-scale (McShane, 1976; Penn, 1977; Kelley, 1979). Less restricted aeration could possibly prevent any tendency towards self heating,

effectively preventing the colonisation of the straw by the contaminant fungi isolated in the previous section.

4.2.2 Materials and methods

Loose barley straw was placed in 5kg lots in four black polythene sacks. 10 litres of 1% w/w ammonia solution were prepared and 100ml of a 1×10^6 spores/ml suspension added. 2.5 litres of the ammonia and spores were added to each sack. Each sack was shaken to thoroughly wet the straw and the excess liquid was discarded.

The same procedure was repeated with four other straw sacks, but an uninoculated ammonia solution was used. This was to investigate the potential of the natural inoculum previously shown to be present in the straw. The sacks were incubated at $29^{\circ}C \pm 2$ in a dark cellar.

After the problems encountered in the previous section it was decided to confine investigations to the centres of the "composts". It was thought that self-heating and its consequences could be rapidly detected and possibly remedied if it occurred. Samples for digestibility estimation and pH measurement were removed from the "compost" centres on days 0, 5, 10 and 15. Temperature of the compost centres was monitored as previously described, and six screened substrate tubes were placed in the central area of each

compost. Two screened substrate tubes were removed from each compost on sampling days following the initial setting up. The straw baits were removed and plated out onto malt extract agar with aureomycin. The fungi isolated by the technique were subcultured and identified.

The sacks were incubated with open tops to permit the escape of excess heat generated if any self heating occurred.

4.2.3 Results

These are presented in Tables 4.3., 4.4. Table 4.3 shows that digestibility increased up to day 10 in both inoculated and uninoculated composts. Digestibility values were maximum in the inoculated composts reaching 57.6 by day 10. By day 15, digestibility values had fallen to levels approaching the original straw.

Temperature remained fairly stable between 29^{o}C and 34^{o}C throughout the experiment. pH declined through approximately one unit through the period of the experiment.

Table 4.4 shows that C. cinereus was the only fungus isolated from the inoculated composts while A. fumigatus and G. roseum were consistently isolated with C. cinereus from the uninoculated composts. Visual inspection of the

TABLE 4.3.

DIGESTIBILITY, TEMPERATURE and pH

OF BAGGED AMMONIATED STRAW COMPOSTS

	SAMPLING DAY	DIGESTIBILITY (%)	TEMPERATURE (%)	pH
INOCULATED	0	45.3 ± .1	29.0 ± .5	9.35 ± .30
	5	49.0 ± .4	31.5 ± .4	8.85 ± .33
	10	57.6 ± .7	30.0 ± .4	8.45 ± .12
	15	46.2 ± .6	33.5 ± .5	8.30 ± .08
UNINOCULATED	0	45.4 ± .3	29.0 ± .3	9.35 ± .04
	5	46.3 ± .8	30.5 ± .1	8.75 ± .25
	10	49.4 ± .8	34.0 ± .6	8.45 ± .16
	15	47.8 .1	30.5 .4	8.20 .23

TABLE 4.4.

FUNGI ISOLATED BY THE SCREENED SUBSTRATE METHOD

FROM BAGGED AMMONIATED STRAW COMPOSTS

SAMPLING DAY	LOCATION OF ISOLATE	
	INOCULATED COMPOST	UNINOCULATED COMPOST
5	C. cinereus	C. cinereus G. roseum A. fumigatus
10	C. cinereus	C. cinereus G. roseum A. fumigatus
15	C. cinereus	C. cinereus G. roseum A. fumigatus

inoculated composts confirmed that C. cinereus had completely ramified the batches of straw composted.

4.2.4 Discussion

From the results obtained it can be seen that C. cinereus grew consistently through the inoculated straw composts giving increased digestibility up to the tenth day. These results reflected the bench top experiment results described in Chapter 3, with digestibility results peaking at 57.6% compared to 58.5% in Chapter 3. The uninoculated composts also produced a material of enhanced digestibility but the maximum level was only 49.4%. This may have been the result of competition for nutrients between the three actively growing fungi isolated from these composts.

Temperatures remained stable in all cases, and it appears that the problem of self heating has been solved by adequate aeration, i.e. leaving the bags open at the top. pH declined steadily reaching the optimum level for C. cinereus growth by, at the most, day 5 (according to the results obtained in Chapter 2). By the end of the experiment the pH was still within the limits for the self-buffering capacity of the fungus measured at between 7.9 and 8.3 by McShane (1976).

The exclusive isolation of C. cinereus from the inoculated composts compared to the mixed population of three species

obtained from the uninoculated composts indicates a swamping effect and possible antagonistic effects by C. cinereus under the alkaline conditions. Alkaline conditions, particularly high ammonia levels, are known to have inhibitory effects on most fungi; indeed it has been shown that ammonia plays an extremely important role in soil fungistasis (Ko and Hora, 1972; and Ko et al., 1974) and it would seem that a similar situation is occurring here. The barley straw used in this investigation has been shown to have at least 22 indigenous mesophilic and thermotolerant species (Kelley, 1979) thus the effects of ammonia added to the straw, coupled with the C. cinereus inoculum had a drastic effect on species diversity.

It appears that provided a suitable inoculum is used in this method of selection for C. cinereus, undesirable contaminant species may be kept to a minimum or excluded completely. It is important to note that the occurrence of A. fumigatus in the uninoculated composts indicates a tolerance to alkaline conditions. This organism is a known pathogen capable of causing respiratory disease in man and other animals, and mycotic abortion in cattle (Lacey, 1975). It is obviously vital that the growth of this fungus is suppressed if the upgraded straw (in terms of its digestibility) is to be used as a ruminant feedstuff. From the results obtained in this investigation it appears that a

swamping inoculum of C. cinereus propagules is necessary to achieve this desired result. Kelley (1979) showed that a swamping inoculum of C. cinereus spores also greatly reduced the number of actinomycetes isolated from bench scale straw treatments. Actinomycetes were not studied in this investigation but it is likely, in view of Kelley's work, that actinomycete populations did not build up to levels indicated as "mouldy" hay by Lacey (1975).

The increases in digestibility obtained by exclusive growth of C. cinereus on the straw were unfortunately short-lived. The falls in digestibility again mirrored the results obtained in Chapter 3. This indicates that the upgraded material must be rapidly fed to animals before it deteriorates, or the deterioration must be prevented.

It has been stated earlier that the upgrading process was planned with economy and ease of operation in mind. The process so far is uncomplicated and remains so if the material produced is fed directly to ruminants. The problem of the decline in quality of the material is only important if the material is required to be stored.

Methods of preventing the growth of the fungus such as drying and pelleting render the whole process uneconomic due to the high energy input required (Wilton, 1980 personal communication). Use of a chemical preservative is a

possibility but a study on the effect of propionic acid on the storage losses of moist hay by Davies and Warboys (1978) indicated that the chemical was difficult to apply effectively and was thus a poor preservative for moist forages.

Concentrations of ammonia sufficient to elevate the pH to preserve the straw are another possibility, but concentrated ammonia is dangerous and unpleasant to use. Also, it is likely that the pH would fall rapidly due to dissipation of the ammonia to the atmosphere, whilst measures to contain the gas would probably be uneconomic to employ. It appears that at present the problem of economically storing fungally upgraded straw without subsequent deterioration remains unsolved.

4.3 CHAPTER CONCLUSIONS

The increases in digestibility obtained from the inoculated straw composts indicate that the large scale upgrading of unsterile straw is feasible. It appears that the requirements for successful growth of *C. cinereus* on straw to give maximum digestibility are a temperature of $29^{o}C \pm 2$, sufficient 1% w/w ammonia solution to create a suitable pH and a swamping inoculum of *C. cinereus* propagules.

The problem of decline in digestibility after the peak has been reached by the tenth day means that storage of the material is not feasible. This problem remains unsolved.

The next chapter will describe the development of the screened substrate tube technique and its application in the investigation of actively growing fungi, particularly C. cinereus.

C H A P T E R F I V E

THE ISOLATION OF ACTIVELY GROWING FUNGI FROM SOIL

5.1 THE DEVELOPMENT OF A TECHNIQUE FOR THE ISOLATION OF ACTIVE STRAW COLONISING FUNGI

5.1.1 Introduction

Having shown C. cinereus to be present on the straw used in the previous study, it was decided to investigate the origin of this natural inoculum. This necessitated the designing of a method to selectively isolate fungi that were actively growing in solid substrates such as straw and soil. The development of the technique is described in this chapter.

The origin of the propagules of C. cinereus on straw is difficult to pinpoint, but since the fungus has been shown to be widespread in arable soils (Kelley, 1979) this may be the most likely source.

The techniques used to isolate fungi from soil are numerous and varied, and all are, in one way or another, selective for fungi with similar physiological characteristics. This selective nature can be put to good use when investigating one particular group of microorganisms, provided the limitations of the techniques used are realised.

The isolation of fungi from soil particles placed on or incorporated into agar media (Waksman, 1916; Warcup, 1950) does not distinguish between actively growing and passive fungi. The dilution plate technique (Waksman, 1927; Warcup, 1960) is similarly misleading (Harley, 1971).

Several techniques have been devised that select for actively growing fungi. These include serial washing to remove surface contamination (Harley and Waid, 1955), direct hyphal plating by teasing fungal fragments from soil (Warcup, 1955) and the use of selective media. These methods are either laborious, impractical or furnish results that can be difficult to interpret (Kelley, 1979).

Chesters (1940) used agar media contained within a glass tube pierced with open-ended invaginated capillaries to isolate actively growing fungi from soil. Thornton (1952) modified this technique to produce screened immersion plates, but the method was selective for fungi only capable of growing under conditions of low oxygen tension (Chesters and Thornton, 1956).

Eggins and Lloyd (1968) developed a screened substrate tube technique where a fibrous cotton cellulosic "bait" was screened from direct contact with the soil by glass fibre fabric tape. On plating out the screened bait it was found that the count of passive microorganisms was lessened but their colonisation of the bait was not prevented (Allsopp and Eggins, 1972).

It was thought that the main limitation of the screened substrate technique was the screening material itself. The glass fibre tape did not provide a standard aperture size,

and thus did not prevent the entry of certain members of the soil fauna, such as mites and nematodes, which could contaminate the bait with the propagules of passive fungi (Allsopp, 1973).

Prior to publication, Edwards, et al. (1980) suggested the use of 106 µm nylon bolting cloth manufactured by Henry Simon Limited, Stockport, Cheshire. The material could withstand autoclaving and was produced in a range of mesh sizes down to 5 µm, thus it was considered a potentially useful material as a screen for the screened substrate tube technique. A range of samples from 106 µm to 5 µm was obtained and incorporated into a modified screened substrate tube technique for the isolation of actively growing fungi.

5.1.2 Materials and methods

Straight pieces of barley straw of the variety "Hassan" were taken, divided longitudinally and cut into strips 2cm long. Two strips were taken and fixed to the outer wall of a glass test tube, 5cm above the bottom of the tube. The strips were fixed with a clear silicone rubber sealant produced by NV Dow Corning, Seneffe, Belgium.

Nylon bolting cloth of a range of standard aperture sizes was obtained, namely 106, 95, 85, 80, 75, 64, 53,

45, 35, 20, 15, 10 and 5 μm. These were cut into strips 5cm by 8cm. A consistent seam of sealant was run onto the tube around the straw baits about 1cm away. A strip of 106 μm cloth was placed over the baits and stuck to the tube with the sealant. The sealant emerged through the pores of the cloth enabling a further layer of cloth from the series to be fixed over the top. Great care was taken to ensure that the sealant did not run onto the straw baits, and also that no corridors of entry at the sides of the sealant barrier were created. This ensured that the only point of access to the bait was through the cloth. The double layer of cloth with the 106 μm as backing ensured that the outer barrier was a known minimum distance from the straw baits.

The sealant on the prepared tubes was allowed to cure for 24 hours before being moistened, wrapped in aluminium foil and autoclaved. The sealant and bolting cloths were specified by their manufacturers as being resistant to the conditions encountered in autoclaving, and were found to be so in this case.

Four tubes of each mesh size were prepared.

15kg of the top 5cm of barley field soil were obtained from the field location described in Chapter 2. This was sieved to remove large stones and placed in a large

polyethylene tank. The soil was maintained at 25°C for one week before starting the experiment. The tubes were placed in the soil, care being taken to pack the soil around the tubes with as even a pressure as possible. The tank was covered and incubated at 25°C. After 72 hours the tubes were removed. The nylon screens were carefully peeled off and the straw baits were removed aseptically and placed onto malt extract agar plates with aureomycin. Plates were incubated at 25°C and inspected daily for fungal colonies. As soon as practically possible the colonies were picked off, subcultured, and subsequently identified.

5.1.3 Results

These are presented in Tables 5.1 and 5.2. The dilution plate technique isolated 19 species of which Pencillium sp., Phoma sp. and Scopulariopsis brevicaulis were isolated solely by this method. 25 species were isolated using straw baits in the screened substrated tube technique. The range of mesh sizes between 106 µm and 15 µm isolated most of the species found on the dilution plates as well as Chaetomium globosum, Cladosporium herbarum, Fusarium culmorum and Zygorrhynchus moelleri. Five species were isolated using the two smallest aperture meshes of 10 µm and 5 µm. These were Coprinus cinereus, two sterile white mycelia bearing clamp connections (F1 and F2), one sterile white mycelium F3 and Sclerotium rolfsii. Nematodes were detected in all

KEY TO TABLE 5.1

1	*Alternaria* sp.
2	*Aspergillus fumigatus*
3	*A. nidulans*
4	*A. niger*
5	*A. versicolor*
6	*Cephalosporium* sp.
7	*Chaetomium globosum*
8	*Cladosporium herbarum*
9	*Coprinus cinereus*
10	*Epicoccum purpurascens*
11	*Fusarium culmorum*
12	*F. oxysporum*
13	*Gliocladium roseum*
14	*Humicola grisea*
15	*Mucor hiemalis*
16	*Penicillium cyclopium*
17	*P. funiculosum*
18	*Penicillium* sp.
19	*Phoma* sp.
20	*Rhizopus* sp.
21	*Scopulariopsis brevicaulis*
22	*Stachybotrys atra*
23	*Trichoderma viride*
24	*Zygorrhynchus moelleri*
25	*Mycelium sterila* F1
26	*Mycelium sterila* F2
27	*Mycelium sterila* F3
28	*Sclerotium rolfsii*

TABLE 5.1

FUNGI ISOLATED FROM BARLEY FIELD SOIL BY DILUTION PLATES AND STRAW SCREENED BY A RANGE OF MESH SIZES

FUNGI ISOLATED	APPEARANCE OF FUNGUS													
	DIL'N PLATE	NYLON SCREEN MESH APERTURE µm												
		106	95	85	80	75	64	53	45	35	20	15	10	5
1	+	+		+	+				+	+		+		
2	+	+	+	+	+	+	+	+	+	+	+			
3	+	+		+				+			+	+		
4	+		+							+		+		
5	+					+	+	+						
6	+		+											
7					+					+	+	+	+	+
8				+					+					
9													+	+
10	+			+				+	+					
11		+		+	+		+	+			+			
12	+	+								+				
13	+			+		+	+				+		+	+
14	+		+			+				+		+		+
15	+				+		+	+						
16	+	+	+					+		+	+	+		
17	+				+			+						
18	+													
19	+													
20	+					+			+			+		
21	+													
22	+	+	+			+								
23	+	+	+	+		+		+		+	+		+	+
24		+							+					
25													+	+
26														+
27														+
28													+	+

TABLE 5.2

OCCURRENCE OF MITES AND NEMATODES IN THE ISOLATIONS

	APPEARANCE OF MITES AND NEMATODES													
		NYLON SCREEN MESH APERTURE um												
	DIL'N PLATE	106	95	85	80	75	64	53	45	35	20	15	10	5
MITES	+	+		+										
NEMATODES	+	+	+	+	+	+	+	+	+	+	+	+		

plates, except those of the 10 µm and 5 µm isolations, after ten days of incubation.

5.1.4 Discussion

It has long been recognised that the dilution plate technique selects for the heavily sporing fungi and the spores themselves, so that spores and hyphae which are more firmly attached to soil particles are less frequently isolated. Warcup (1957) showed that 80% of the colonies obtained by this technique arise from spores. This is reflected by the results of the present dilution series in that the majority of fungi isolated were heavily sporing Fungi Imperfecti.

Three species were isolated by the dilution plates alone, whilst nine species were isolated solely by the screened substrate method. It is important to note that five of these nine species were isolated using the two smallest mesh sizes and that three of them were basidiomycetes. Warcup (1959) failed to isolate any basidiomycetes from wheatfield or pastureland soils using dilution plates, although he did isolate a number of species from the same plots by direct hyphal plating methods.

The occurrence of basidiomycetes on straw baits screened from contamination by soil particles seems to indicate their

selective isolation as actively growing species. The isolation of five species of fungi by the technique using 10 µm and 5 µm mesh sizes alone suggests the active passage of these organisms through the fine pores of the mesh barriers to colonise the straw bait. The change in species isolated when the two finest meshes were used compares favourably to the absence of nematodes in these isolations. It is possible that the fungi isolated using the larger mesh sizes were simply carried across the barrier as passive propagules adhering to the bodies of nematodes and mites small enough to penetrate the pores of the mesh. In this manner, passive spores, particularly of the heavily sporing fungi could gain access to a substrate suitable for growth without prior germination.

From the results obtained it appears that actively growing fungi are capable of penetrating the 10 µm and 5 µm meshes to colonise straw baits, from which they can be subsequently isolated. The occurrence of C. cinereus, isolated from soil without any amendment with ammonia is significant. This indicates that C. cinereus in this experiment, was an actively growing fungus capable of colonising buried straw. This implies that C. cinereus has an active role in straw decomposition in the field situation.

5.1.5 Conclusions

The screened substrate tube technique selectively isolated nine species of actively growing fungi from the soil when screens of 10 μm and 5 μm were used.

Screens of greater pore size than 10 μm allowed entry of members of the soil fauna which probably carried passive spores of other fungi onto the straw baits where they germinated.

Three basidiomycetes were isolated, including *C. cinereus*.

The next section describes the use of the screened substrate tube technique with 5 μm mesh for the isolation of actively growing *C. cinereus* from arable soil.

5.2 THE ISOLATION OF ACTIVELY GROWING C. CINEREUS FROM SOIL

5.2.1 Introduction

The results from the previous section indicated that active mycelium of *C. cinereus* was present in the soil used for the preliminary testing of the screened substrate tube technique. It was thought necessary to fully investigate

the effectiveness of this technique for the isolation of C. cinereus from soil.

It is possible that some isolates of C. cinereus could be missed by the plating out of straw baits, due to overgrowth of other actively growing fungi. Kelley (1979) devised a technique involving a 1% ammonia enrichment system which effectively isolated C. cinereus from straws and soils, although it gave no indication of the previous activity or passivity of the isolate. This technique, which is fully described in Appendix Ie was used to detect the presence of C. cinereus on straw baits, thus indicating the level of activity of the fungus in straw colonisation.

5.2.2 Materials and methods

The same soil burial system and incubation temperature was used as in the previous reaction. 24 screened substrate tubes incorporating 2 baits each were prepared as described previously. 5 μm mesh was used in all cases as it was thought this mesh size gave the maximum possible protection from contamination without preventing fungal colonisation of the baits.

These tubes were placed in the soil burial tank, incubated and removed at 6 hourly intervals over the first day and 12 hourly intervals up to the fifth day. The straw

baits were aseptically removed from the tubes and colonisation by C. cinereus was ascertained by the method of Kelley (1979).

5.2.3 Results

The results are presented in Figure 5.1. It was found that straw bait colonisation took place after 48 hours of immersion in the soil. Percentage frequency of isolation increased with time of immersion.

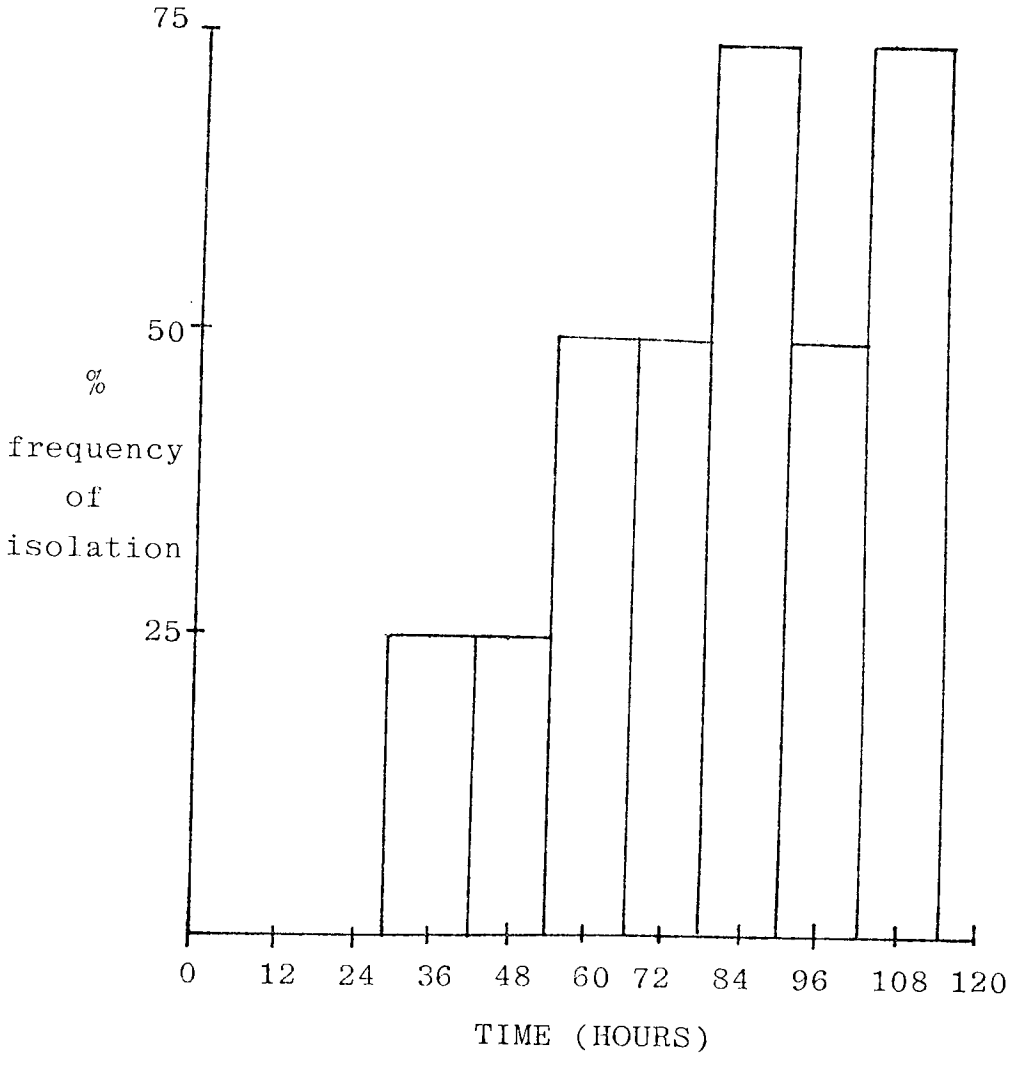
5.2.4 Discussion

From the results obtained it can be seen that actively growing C. cinereus can be isolated using a combination of the screened substrate tube technique and subsequent ammonia enrichment to encourage its further growth on the straw baits.

The isolation of C. cinereus from straws and soils using ammonia enrichment enabled Kelley (1979) to establish a strong correlation between fields under cereal crops and the occurrence of the fungus. The technique did not, however, distinguish between actively growing hyphae and spores or sclerotia. The combination of substrate screening followed by ammonia enrichment appears to be a method by which the role of C. cinereus in the successional colonisation of

FIGURE 5.1

THE ISOLATION OF ACTIVELY GROWING C. CINEREUS FROM BURIED STRAW AGAINST TIME



buried straw could be investigated, both in the laboratory or in the field.

5.3 CHAPTER CONCLUSIONS

It has been shown that the screened substrate tube technique using 5 μm mesh as a screen enabled actively growing fungi to be isolated from soil. C. cinereus has been shown to be an active coloniser of buried straw.

An effective technique for the isolation of actively growing C. cinereus from soil has been developed using screened substrates and subsequent ammonia enrichment to detect the presence of the fungus. It is thought that this combination of techniques could aid investigations into the origin and activity of C. cinereus in arable soil.

The next chapter describes the in-field application of these techniques in an investigation of the activity of the fungi of a barley field soil.

C H A P T E R S I X

AN INVESTIGATION OF THE ACTIVITY OF STRAW COLONISING FUNGI IN A BARLEY FIELD SOIL

6.1 INTRODUCTION

The results obtained in the previous chapter indicated that the screened substrate tube technique isolated actively growing fungi from soil. The ammonia enrichment technique developed by Kelley (1979) enabled the presence of actively growing *C. cinereus* mycelium to be detected when used in conjunction with the above technique. It was thought that use of these techniques in an investigation of the colonisation of barley straw by soil fungi, especially *C. cinereus*, in the field could serve several purposes:-

1. The origin of *C. cinereus* propagules encountered on harvested straw in the straw biodegradation process could be indicated.

2. The colonisation of straw in soil contact by fungi could be further elucidated.

3. The in-field isolation programme would be an adequate test of the new technique.

The work described in Chapter 5 showed that *C. cinereus* appeared on buried straw after 2 days. Thus it was decided to employ a short term isolation programme with frequent sampling rather than a longer period experiment. It was

thought that a 3 - 4 day in-field experiment where environmental conditions were very variable would be sufficient for the purposes of the investigation. Longer period experiments of this type would probably be more valuable conducted under more controlled conditions.

6.2 MATERIALS AND METHODS

45 screened substrate tubes incorporating 2 straw baits each were prepared as described in the previous chapter. The tubes were moistened, wrapped in aluminium foil in groups of 5 and autoclaved. At the barley field site described in Chapter 2 the tubes were carefully inserted into the soil with the aid of a trowel, ensuring that the baits were buried at approximately 5cm depth. The tubes were spaced 10 - 20cm apart within an area of approximately $4m^2$.

Air temperatures and soil temperatures at 5cm depth were taken using a mercury in glass thermometer when sets of tubes were removed from the soil. pH was measured by the method of Tansey and Jack (1976), where 1g of soil was mixed with 1ml of distilled water, shaken and the pH of the suspension taken with a glass electrode and Pye model 78 pH meter. The state of cultivation of the field or state of the crop was noted.

Groups of 5 tubes were removed at 6 hourly intervals over the first day and at 12 hourly intervals subsequently. The 2 baits from each tube were removed aseptically; one was placed on a previously prepared malt extract agar plate while the other was placed in a sterile universal bottle. All plates and universal bottles were placed in a refrigerator at 4°C until the end of the sampling period when the plates were incubated at 25°C. Fungi arising on the straw baits were isolated as soon as possible and identified. The straw baits in universal bottles were examined for the presence of C. cinereus by the method of Kelley (1979).

This experimental programme was conducted 4 times, in August and October 1978 and February and May 1979.

6.3 RESULTS

The results are presented in Tables 6.1 to 6.5. It can be seen that the greatest number of actively growing fungal species were isolated in October 1978. The least number of species were isolated in February 1979. C. cinereus was isolated at a frequency of 20 - 40% after 48 hours soil burial except in February when it was not isolated. Gliocladium roseum was isolated after 18 hours in May and August, 24 hours in October and 48 hours in February. In all cases its frequency of isolation increased with time of soil burial. A similar response was shown by Trichoderma

TABLE 6.1

SOME CHARACTERISTICS OF THE BARLEY FIELD UNDER INVESTIGATION

MONTH	STATE OF CULTIVATION OR CROP	pH
August	Mature barley crop	$6.28 \pm .24$
October	Crop and straw harvested stubble burnt and ploughed in	$6.9 \pm .43$
February	Ploughed and harrowed	$6.59 \pm .52$
May	Young barley crop	$6.88 \pm .31$

TABLE 6.2.

PERCENTAGE FREQUENCY OF FUNGAL SPECIES ISOLATED FROM A BARLEY FIELD SOIL IN AUGUST 1978 USING SCREENED SUBSTRATE TUBES

FUNGAL SPECIES	FREQUENCY OF ISOLATION (%)								
Coprinus cinereus						20	40	40	20
Chaetomium globosum					20				20
Gliocladium roseum			60	60	60	80	100	100	100
Humicola grisea				20		20			40
Mycelium Sterila F1	20		20		20				
Mycelium Sterila F2									20
Sclerotium rolfsii								20	20
Trichoderma viride			20	20	60	60	40	40	60
Zygorrhynchus moelleri			20	20			40	40	40
Period of Burial (Hours)	6	12	18	24	36	48	60	72	84
Soil temp. at tube removal (^{o}C)	15.1 ± 0.7	13.0 ± 0.3	15.1 ± 0.7	16.6 ± 0.2	16.6 ± 0.2	16.6 ± 0.2	12.2 ± 0.8	12.2 ± 0.8	12.2 ± 0.8
Air Temp (^{o}C)	15.5	12.0	15.5	16.5	16.5	16.5	14.0	14.0	14.0

TABLE 6.3

PERCENTAGE FREQUENCY OF FUNGAL SPECIES ISOLATED FROM A BARLEY FIELD SOIL IN OCTOBER 1978 USING SCREENED SUBSTRATE TUBES

FUNGAL SPECIES	FREQUENCY OF ISOLATION (%)								
Coprinus cinereus						20	40	40	40
Chaetomium globosum					20				
Cladosporium herbarum			20			20			
Fusarium oxysporum				20	20	40		20	40
Gliocladium roseum				40	60	60	80	60	80
Humicola grisea					40	40	40		
Mycelium Sterila F1		20		20					
Mycelium Sterila F2	20					20			
Penicillium cyclopium				20			20		
Sclerotium rolfsii								20	40
Trichoderma viride				40	40	60	40	60	60
Zygorrhynchus moelleri					40		20	60	20
Period of Burial (Hours)	6	12	18	24	36	48	60	72	84
Soil temp at tube removal (^{o}C)	9.2 ± 0.6	7.1 ± 0.3	9.2 ± 0.6	10.0 ± 0.7	10.0 ± 0.7	10.0 ± 0.7	10.5 ± 0.8	10.5 ± 0.8	10.5 ± 0.8
Air temp (^{o}C)	10.5	8.0	10.5	12.5	12.5	12.5	11.0	11.0	11.0

TABLE 6.4.

PERCENTAGE FREQUENCY OF FUNGAL SPECIES ISOLATED FROM A BARLEY FIELD SOIL IN FEBRUARY 1979 USING SCREENED SUBSTRATE TUBES

FUNGAL SPECIES	FREQUENCY OF ISOLATION (%)								
Fusarium culmorum					20			20	40
Gliocladium roseum						20	60	40	60
Mycelium Sterila F1									20
Trichoderma viride					20		20	40	60
Period of Burial (Hours)	6	12	18	24	36	48	60	72	84
Soil temp. at tube removal (^{O}C)	4.3 ± 0.8	3.9 ± 0.6	4.3 ± 0.8	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.3	3.6 ± 0.7	3.6 ± 0.7	3.6 ± 0.7
Air temp. (^{O}C)	5.0	3.5	5.0	4.5	4.5	4.5	2.0	2.0	2.0

TABLE 6.5.

PERCENTAGE FREQUENCY OF FUNGAL SPECIES ISOLATED FROM A BARLEY FIELD SOIL IN MAY 1979 USING SCREENED SUBSTRATE TUBES

FUNGAL SPECIES	FREQUENCY OF ISOLATION (%)								
Coprinus cinereus						20	20	20	20
Chaetomium globosum					20		20		
Gliocladium roseum			20	20	60	80	60	80	80
Humicola grisea			20			20			20
Mycelium Sterila F1			20		20	20			20
Mycelium Sterila F2				20					
Trichodema viride			20			20	40	40	80
Zygorrhynchus moelleri				20	40	40	40	20	60
Period of Burial (Hours)	6	12	18	24	36	48	60	72	84
Soil Temp at Tube Removal (^{o}C)	14.4 ± 0.2	12.3 ± 0.3	14.4 ± 0.2	13.7 ± 0.2	13.7 ± 0.2	13.7 ± 0.2	11.8 ± 0.4	11.8 ± 0.4	11.8 ± 0.4
Air Temp (^{o}C)	17.0	11.5	17.0	16.0	16.0	16.0	10.5	10.5	10.5

viride and Zygorrhynchus moelleri. Cladosporium herbarum and Penicillium cyclopium were both isolated only in October, at 20% frequency from 2 straw baits. Fusarium oxysporum was similarly only isolated in October but showed an increasing frequency of colonisation of straw baits from 20% after 24 and 36 hours soil burial and 40% subsequently.

Soil temperatures and air temperatures varied markedly with the month of sampling, the lowest temperatures being recorded in February and the highest in May. Soil temperatures at the 5cm depth were found to be fairly similar to air temperatures.

Prolonged incubation of up to 14 days at 25^{o}C of the malt extract agar plates used for initial isolations of fungi showed occasional contamination, with small numbers of nematodes, of baits buried for 84 hours. It was thought that the nematodes entered as very small stages and probably did not carry any significant numbers of passive fungal propagules. No nematodes were detected on baits buried in soil for less than 84 hours.

6.4 DISCUSSION

The isolation of only 4 fungal species in February compared to 12 in October indicates a considerable seasonal

activity in some species of fungi. It is difficult to separate the cause of this phenomenon into any single category as several factors may have been involved. Temperature may have been important as well as tillage and state of growth of the barley crop. Organic matter content, moisture content, pH, oxygen tensions and carbon dioxide tensions and other less obvious factors such as antagonism between species may have been in part responsible. Whatever the factors involved it is very interesting to observe the variable occurrence of these fungi as actively growing straw colonisers in the field situation.

The isolation of C. cinereus as an actively growing species in barley field soil, albeit at a low frequency of occurrence compared to certain other species, is important when considering the origin of C. cinereus in harvested straw. Kelley (1979) showed C. cinereus to be almost ubiquitously present in cereal field soils but her methods did not distinguish between actively growing mycelium and passive propagules. The above results indicate that some of the isolations obtained by Kelley (1979) were probably from actively growing mycelium in the soil.

It is possible that active mycelium of C. cinereus, particularly near the soil surface, could produce chlamydospores and sclerotia that could be transmitted to the standing crop. Rain splashes, wind or the disturbance of

harvesting could effect this, producing a dormant inoculum on harvested straw capable of active colonisation when suitable conditions arose.

It is also possible that C. cinereus could complete its full life cycle in the waste straw that frequently accumulates at the edges of cereal fields. In this way spores could be spread over the field as a diffuse airborne inoculum. Unfortunately the basidiocarps of C. cinereus are ephemeral, thus it is difficult to observe the phase of sexual reproduction. Closer inspection of peripheral litter in cereal fields during autumn would probably show the presence of C. cinereus basidiocarps, in view of the fact that it is active in straw decomposition in cereal field soils.

Chaetomium globosum was isolated occasionally during the experiment except in February. This is the commonest of the Chaetomium species, especially on cellulosic substrates. It has frequently been isolated from straw composts (Eastwood, 1952; Chang and Hudson, 1967).

Two species of Fusarium were isolated during the experiment, Fusarium culmorum during February only and Fusarium oxysporum during October only. Both were found to increase in frequency of isolation with time of soil burial, indicating a fairly high activity in the soil. F. culmorum

has a very wide range of host plants and is known mainly as a pathogen of cereals. It has been shown to have a high competitive, saprophytic ability (Butler, 1953; Lucas, 1955) which may account for the small number of other species isolated during February. F. oxysporum is very widely distributed, occurring on numerous host plants and in very varied soils. Warcup (1957) showed that increased frequency of the fungus occurred after ploughing the soil. This also appears to be the case during the isolations of October; after stubble burning and ploughing the fungus appeared as an active component of the barley field soil.

Gliocladium roseum is a common soil fungus and coloniser of rotting plant materials. It was the commonest fungus isolated during the experimental programme, indicating that it was a highly active colonist of buried straw. It was active in this respect throughout the four sampling periods with the earliest isolations occurring in August after 18 hours of soil burial. This organism has previously been shown to respond to selective techniques for cellulolytic fungi (Dickinson and Pugh, 1965), and with its fast growth rate it is probably one of the first fungi to colonise straw in contact with the soil.

Humicola grisea was isolated occasionally during the experiment except in February. The fungus is widespread in arable soils and can often be isolated by selective

techniques on cellulosic substrates (Tribe, 1957). The sporadic isolations of this fungus may indicate the scarcity of the active mycelium compared to other species such as G. roseum. Early isolations of a fungus would not necessarily indicate rapid growth, as the straw bait could have been placed in close proximity to any of the actively growing species in the soil. Scarcity could be indicated by gaps in the isolation sequence, but this could also be brought about by inter specific antagonism. From the results obtained there appears to be little evidence of fungal antagonism against H. grisea on the straw baits.

The occurrence of the two sterile white mycelia bearing clamp connections, F1 and F2, indicated activity that is not usually demonstrated by more conventional techniques. Warcup (1957) isolated 10 basidiomycetes from wheat field soil by micro-manipulation of active hyphae from soil with fine instruments. This tedious exercise did not, however, give any indication of the capacity for colonisation of substrates by these fungi. The results obtained by the screened substrate tube method may indicate the opportunistic nature of these species when confronted with a readily available, uncolonised substrate. The gaps between isolations of these two species indicate either scarcity of active mycelium, its injury during tube insertion in the soil, or antagonism by other colonising fungi.

The isolations of Cladosporium herbarum and Penicillium cyclopium during October showed that both species may have been affected by post harvest stubble burning and ploughing, in a similar fashion to F. oxysporum. Both species are common soil fungi, although the very low frequency of their occurrence indicates a low level of activity in the early colonisation of straw.

Sclerotium rolfsii was isolated after 72 hours of soil burial in August and October. This indicates that the fungus is probably a later coloniser of buried straw than species such as G. roseum.

Trichoderma viride is used here as a species aggregate to include all Trichoderma isolates with green conidia (Webster, 1964; Rifai, 1969) and was isolated during all four sampling periods. This is one of the most widely distributed soil fungi. It has frequently been isolated from soils using conventional isolation techniques, and it occurs as an early coloniser on numerous plant roots. Bruehl and Lai (1966) showed that straw colonised by T. viride could only be occupied by other fungi with difficulty. The fungus was isolated as one of the earliest colonisers in all cases but no effects of antagonism towards other fungi could be demonstrated.

Zygorrhynchus moelleri is one of the commonest Mucoraceae, and has a worldwide distribution in soil (Hesseltine and Mehrotra, 1959). Thornton (1952) found Z. moelleri to be fairly easily isolated using "screened immersion tubes". This is also reflected in this study where it was commonly isolated after 24 hours of soil burial, and with increasing frequency subsequently, although it was not isolated at all during February. Z. moelleri appears to be an active early coloniser of straw buried in the soil.

The results obtained from the isolation programme suggest a pattern in the fungal colonisation of buried straw. The occurrence of certain fungi varied with the season, notably with only 4 fungi isolated during February. Disturbance of the soil by ploughing probably influenced the isolation of F. oxysporum and possibly also C. herbarum and P. cyclopium. G. roseum, T. viride and Z. moelleri were frequently among the first species to colonise the straw, although the basidiomycetes F1 and F2 were isolated very soon after soil burial in October. S. rolfsii and C. cinereus appeared to be fairly consistent later colonisers of the straw.

Malik and Eggins (1969) detected a colonisation pattern on perfused cellulose baits in pasture soil where F. solani and T. viride were early colonisers and G. roseum, Paecilomyces varioti, Arthrobotrys sp. and Gelasinospora

cerealis were later developers. This experiment was conducted over a period of 35 days, however it is important to note that F. solani and T. viride were isolated after 12 hours.

The isolation of different early colonisers from pasture and cereal soils using cellulosic baits may reflect a different range of fungal flora, particularly the early colonisers in the two soils. Use of 5 µm mesh as a barrier may prevent the most rapid colonisation of baits encountered by Malik and Eggins (1969) who used glass fibre sleeving as a screen. This could also indicate the prevention of early contamination of the bait by soil fauna by 5 µm mesh, as opposed to glass fibre sleeving which has been shown by Allsopp (1973) to be penetrated fairly readily by nematodes.

6.5 CHAPTER CONCLUSIONS

It has been shown that certain fungal species commonly occur as active colonisers of straw buried in barley field soil. Of these species, G. roseum, T. viride and Z. moelleri were the most frequently isolated early colonisers while C. cinereus and S. rolfsii occurred as later colonisers. The occasional isolation of certain other species, notably the sterile basidiomycetes F1 and F2, may have reflected their relative scarcity compared to the above species. Some

species such as F. oxysporum may have been increased in activity by the physical disturbance of the soil during ploughing.

The occurrence of C. cinereus as an actively growing species in barley field soil indicated that the propagules of C. cinereus in harvested straw probably originated from the crop soil.

C H A P T E R S E V E N

AMMONIA UTILISATION BY *COPRINUS CINEREUS* AND ITS ON FARM PRODUCTION FOR USE IN STRAW BIODEGRADATION

7.1 AMMONIA UTILISATION BY C. CINEREUS

7.1.1 Introduction

One of the main aims of the work presented in this thesis has been to investigate the potential of C. cinereus as the fungal agent in a straw biodegradation process. The chief method of inducing consistent and rapid growth of the organism on unsterile straw involved the use of ammonia solutions. These investigations and other previous work have indicated that C. cinereus is capable of utilising and exploiting ammonia at concentrations that are inhibitory to most other fungi.

In this chapter, the effects of ammonia on C. cinereus, its production and utilisation by the organism and the availability of ammonia on the farm for use in straw biodegradation will be investigated. It is hoped that this study will give some indication of the ammonia utilisation capabilities of C. cinereus, as well as the feasibility of using farm-produced ammonia in a straw biodegradation process.

McShane (1976) showed that C. cinereus had the capacity to alter the pH of nutrient media to between 7.9 and 8.3 when the carbon to nitrogen ratio was 25:1 or less. Since this was within the pH limits for optimal growth of the fungus, he suggested that C. cinereus exhibited a capacity for self-buffering by excretion of metabolites. It was

also shown in Chapter 2 that the pH of straw tended to shift towards alkalinity during growth of C. cinereus. In view of this it was decided to conduct a simple pH monitoring experiment to investigate the capacity of C. cinereus to modify the pH of straw with and without ammonia amendment.

7.1.2 Materials and methods

Barley straw of the variety used in the previous chapters was chopped to approximately 2cm length. Approximately 30g of chopped straw was placed in a 2.5 l glass beaker and 10ml of distilled water added. The beaker was topped with aluminium foil and autoclaved. Fifteen beakers of sterile straw were prepared and divided into 5 replicates of 3 treatments.

1. A solution of 1% w/w ammonia was prepared and charged with a spore suspension of C. cinereus to give a final spore count of 1×10^4 spores/ml ammonia solution. 1 litre of the suspension was aseptically added to the straw in the beaker and thoroughly mixed. Excess liquid was decanted, the foil cap replaced and the beaker incubated at $30^{\circ}C$.

2. A similar operation was carried out except that the spore suspension was omitted.

3. A similar operation was carried out except that distilled water was used in place of ammonia solution

to prepare the final spore suspension.

1g samples were aseptically removed from each treatment and placed in a beaker. 2ml distilled water were added and stirred through the sample. Excess liquid was allowed to drain off and after 30 minutes the pH of this liquid was taken using a glass electrode and Pye Model 78 pH meter.

7.1.3 Results

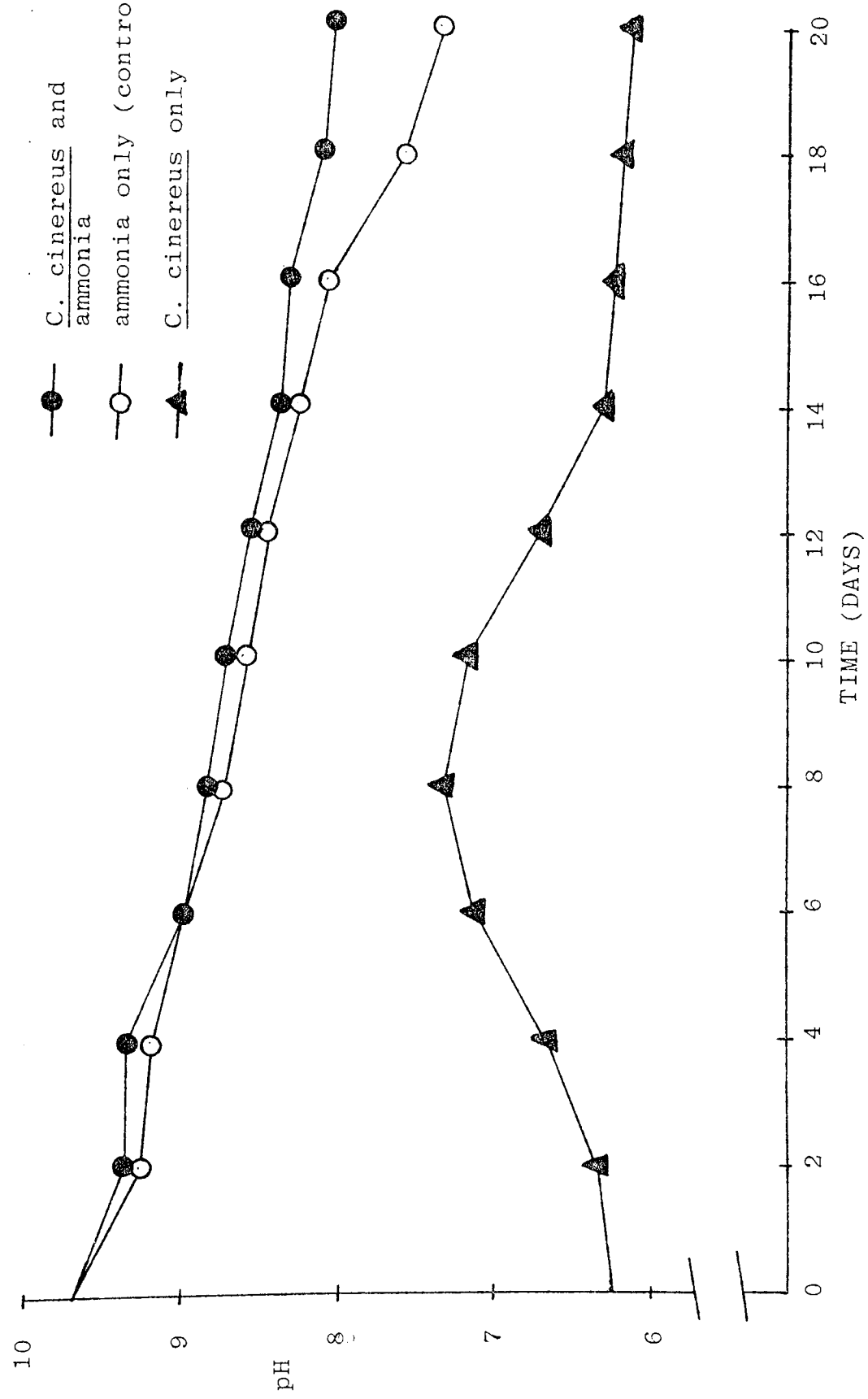
The results are presented in Figure 7.1. The pH of inoculated straw amended with ammonia remained at a higher level towards the end of the experiment than the control. The final pH, after 20 days incubation, of the inoculated ammoniated straw was 8.14 compared to 7.41 in the control. The pH of the unamended, inoculated straw rose from 6.21 initially to a maximum of 7.33 after 8 days, eventually stabilising around the starting pH after 14 days.

7.1.4 Discussion

From the results obtained it can be seen that *C. cinereus* exhibits a capacity to increase the pH of its growth substrate, and where conditions permit, to maintain that pH at a level highly suitable for its growth. The mechanism by which this is effected is not fully understood but Milne (1975) suggested that it could be brought about by ammonia production.

FIGURE 7.1

THE pH OF BARLEY STRAW WITH AND WITHOUT AMMONIA DURING GROWTH OF C. CINEREUS



He showed that urea in the cytoplasm of the mycelium is broken down by a urease to carbamate and ammonia. The ammonia is assimilated by the cell for growth and the carbamate is excreted from the mycelium by active transport. The carbamate then breaks down in the substrate to form ammonia and carbon dioxide. This ammonia production by C. cinereus probably accounts for the increases in pH recorded during the previous experiment.

From electrochemistry it is known that as pH falls, the proportion of ammonium NH_4^+ to ammonia NH_3 increases. This means that at low pH, less ammonia is available for uptake than at a high pH, as it is a charged ion and will not pass through the cell membrane by passive diffusion. By comparison of ammonia uptake at high and low pH, Milne (1975) concluded ammonia uptake by C. cinereus to be by passive diffusion.

From the results obtained by monitoring the pH of straw during C. cinereus growth it appears that ammonia is used as a nitrogen source, particularly at high pH. Some ammonia is returned to the substrate by decomposition of metabolites and an alkaline pH is maintained. The gradual decline in pH during C. cinereus growth may be accounted for by the loss of ammonia through the gaseous phase, as shown by the uninoculated control, and also by the "fixing" of nitrogen in the form of fungal protein.

Moore and Jirjis (1976) demonstrated that ammonium has a fundamental regulatory influence on the progress of sclerotium morphogenesis in C. cinereus. Sporophores and sclerotia are both produced by the dikaryon on artificial media and are apparently alternate outcomes of a single initiating process. Competition for nutrients between these two pathways appears to be regulated by ammonia production from the sporophore, to delay sclerotium maturation in favour of the production of large numbers of genetically recombinant spores. Stewart and Moore (1974) showed that the concentration of ammonia in the medium increased by a factor of three over the four to five days between first appearance of sporophore initials and first discharge of mature spores. Moore and Jirjis (1976) deduced that this activity and the dependence of sclerotial maturation on ammonia concentration resulted in the sequencing of the developmental alternatives. Preference will be given to sporophore development and only when conditions are unfavourable to this more demanding pathway will sclerotium maturation be released from its inhibition.

Further work by Moore (personal communication) has shown ammonia production to be a regular occurrence when C. cinereus is grown on a range of organic nitrogen sources. Moore has also shown that ammonia is produced by both monokaryons and dikaryons in the vegetative and sporulating stages of growth. The significance of this activity in terms of the regulation of development in C. cinereus is not understood but it may

be of importance in understanding the basic ecology of the organism.

Ammonia has been shown to have an active role in fungistasis in certain alkaline soils (Ko and Hora, 1972; Ko et al., 1974) and Schippers and Palm (1973) found it to be an effective fungistat in soils amended with chitin. The results obtained by Kelley (1979) and in Chapter 4 of this thesis suggest that a similar situation occurs when 1% ammonia solution is added to straw. The ammonia inhibits the growth of other fungi so that C. cinereus does not have to compete. Kelley (1979) showed that this effect is not conferred simply by a high pH or the combination of pH and a nitrogen source. It appears to be due to the ability of C. cinereus to tolerate levels of ammonia that are inhibitory to other fungi.

The natural environment of C. cinereus includes accumulations of plant debris (Quli, 1978) straw composts, agricultural soils and farmyard manures. It could be expected that C. cinereus would be able to tolerate and utilise high levels of ammonia which are frequently encountered in these situations. Also, the production of ammonia by C. cinereus may further prevent the growth of competitive organisms on the substrate by a fungistatic effect.

Sagara (1975) isolated other fungi usually classed as coprophiles by the use of ammonia. The observation that

C. cinereus can be isolated from straw and soils using ammonia infers that C. cinereus can be placed in Sagara's chemo-ecological grouping of "ammonia fungi". In view of the wide distribution of C. cinereus and the extent of Sagara's isolation programme it is a little surprising that he did not encounter this organism.

7.2 THE PRODUCTION OF AMMONIA FROM LIVESTOCK WASTES FOR USE IN STRAW BIODEGRADATION

As previously mentioned in Chapter 1, it was thought that the straw upgrading process could be linked to an animal slurry treatment technique which provided a source of ammonia. The slurry treatment technique is a physico-chemical method based on the ammonia stripping process developed by Callahan et al. (1976) for the denitrification of domestic waste water.

In wastes containing organic nitrogen, ammonium ions exist in equilibrium with ammonia and hydrogen ions and this equilibrium is pH dependant. At pH 7 and below only ammonium ions in true solution are present whilst at pH 12 only dissolved ammonia gas is present. Callahan et al. (1967) showed that optimum gaseous ammonia removal occurs between pH 10.8 and 11.5 when the waste water is vigorously agitated. The increase in pH can be achieved by the addition of either agricultural grade hydrated lime or quick

lime to the waste water. The liming process encourages the release of ammonia gas and separates out the phosphates, organic carbon and any insoluble nitrogen.

The release of ammonia from solution depends on the surface tension at the air-water interface and the difference in ammonia concentration between the water and the air. The air-water contact provided by the drop formation and reformation in a stripping tower was found to be an efficient method of removing ammonia gas from solution (Callahan et al., 1967).

When the ammonia stripping process is applied to farm animal waste it is necessary to separate the solid and liquid fractions using a filter or a mechanical separating device. Seal (1973) found that it was possible to effectively use straw bales as a filter for animal waste separation.

A pilot scale ammonia stripping tower designed for animal waste treatment was constructed by Seal (personal communication) which removed 92% of the dissolved ammonia from pig effluent when operated at a flow rate of 1 litre of liquid per minute. Analyses of ammoniacal nitrogen for removal in a range of pig slurries gave a figure of 65% of the total nitrogen content. For beef cattle slurry the figure is approximately 40% of the total nitrogen content (Agricultural Research Council, 1976). The remaining

nitrogen can be removed in the lime coagulation process to yield a final effluent very low in nitrogen.

On this basis, from Table 7.1, it is thus possible to obtain 1.74g of ammonia per litre of cattle slurry or 3.70g of ammonia per litre of pig slurry. Continuous operation of the process could thus treat the effluent from 25 cows per day or 320 pigs per day, producing 2.50kg and 5.33kg of ammonia respectively. It is likely that an economy of scale could be obtained if the stripper were built to plant scale and that the efficiency of ammonia removal would increase. The stripped ammonia could probably be re-dissolved in water to form a manageable commodity for dilution to 1% w/w for use in straw biodegradation.

The settled lime separated solids from treated pig effluent have been shown to have a fertiliser and soil conditioning value. An analysis of the lime separated solids compared to the liquid supernatant after stripping is shown in Table 7.2.

It can be seen that phosphorus in particular is concentrated in the sludge fraction, as is potassium but to a lesser extent. The organic non-ammoniacal nitrogen is also concentrated in this fraction leaving only 0.1% in the liquid after stripping. Calcium constituted nearly 43% in the sludge with only 0.01% in the supernatant, indicating good separation.

TABLE 7.1

VOLUME AND NITROGEN CONTENT OF CATTLE AND PIG WASTE

ANIMAL	QUANTITY (litres)	TOTAL NITROGEN (g)	NITROGEN/LITRE (g) ℓ^{-1}
cow	56.8	222	3.90
pig	4.5	23	5.11

(Wheatland and Borne, 1970)

TABLE 7.2

NUTRIENT CONTENT OF LIME SEPARATED SOLIDS AND LIQUID SUPERNATANT

(% in fresh material)

SAMPLE	SLUDGE	SUPERNATANT
DRY MATTER (%)	99.8	2.7
NITROGEN (%)	0.80	0.10
PHOSPHATE (%)	0.71	0.01
POTASH (%)	1.70	0.77
CALCIUM (%)	42.80	0.01
MAGNESIUM (%)	0.21	0.002

Analyses by the Ministry of Agriculture, Fisheries and Food, 1974

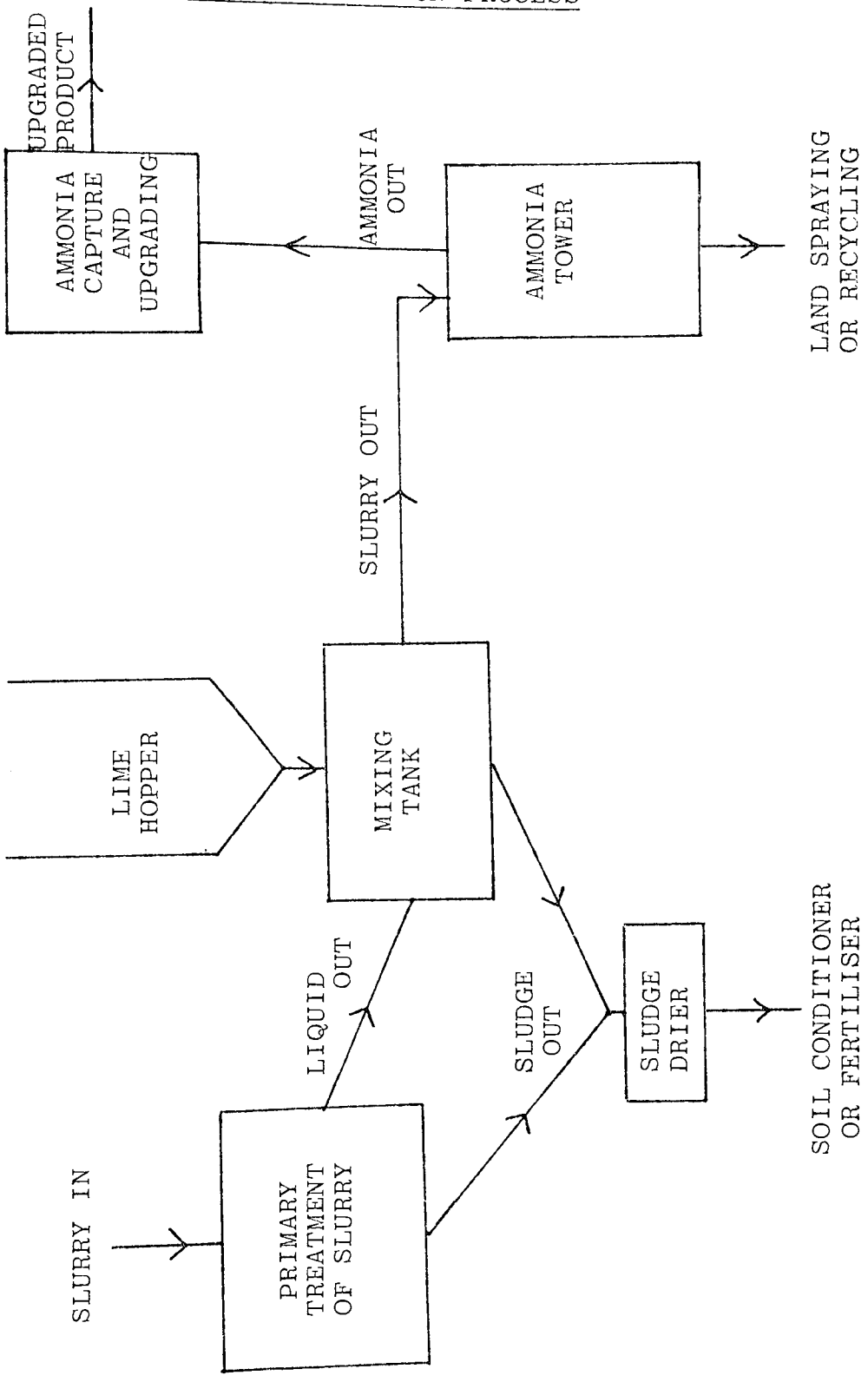
The value of the sludge is based on current (1980) U.K. prices for nitrogen, phosphorus and potassium and has been calculated to have a fertiliser value of £4.85 per ton. In addition to this figure the value of the lime as a soil conditioner must be taken into consideration, and this would make the finalvalue about £14.85 per ton. The value of stripped ammonia may be calculated as 25p per kilogram from fertiliser suppliers latest prices.

Figure 7.2 shows a flow diagram of an animal waste treatment system incorporating an ammonia stripping tower and biodegradation scheme for utilisation of the stripped ammonia. Such a system could be under automatic control to produce a well defined fertiliser and soil conditioner plus ammonia for the straw biodegradation process. The removal of ammonia via the gaseous phase from the animal waste reduces pathogen problems and prevents any carry over of chemicals, antibiotics and growth promoters into the upgraded feedstuff. These have been found to be severe problems in the feeding of whole wastes back to animals (Brady, 1969).

The economic operation of such a system depends on several factors including the scale of the treatment system, equipment installation, maintenance and the cost of power, raw materials and labour. This has to be offset by the efficient treatment of the waste, the value of the lime sludge and the value of the upgraded straw if the stripped ammonia is used in the biodegradation process.

FIGURE 7.2

A FLOW DIAGRAM OF THE ANIMAL SLURRY TREATMENT SYSTEM AND BIODEGRADATION PROCESS



The amount of ammonia produced from cattle slurry by the pilot scale process is sufficient to amend 250kg of straw per day or 10kg of straw per cow, while pig slurry provides enough to amend 533 kg of straw per day. It has been shown in previous chapters that a material equivalent in digestibility to good quality hay can be obtained by growth of C. cinereus on ammoniated straw, although the protein content is too low for it to be used as a complete diet. Taking, for simplicity the upgraded material to be equivalent in value to good quality hay, the present prices of barley straw and good quality hay are £25 and £50 per tonne respectively (Farmers Weekly November 21st 1980). This, therefore indicates an approximate doubling of the value of the straw, although approximately 10% by weight is lost during the growth of C. cinereus to produce the upgraded material. Economic evaluation of the whole system is very difficult requiring a detailed investigation of input and returns which would depend very much on individual farm requirements. Such an investigation is beyond the scope of this study.

The most likely situation to be found on the farm would be the recycling of nitrogen from ruminants, especially as the biodegraded straw is too low in protein to be fed to monogastric animals. This does not, however, preclude the use of ammonia from pig slurry to produce cattle feed. It can be seen that this type of treatment and upgrading system

could only function effectively on a mixed farm or where livestock and cereal specialst concerns overlap. Separation of the supplies of straw and ammonia confronts the problem of transportation of the raw materials to a central processing area. As mentioned in Chapter 1, straw is difficult and expensive to transport. The best solution would probably be to transport concentrated stripped ammonia to the straw biodegradation site, although this could also be prohibitively expensive.

The problems encountered with animal waste from nil or minimal grazing farms become rather similar to those of industrial and human domestic waste management. Concentration of production necessitates treatment processes that must be installed whether they give economic returns or not. The process described above could possibly give such returns but it is necessary to gauge them by considering the whole cycle of nutrient movement, and not just the solution of the immediate problem of waste accumulation.

7.3 CHAPTER CONCLUSIONS

The slow decline in pH of ammonia amended straw and the increase in pH of unamended straw indicate that C. cinereus is capable of modifying the pH of its surroundings, provided sufficient available nitrogen is present, to levels that are most conducive to its growth. It is most likely that these

alkaline pHs are the result of ammonia production by the fungus.

The study on the possibility of stripping ammonia from livestock waste indicated that sufficient ammonia could be produced for use in a straw biodegradation process to amend 10kg of straw per day per head of cattle producing the waste. The economics of the combined system are difficult to establish but in view of the valuable material produced it is possible that it is a viable alternative to conventional waste treatment operations and present straw disposal practices.

C H A P T E R E I G H T

DISCUSSION, CONCLUSIONS AND PROPOSALS FOR FUTURE WORK

8.1 DISCUSSION AND CONCLUSIONS

The work reported in this thesis should be viewed from both the pure and the applied approach. The pure aspects of the study are the contributions made to understanding the activity of C. cinereus both on straw and in cereal field soil. It has been shown that the responses of C. cinereus in terms of straw decomposition vary markedly with increasing concentrations of ammonia added to the substrate. Information has also been gained on the ecology of other fungi which have been isolated during the course of the work.

The applied aspect of the study is the interpretation and upscaling of the above work to investigate the feasibility of its application in a straw biodegradation process, as suggested at the beginning of the project. The results indicate that it is possible to produce an upgraded material provided certain parameters within the composting process are controlled. The results also point to certain problems which may be found in the on farm application of the process.

From the pure research point of view, the development of the modified screened substrate tube technique for fungal isolation could be of value in the investigation of actively growing fungi in opaque substrates. The isolation of actively growing C. cinereus mycelium from barley field soil

indicated that cereal field soil was the most likely origin for the propagules of C. cinereus found in harvested straw.

It has been shown that C. cinereus selectively decomposes hemicellulose and this activity increases with increasing ammonia concentration up to levels at which ammonia begins to become inhibitory. C. cinereus has also been shown to decompose cellulose but to a lesser degree than hemicellulose, possible because hemicellulose is the first polysaccharide to be encountered by the fungus in its ramification of the straw cells. It could be suggested that the responses of C.cinereus to ammonia amendment, in terms of straw decomposition, reflect the activity of the fungus in its habitats of dung heaps and other such environments in which free ammonia is present. The induction and promotion of C. cinereus growth by ammonia suggests that it is of importance in the metabolism of the fungus. The work of Moore and Jirjis (1976) and subsequent work by Moore has shown that ammonia is produced by the vegetative mycelium and also by sporophores, where its production correlates with inhibition of formation and maturation of sclerotia. Tolerance to ammonia and its production by C. cinereus could also be implicated in its use as a fungistat, thereby conferring a competitive advantage to the organism within the substrate. Although it has not been conclusively demonstrated, it appears that ammonia plays an important role in the ecology of C. cinereus, and possibly other fungi, in composting organic material, but

its precise functions are as yet unclear.

Application of the responses of C. cinereus to ammonia addition in straw has shown that it may be used to advantage in producing a material of enhanced feed value to ruminants. It has been shown that hemicellulose and cellulose decomposition correlate closely with digestibility increases in the short term. This increase in digestibility is probably a result of disruption of much of the remaining cell wall material, allowing increased access to the normally shielded carbohydrates, by the rumen microflora. This explanation is supported by the decline in digestibility recorded later which is closely aligned to further decrease in cell wall carbohydrates. Lignin decomposition or modification, although not demonstrated by the techniques employed, was inferred by comparison of hemicellulose and digestibility values from selected pH ranges. The results indicated that an undetected alteration of straw structure partially contributed to digestibility increases, particularly in the alkaline pH ranges. It is possible that the shielding properties of lignin were modified by fungal activity, thus allowing further access to the cell wall components by rumen micro-organisms.

Upscaling of the work has shown that it is possible to produce an upgraded material on the large scale provided aeration is sufficient to prevent the occurrence of self

heating. Bound bales were found to be unsuitable as their centres became colonised by contaminant fungi and also became self heating. This resulted in rapid decreases in digestibility of the straw in the bale centres.

The use of loose straw in black polythene sacks was found to be successful for production of a uniformly upgraded material, provided a swamping inoculum of C. cinereus spores was added to straw amended with 1% w/w ammonia solution. Incubation at 29°C ± 2 for 10 days was found to produce a material equivalent in digestibility to good quality hay although the protein content was less than the 12% minimum recommended by the A.R.C. (1965) to sustain the microbial activity of the rumen.

It has also been shown that digestibility of the material declined rapidly after peaking on the tenth day, which could present severe problems if the material was to be stored. No economically feasible method of storing the material appears to be available, thus it seems the most effective method on the farm involves batch composting of straw to feed fresh to ruminants while the digestibility remains enhanced.

At current prices the increase in digestibility of straw reflects a doubling of its intrinsic value as a feedstuff, as indicated in Chapter 7. It has been shown that

livestock wastes can be treated by a physico-chemical method to supply sufficient ammonia to upgrade a minimum of 10kg of straw per cow producing the waste. The economics of such a nutrient recycling scheme are very difficult to establish, but in terms of efficient waste treatment and the production of valuable fertiliser and feedstuff it could be an effective system for use on the mixed farm.

This work has served to indicate the possibility of using the physiological characteristics of C. cinereus for production of a ruminant feedstuff from barley straw. By exploiting some of the naturally occurring limiting factors found in composts it has become possible to achieve this desired result although further research and refinement is still required before the process can be operated efficiently on the farm.

The conclusions can be summarised as follows:-

1. C. cinereus is physiologically adapted for the colonisation of straw amended with ammonia, with the pH optimum for its growth at 30^{o}C lying between pH 8 and 9.

2. It decomposes hemicellulose and cellulose in straw but hemicellulose is preferentially attacked. This activity coupled with probable modification of lignin structure may account for digestibility increases recorded after 10 days

incubation. Digestibility values decline after peaking at day 10, probably due to fungal utilisation of carbohydrate previously made available to rumen micro-organisms.

3. Larger scale trials have shown that it is possible to upgrade loose straw to digestibility values equivalent to good quality hay. Protein content of the material is less than the requirement for normal rumen function, thus the upgraded straw is not adequate as a complete diet.

4. It has been shown that it is feasible to link the straw biodegradation process to an animal slurry treatment system which serves as an ammonia source. The economic aspects of this scheme are unclear.

5. C. cinereus has been shown to be an active component of the fungus flora of a barley field soil, by use of a modified screened substrate immersion technique. This is important in understanding the origin of the propagules of C. cinereus in harvested straw, indicating that they arise from the soil.

8.2 CRITICISMS AND PROPOSALS FOR FUTURE WORK

One of the main criticisms which could be levelled at this work is the validity of interpreting biochemical digestibility estimations in terms of animal digestion. The

techniques used were the best available at the time for this type of analysis, but undoubtedly the absolute criterion for assessment of digestibility, nutritional value and palatability lies in a full scale animal feeding trial. Such an experiment was beyond the scope of this project but must be among the next steps taken to establish the full effectiveness of the scheme as an on farm process.

Before this type of trial can be effected it will be necessary to produce biodegraded straw on a scale sufficient to supply the requirements of the test animals. This work will be of great value in appreciating the problems associated with full scale straw upgrading such as possible changes in pH, temperature, oxygen and carbon dioxide tensions which could grossly affect the growth and dominance of C. cinereus.

The problem of storage of the upgraded material is difficult to solve economically, and until a cheap solution is found the only alternative will be to compost in batches as mentioned earlier. Further research in preserving this and other wet forages could be of great benefit in the future to farmers viewing this type of process as a silage supplementation or replacement scheme.

The use of ammonia to encourage the growth of C. cinereus in straw raises the question of the danger to ruminants of ingestion of excessive amounts of ammonia. Urea has to

be fed at a low level to avoid excessive ammonia production in the rumen and resultant toxicity (Loosli and McDonald, 1968). It is unlikely that ammonia levels in biodegraded straw will be dangerously high since silage, with relatively high ammonia levels, has been fed to cattle very successfully. Also, as mentioned in Chapter 1, anhydrous ammonia is used in Norway to chemically increase straw digestibility with no apparent harmful effects to livestock (Arnason and Mo, 1977).

A second health factor to be considered is the production of mycotoxins in the upgraded material. Fungi known to produce toxins have been shown to be present on straw, for example Aspergillus fumigatus, thus it is very important that the upgrading process prevents the growth of all fungi except C. cinereus. Ammonia has proved to be successful in inactivating aflatoxin in corn (Brekke et al., 1977). Fungi and actinomycetes can be dangerous in other ways. A. fumigatus can cause Aspergillosis in the lungs of man and livestock and has been associated with mycotic abortions in cattle. Actinomycetes are known to cause allergic reactions in man, for example "Farmers' Lung" disease thus it is vitally important that these factors are controlled. Further investigations into the occurrence of these organisms during straw biodegradation is obviously a prerequisite to full scale operation of the process.

It would be of great interest to investigate the use of other species or mutations of C. cinereus for straw

biodegradation. Selection of characteristics such as lignin decomposition or hemicellulose utilisation could result in more effective straw biodegradation although other factors such as resistance to competitive organisms and ammonia tolerance would also need to be further investigated.

Clearly there is need and scope for further investigations into the project as a whole. The health and safety aspects of the process require close scrutiny while the problems of economic production, storage and feeding of the material are only likely to be solved by full farm scale trials.

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A P P E N D I C E S

APPENDIX Ia

THE CULTURE OF C. CINEREUS AND PRODUCTION OF SPORE SUSPENSIONS

Axenic cultures of C. cinereus were maintained in aluminium foil topped 1 lb size jam jars on 30ml of potato maltose agar medium (Kemp and Watling, 1977). Cultures were incubated in an unlit incubator at 30°C for 5 days after which they were kept at ambient temperature, 20°C ± 2, on the bench top.

The appearance of sporophores was closely monitored, and mature caps were aseptically removed with forceps just before the onset of autolysis - when the outer edges of the caps became grey. Each cap was bisected longitudinally, the remains of the stipe removed, and placed gills downward in a petri dish. After spore discharge and autolysis the remaining cap tissue was aseptically removed and the autolytic fluid containing the spores allowed to dry. Spore prints were stored in a refrigerator at 4°C until required.

A spore suspension was prepared by suspending a spore print in 100ml of distilled water containing 0.05g/l Tween 80 as a wetting agent. A haemocytometer was used to count the spores per unit volume of the concentrate. Distilled water was then added to produce the final spore inoculum concentration.

APPENDIX Ib

DETERMINATION OF THE PEPSIN-DIASTASE SOLUBLE FRACTION

The weighed ethanol-benzene extracted straw sample was placed in a 100ml flask and 40ml of 1% pepsin in 0.1M HCl were added. The flask was stoppered and incubated for 12 hours at 40°C. After incubation the residue was filtered onto a pre-weighed Whatman Number 1 filter paper and washed 3 times with hot distilled water. The filter paper and sample were dried at 85°C for 24 hours.

A buffer solution of pH 4.45 was prepared by mixing 3 volumes of 0.2M acetic acid with 2 volumes of 0.2M sodium acetate solution. 1g of powdered thymol was added to 1 litre of the solution as a preservative.

The dry pepsin treated sample was transferred to a 100ml flask and 10ml of distilled water added. The flask was heated for 30 minutes on a boiling water bath to gelatinise starch. A glass funnel was inserted into the neck of the flask to minimise the loss of water. After cooling, 10ml of buffer solution and 10ml of 0.5% diastase solution were added to the flask. The flask was stoppered tightly and incubated at 37°C for 44 hours. The residue was filtered onto a pre-weighed Whatman Number 1 filter paper and washed 3 times with hot distilled water. The filter

paper and sample were dried at 85°C for 24 hours and then weighed. The weight loss was taken as the amount of sample solubilised by the action of pepsin and diastase.

The method used was originally described by Weinmann (1947) for the determination of total available carbohydrate in plants. The pepsin stage was employed largely to prevent protein binding with lignin to give artificially high lignin values (Ellis et al., 1946).

APPENDIX Ic

THE DETERMINATION OF CELLULOSE AND LIGNIN

The weighed alkali extracted straw sample was placed in a 250ml Soxhlet flask and 20ml of 72% sulphuric acid solution added at 20°C. The flask was maintained at 20°C for 2 hours in a water bath. 125ml of distilled water was added and the sample was filtered onto a Whatman Number 1 filter paper. The residue was washed with hot distilled water and then placed back in the Soxhlet flask by washing through a glass funnel with 3% sulphuric acid. The volume was made up to 150ml of sulphuric acid and refluxed for 2 hours. The residue was filtered and washed with hot distilled water until acid-free. The dry weight of the residue was taken as lignin and the cellulose content determined by difference.

The method used was originally described by Ellis et al., (1946).

APPENDIX Id

THE MICRO KJELDAHL METHOD FOR THE DETERMINATION OF NITROGEN

Reagents

1. Catalyst

K_2SO_4 = 80g

$CuSO_4$ = 20g

$Na_2SeO_4 10H_2O$ = 0.34g

These chemicals were mixed and ground to a fine powder.

2. Concentrated sulphuric acid ('Analar' grade).

3. 40% w/v sodium hydroxide.

4. 4% w/v boric acid.

About 0.15g dried ground sample were accurately weighed and placed in a 30ml digestion flask. 4ml concentrated sulphuric acid and sufficient catalyst to cover the end of a micro-spatula were added. The mixture was digested for about 3 hours until the liquid was colourless, and then it was allowed to cool. The nitrogen in the form of ammonia was liberated using 40% sodium hydroxide in a Markham still

and collected in 10ml boric acid with 1 drop of bromocresol purple indicator. The boric acid was titrated against $\frac{M}{50}$ hydrochloric acid and the following equation used to determine the nitrogen:

$$\% \text{ nitrogen in sample} = \frac{\text{Titre volume x } 0.028}{\text{sample weight}}$$

The crude protein content of the sample was calculated by multiplying the nitrogen content by 6.25.

APPENDIX Ie

THE METHOD FOR DETECTING THE PRESENCE OF C. CINEREUS ON STRAW

Five pieces of straw internode about 3 cm long were placed in a universal bottle and 5 drops of distilled water added. A Durham tube was placed open end upwards in the bottle and the cap was replaced. The system was sterilised by autoclaving for 20 minutes.

After cooling the Durham tube was aseptically filled with 1% w/w sterile ammonia solution. The straw piece suspected of supporting C. cinereus mycelium was aseptically placed on the straw internodes. The universal bottle cap was replaced loosely and the system incubated at $30^{o}C$ for 5 days. The presence of white mycelium on the straw after this time was taken to indicate the presence of C. cinereus. This was later confirmed by the occurrence of microsclerotia after subculturing onto potato maltose agar, and the presence of clamp connections on hyphae examined microscopically.

The method used was a slightly modified version of the technique devised by Kelley (1979).

APPENDIX If

MEDIA USED IN EXPERIMENTAL WORK

1. Potato Maltose agar

Cooked potatoes	200g
Maltose	5g
Agar	10g
Distilled water to	1 litre

Potatoes are peeled, boiled and squeezed through muslin before adding to the other ingredients.

2. Malt Extract agar (Oxoid).

Malt extract	30g
Mycological peptone	5g
Agar	15g
Distilled water	1 litre
Plus	
Aureomycin	6 µg/ml medium

A P P E N D I X II

STATISTICAL ANALYSIS OF RESULTS

APPENDIX II

ETHANOL-BENZENE SOLUBLE FRACTIONS

Day 5

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	5.76 ± .25	5.49 ± .16	5.73 ± .21	5.79 ± .33	5.69 ± .14
6 to 7	5.66 ± .31	5.62 ± .33	5.39 ± .04	5.81 ± .35	5.62 ± .17
7 to 8	5.70 ± .31	5.70 ± .22	5.84 ± .38	5.77 ± .20	5.75 ± .06
8 to 9	5.63 ± .17	5.54 ± .19	5.57 ± .21	5.59 ± .21	5.58 ± .03
9 to 10	5.49 ± .15	5.61 ± .18	5.76 ± .25	5.74 ± .21	5.65 ± .13

Day 10

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN SD
5 to 6	5.59 ± .17	5.73 ± .23	5.75 ± .08	5.76 ± .14	5.71 ± .08
6 to 7	5.75 ± .06	5.74 ± .24	5.63 ± .15	5.78 ± .10	5.73 ± .07
7 to 8	5.69 ± .21	5.58 ± .11	5.67 ± .13	5.79 ± .04	5.68 ± .09
8 to 9	5.76 ± .07	5.74 ± .10	5.70 ± .13	5.73 ± .11	5.73 ± .03
9 to 10	5.78 .15	5.68 .08	5.63 .19	5.61 .04	5.68 .08

Day 15

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN SD
5 to 6	5.83 ± .03	5.78 ± .04	5.74 ± .06	5.76 ± .09	5.78 ± .04
6 to 7	5.89 ± .10	5.74 ± .04	5.72 ± .03	5.81 ± .09	5.79 ± .08
7 to 8	5.83 ± .11	5.86 ± .06	5.84 ± .08	5.88 ± .15	5.85 ± .02
8 to 9	5.80 ± .08	5.78 ± .02	5.82 ± .07	5.79 ± .07	5.80 ± .02
9 to 10	5.81 ± .08	5.82 ± .12	5.84 ± .11	5.87 ± .02	5.84 ± .03

Specimen Analysis of Variance from Samples from Day 5, from 8 to 9 pH range

Replicate	As 2	As 3	As 4	As 6
1	5.55	5.43	5.62	5.45
2	5.32	5.38	5.78	5.76
3	5.81	5.62	5.74	5.33
4	5.66	5.41	5.39	5.58
5	5.71	5.84	5.32	5.82
Totals	28.05	27.68	27.85	27.94
Grand Total	111.52			
Means	5.63	5.54	5.57	5.59
Sum of Squares	157.5007	153.3874	155.2949	156.2978
	a1	a2	a3	a4
A = 622.4808				
Square Col. totals =	157.3605	153.2365	155.1245	156.1287
No. observations				
	b1	b2	b3	b4
B = 621.8502				
difference (a - b)	0.1402	0.1509	0.1704	0.1691

(A-B) = 0.6306

$\frac{(\text{Grand Total})^2}{\text{No. observations}} = D = 621.8355$

Total SOS = A - D = 622.4808 - 621.8355 = 0.6353

SOS Between Treatments = B - D = 621.8502 - 621.8355 = 0.0147

Residual SOS = A - B = 0.6306

Table of Analysis of Variance

Source of Variance	Sum of Squares	°Freedom	Mean Square
Between treatments	0.0147	u - 1 = 3	0.0049
Residual	0.06306	u(V-1)= 16	0.039
Total	0.6453	uV - 1 = 19	0.034

Variance Ratio (F)

$F = \frac{\text{Between treatments mean square}}{\text{Residual Mean Square}} = \frac{0.0049}{0.039}$

= 0.13

Variance Ratio of Samples over pH Ranges Investigated

pH Range	Variance Ratio (F)		
	Day 5	Day 10	Day 15
5 to 6	0.28	0.32	0.55
6 to 7	0.35	0.61	0.43
7 to 8	0.15	0.24	0.44
8 to 9	0.13	0.32	0.41
9 to 10	0.17	0.49	0.67

From tables, F = 1.7 at p = 0.2 level, therefore variance due to strain difference over the pH range investigated is not significant.

PEPSIN-DIASTASE SOLUBLE FRACTIONS

Day 5

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	8.30 ± 3.5	8.35 ± .41	8.30 ± .12	8.21 ± .23	8.29 ± .06
6 to 7	8.65 ± .33	8.75 ± .30	8.73 ± .22	8.82 ± .49	8.74 ± .07
7 to 8	9.54 ± .20	9.12 ± .57	9.66 ± .41	9.47 ± .21	9.45 ± .23
8 to 9	10.70 ±.46	11.03 ±.15	10.87 ±.30	10.74 ±.42	10.84 ±.15
9 to 10	9.52 ± .32	9.74 ± .33	9.70 ± .42	9.63 ± .25	9.65 ± .10

Day 10

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	8.44 ±.15	8.40 ±.23	8.62 ±.13	8.53 ±.04	8.50 ±.10
6 to 7	9.27 ±.17	8.95 ±.19	9.24 ±.16	9.39 ±.18	9.21 ±.19
7 to 8	10.23 ±.21	10.10 ±.11	10.21 ±.12	10.11 ±.23	10.16 ±.07
8 to 9	12.60 ±.17	12.63 ±.14	12.53 ±.14	12.65 ±.16	12.60 ±.05
9 to 10	12.52 ±.26	12.41 ±.14	12.39 ±.26	12.68 ±.16	12.50 ±.13

Day 15

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	9.50 ±.55	9.99 ±.64	9.66 ±.62	9.23 ±.56	9.60 ±.32
6 to 7	10.33 ±.62	9.84 ±.66	9.90 ±.43	10.44 ±.49	10.13 ±.30
7 to 8	12.47 ±.79	12.73 ±.21	12.62 ±.24	12.32 ±.35	12.53 ±.18
8 to 9	13.58 ±.31	13.66 ±.39	13.55 ±.41	13.30 ±.62	13.52 ±.16
9 to 10	11.75 ±.44	11.52 ±.24	11.77 ±.19	11.13 ±.18	11.54 ±.30

Specimen Analyis of Variance of Samples from Day 10, from pH range

Replicate	As 2	As 3	As 4	As 6
1	12.52	12.83	12.33	12.84
2	12.50	12.72	12.50	12.79
3	12.73	12.54	12.62	12.52
4	12.84	12.55	12.69	12.48
5	12.43	12.50	12.52	12.62
Totals	63.02	63.14	62.66	63.25
Grand Total	252.07			
Means	12.60	12.63	12.53	12.65
Sum of Squares	794.4238	797.4114	785.3298	800.
	a1	a2	a3	a4
A = 3177.3799				
Square Col. totals =	794.3041	797.3319	785.2551	800.1125
No.Observations	b1	b2	b3	b4
B = 3177.0036				
Difference(a-b)	0.1197	0.0795	0.0747	0.1024

(A-B) = 0.3763

$\frac{(\text{Grand Total})^2}{\text{No.observations}} = D = 3176.9642$

Total SOS = A - D = 0.4157

SOS Between Treatments = B - D = 0.0394

Residual SOS = A - B = 0.3763

Table of Analysis of Variance

Source of Variance	Sum of Squares	^{o}Freedom	Mean Square
Between treatments	0.0394	u - 1 = 3	0.0131
Residual	0.3763	u(V-1)= 16	0.0234
Total	0.4157	uV - 1 = 19	0.0219

Variance Ratio (F)

$F = \frac{\text{Between treatments mean square}}{\text{Residual Mean Square}} = \frac{0.0131}{0.0235}$

= 0.56

Variance Ratio of Samples over pH Ranges Investigated

pH range	Variance Ration (F)		
	Day 5	Day 10	Day 15
5 to 6	0.55	0.92	0.39
6 to 7	0.39	0.62	0.44
7 to 8	0.36	0.63	0.80
8 to 9	0.64	0.56	0.88
9 to 10	0.73	0.64	0.37

From tables, F 1 =.7 at p = 0.2 level, therefore variance due to strain difference over the pH range investigated is not significant.

HEMICELLULOSE FRACTIONS

Day 5

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	33.39 ± 0.16	33.45 ± .95	33.64 ± .47	33.55 ± .63	33.50 ± .11
6 to 7	32.28 ± 0.93	32.64 ± 1.20	32.79 ± .61	32.35 ± .89	32.57 ± .26
7 to 8	31.31 ± 0.64	31.20 ± .35	31.33 ± .98	31.60 ± .60	31.36 ± .17
8 to 9	30.52 ± 0.62	30.58 ± .67	30.21 ± .95	30.02 ± .55	30.33 ± .26
9 to 10	31.08 ± 0.61	30.92 ± .39	30.98 ± .67	31.23 ± .32	31.05 ± .14

Day 10

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	29.80 ± .55	29.84 ± .57	29.63 ± .62	29.65 ± .19	29.73 ± .11
6 to 7	28.24 ± .33	28.45 ± .43	28.32 ± .35	28.55 ± .50	28.39 ± .14
7 to 8	27.49 ± .49	27.43 ± .17	27.61 ± .18	27.67 ± .64	27.55 ± .11
8 to 9	25.92 ± .43	25.61 ± .30	25.88 ± .29	25.79 ± .42	25.80 ± .14
9 to 10	26.67 ± .58	26.33 ± .62	26.70 ± .14	26.45 ± .39	26.54 ± .18

Day 15

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	27.54 ± .22	27.77 ± .61	27.86 ± .55	27.50 ± .60	27.67 ± .18
6 to 7	26.49 ± .11	26.13 ± .57	26.83 ± .10	26.14 ± .56	26.37 ± .36
7 to 8	23.49 ± .20	23.52 ± .44	23.21 ± .39	23.11 ± .81	23.36 ± .17
8 to 9	21.92 ± .53	22.16 ± .30	21.84 ± .15	21.91 ± .16	21.96 ± .14
9 to 10	23.94 ± .46	23.87 ± .32	24.26 ± .17	23.74 ± .54	23.95 ± .22

Specimen Analysis of Variance of Samples from Day 10 from 8 to 9 pH range

Replicate	As 2	As 3	As 4	As 6
1	25.95	25.73	25.62	25.81
2	26.24	25.70	26.23	25.39
3	26.39	25.29	26.08	25.94
4	25.70	25.33	25.94	26.40
5	25.31	26.02	25.55	25.42
Totals	129.59	128.07	129.42	128.96
Grand Total	516.04			
Means	25.92	25.61	25.88	25.79
Sum of Squares	3359.4583	3280.7563	3350.2498	3326.8282
	a1	a2	a3	a4

A = 13317.2926

Square Column Totals

No. Observations	3358.7136	3280.3850	3349.9073	3326.1363
	b1	b2	b3	b4

B = 13315.1422

difference (a - b)	0.7747	0.3713	0.3425	0.6919

(A-B) = 1.1504

$\frac{(\text{Grand Total})^2}{\text{No. observations}}$ = D = 13314.8641

Total SOS = A - D = 2.4285

SOS Between Treatments = B - D = 0.2781

Residual SOS = A - B = 1.1504

Table of Analysis of Variance

Source of Variance	Sum of Squares	°Freedom	Mean Square
Between treatments	0.2781	u - 1 = 3	0.0927
Residual	1.1504	u(V-1)= 16	0.0719
Total	1.4285	uV - 1 = 19	0.0752

Variance Ratio (F)

$$F = \frac{\text{Between treatments mean square}}{\text{Residual Mean Square}} = \frac{0.0927}{0.0719}$$

$$= 1.29$$

Variance Ratio of Samples over pH Ranges Investigated

pH Range	Variance Ratio (F)		
	Day 5	Day 10	Day 15
5 to 6	1.21	1.21	1.56
6 to 7	1.53	1.27	1.65
7 to 8	1.36	1.19	1.50
8 to 9	1.57	1.29	1.33
9 to 10	1.24	1.44	1.57

From tables, F = 1.7 at p = 0.2 level, therefore variance due to strain difference over the pH range investigated is not significant.

CELLULOSE FRACTIONS

Day 5

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	42.46 ± .29	42.59 ± .51	42.36 ± .42	42.43 ± .41	42.46 ± .10
6 to 7	42.01 ± .18	41.83 ± .12	41.74 ± .39	41.90 ± .36	41.88 ± .10
7 to 8	40.97 ± .12	41.32 ± .43	41.22 ± .14	41.11 ± .16	41.16 ± .15
8 to 9	40.00 ± .33	40.32 ± .30	40.04 ± .46	39.87 ± .36	40.06 ± .19
9 to 10	40.13 ± .44	40.18 ± .21	40.45 ± .50	40.29 ± .29	40.26 ± .14

Day 10

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	40.21 ± .33	40.35 ± .31	40.29 ± .45	40.54 ± .33	40.35 ± .14
6 to 7	38.81 ± .44	39.14 ± .40	39.18 ± .39	38.84 ± .16	38.99 ± .19
7 to 8	37.71 ± .38	37.42 ± .22	37.52 ± .15	37.45 ± .19	37.53 ± .13
8 to 9	36.41 ± .14	36.50 ± .33	36.79 ± .39	36.52 ± .37	36.56 ± .16
9 to 10	36.61 ± .28	36.43 ± .60	36.61 ± .44	36.83 ± .35	36.62 ± .16

Day 15

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	37.57 ± .44	37.52 ± .36	37.81 ± .33	37.69 ± .49	37.65 ± .13
6 to 7	36.25 ± .51	36.08 ± .45	36.29 ± .32	35.91 ± .43	36.13 ± .17
7 to 8	33.99 ± .42	34.24 ± .61	33.85 ± .13	33.74 ± .62	33.96 ± .22
8 to 9	32.04 ± .18	32.10 ± .55	32.00 ± .45	31.89 ± .33	32.01 ± .08
9 to 10	33.57 ± .19	33.65 ± .10	33.39 ± .52	33.76 ± .12	33.59 ± .16

Specimen Analysis of Variance of Samples from Day 10, from 8 to 9 pH Range

Replicate	As 2	As 3	As 4	As 6
1	40.39	40.63	40.43	39.33
2	40.20	40.59	39.63	39.71
3	39.52	40.08	39.54	40.22
4	39.88	40.33	40.02	40.13
5	40.01	39.96	40.57	39.98
Totals	200.00	201.59	200.19	199.37
Grand Total	801.15			
Means	40	40.32	40.04	39.87
Sum of Squares	8000.4370	8128.0619	8016.0587	7950.1987
	a1	a2	a3	a4

A = 32094.7563

Square Column Totals

	8000	8127.7056	8015.2072	7949.6794
No.observations				
	b1	b2	b3	b4

B = 32092.5922

Difference (a - b)	.4370	.3563	.8515	.5193

(A-B) = 2.1641

$\frac{(\text{Grand Total})^2}{\text{No.observations}}$ = D = 32092.0661

Total SOS = A - D = 2.6902

SOS between treatments = B - D = 0.5261

Residual SOS = A - B = 2.1641

Table of Analysis of Variance

Source of Variance	Sum of Squares	°Freedom	Mean Square
Between treatments	0.5261	u - 1 = 3	0.1754
Residual	2.1641	u(V-1)= 16	0.1353
Total	2.6902	uV - 1 = 19	0.1415

Variance Ratio (F)

$F = \frac{\text{Between treatments mean square}}{\text{Residual mean square}} = \frac{0.1754}{0.1353}$

= 1.30

Variance Ratio of Samples over pH Range Investigated

pH Range	Variance Ratio (F)		
	Day 5	Day 10	Day 15
5 to 6	0.85	1.09	1.07
6 to 7	0.89	1.45	1.11
7 to 8	1.30	1.04	1.53
8 to 9	1.46	1.30	0.35
9 to 10	1.23	1.30	1.26

From tables F = 1.7 at p = 0.2 level, therefore variance due to strain difference over the pH range investigated is not significant

LIGNIN FRACTIONS

Day 5

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	8.10 ± .34	8.23 ± .58	8.19 ± .16	8.11 ± .34	8.16 ± .06
6 to 7	7.99 ± .26	7.84 ± .61	7.93 ± .56	7.72 ± .37	7.87 ± .12
7 to 8	7.91 ± .53	7.79 ± .54	7.71 ± .11	8.18 ± .42	7.90 ± .21
8 to 9	8.24 ± .49	8.02 ± .50	8.00 ± .63	7.54 ± .28	7.95 ± .29
9 to 10	8.30 ± .57	8.16 ± .33	8.33 ± .68	7.93 ± .24	8.18 ± .18

Day 10

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	8.30 ± .73	8.32 ± .32	8.15 ± .32	8.15 ± .18	8.17 ± .20
6 to 7	8.12 ± .22	7.77 ± .54	7.95 ± .23	7.98 ± .51	7.96 ± .14
7 to 8	7.65 ± .22	7.83 ± .59	7.81 ± .69	7.99 ± .35	7.82 ± .14
8 to 9	8.02 ± .50	7.97 ± .37	8.04 ± .54	7.99 ± .64	8.01 ± .03
9 to 10	7.91 ± .64	7.85 ± .33	8.24 ± .61	8.15 ± .52	8.05 ± .18

Day 15

	% ORIGINAL DRY WEIGHT				
pH	As 2	As 3	As 4	As 6	MEAN ± SD
5 to 6	7.95 ± .20	7.98 ± .35	7.96 ± .27	7.81 ± .24	7.93 ± .08
6 to 7	7.88 ± .29	8.10 ± .19	7.99 ± .38	7.73 ± .36	7.93 ± .16
7 to 8	7.85 ± .47	8.00 ± .51	8.13 ± .64	7.79 ± .29	7.94 ± .15
8 to 9	8.21 ± .68	7.63 ± .55	8.08 ± .13	7.91 ± .19	7.96 ± .25
9 to 10	8.33 ± .54	7.84 ± .28	8.21 ± .67	7.65 ± .53	8.01 ± .32

Specimen Analysis of Variance of Samples from Day 10, from 8 to 9 pH Range

Replicate	As 2	As 3	As 4	As 6
1	7.63	8.17	7.33	7.43
2	7.99	8.45	7.61	7.99
3	8.13	7.68	8.37	8.88
4	8.80	7.55	8.39	7.35
5	7.55	8.02	8.52	8.32
Totals	40.10	39.87	40.22	39.97
Grand Total	160.16			
Means	8.02	7.97	8.04	7.99
Sum of Squares	322.5964	318.4567	324.6804	321.1443
	a1	a2	a3	a4

A = 1286.8778

Square Column Totals

	321.6020	317.9234	323.5297	319.5202
No. observations	b1	b2	b3	b4

B = 1282.5753

Difference (a - b)	0.9944	0.5333	1.1507	1.6241

(A-B) = 4.3025

$\frac{(\text{Grand Total})^2}{\text{No. observations}}$ = D = 1282.5613

Total SOS = A - D = 4.3165

SOS Between Treatments = B - D = 0.014

Residual SOS = A - B = 4.3025

Table of Analysis of Variance

Source of Variance	Sum of Squares	°Freedom	Mean Square
Between treatments	0.014	u - 1 = 3	0.0047
Residual	4.3039	u(V-1) = 16	0.2689
Total	4.3039	uV - 1 = 19	0.2265

Variance Ratio (F)

$$F = \frac{\text{Between treatments mean square}}{\text{Residual Mean Square}} = \frac{0.0047}{0.2689}$$

$$= 0.017$$

Variance Ratio of Samples over pH Range Investigated

pH Range	Variance Ratio (F)		
	Day 5	Day 10	Day 15
5 to 6	0.04	0.66	0.44
6 to 7	0.63	0.33	0.87
7 to 8	0.82	0.48	0.80
8 to 9	1.11	0.02	1.18
9 to 10	0.69	0.61	1.24

From tables F = 1.7 at p = 0.2 level, therefore variance due to strain difference over the pH range investigated is not significant.