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UTILIZATION OF CELLULOSE AND HEMICELLULOSE OF PIG FAECES BY *TRICHODERMA VIRIDE*

(with a summary in Dutch)

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1. INTRODUCTION

1.1. General introduction

The breakdown by enzymic hydrolysis of polysaccharides (cellulose, hemicellulose etc.) of plant cell walls has recently become a major area of intensive research. The threat of world food and energy shortages has initiated a number of research activities aimed at producing food, feed and fuel from renewable resources (Srinivasan, 1975; Heichel, 1975; Peitersen, 1975a). Also the increasing quantities of industrial, domestic and agricultural wastes, produced by the human society, has raised the need for methods to convert these materials by technological means into useful products (Harmon, 1973; Schellart, 1975; Sloneker, 1976; Stone, 1976). Consequently, a resource is available all over the world which is renewed annually by the gigantic process of building up plant material. Photosynthesis on earth results in 155 milliard tons of dry weight of primary production a year (Bassham, 1975) of which about two thirds is produced on land and one third in the oceans which constitute 70% of the earth's surface. The major part of plant material is produced in forests (Satchell, 1974) which are the traditional sources of timber and wood pulp for paper. The 2.7% of cultivated land which accounts for 5.9% of the primary production is needed entirely for agriculture. In total, 10% of the photosynthesis product from the cultivated land is used by man; one third of this amount comes available as waste (Bevers, 1975).

The interest in the research on the use of plant cell wall polysaccharides is growing with increasing shortage of the world energy and food supply and increasing problems in waste handling.

The energy crisis of 1973 forced the development of methods for energy generation which are less dependent on fossil fuels. Direct combustion of oil remains the principal means of providing energy which is expected to continue for at least several decades, regardless of technological breakthroughs, because of present resource investment in fossil fuel-powered equipment. Direct combustion of wood, and agricultural and urban wastes remains a large actual or potential energy source and could be increased to meet a much greater demand for energy. Pyrolysis was one of the first methods used to produce solid, liquid and gaseous fuels and other useful by-products from wood, agricultural wastes, coal and petroleum. Pyrolysis of the organic fraction of urban refuse yields about 20% solid char, 40% fuel oil and 27% combustible gas. Cellulose-containing materials with a high content of water and impurities can be converted by microbial and enzymic methods into numerous volatile fuels that can be separated by distillation or stripping, e.g. methane, hydrogen and ethanol (Wise et al., 1975).

Robertson (1920) was the first to call attention to the possibility of microbial conversion of inorganic nitrogen and carbohydrate materials, such as straw,

saw-dust or plant residues, to protein. PRINGSHEIM and LICHTENSTEIN (1920) reported the feeding of animals with *Aspergillus fumigatis* grown on straw supplemented with inorganic nitrogen fertilizer. During world war II, fungi were grown by the submerged-culture technique in Germany and fed to human populations (BUNKER, 1963). In the USSR cellulose from forestry and agricultural wastes is hydrolysed to prepare substrates for the production of food yeast (HOSPODA, 1966). In feeding ruminants there is a great interest in improving digestibility of roughages by treatments which make the hemicellulose and cellulose of the plant cell more accessible to enzymes of the microflora of the rumen. In the Netherlands, intensive dairy cattling is increasing whereas the total area of grassland is decreasing. Prices of roughages are increasing and the interest in using by-products of industrial processes as roughages is also increasing (JANSE, 1975). Manure, pulverized wood and agricultural wastes such as bagasse are materials that can be successfully added in substantial quantities to ruminants feed. Non-ruminant domestic animals, such as pigs and poultry, hardly digest cellulose but they can use protein from microorganisms grown on cellulose-derived sugars. It can be concluded that microbial or enzymic treatment of plant cell material may be of great importance in producing food or food additives in the future.

A further reason for increasing interest in research to convert cellulosic materials into useful products is the need to avoid pollution by the enormous production of wastes in modern human society. In the Netherlands municipal waste contained about 40–50% cellulose in 1974. Most of the cellulose of urban waste is derived from paper together with wastes of crops and fruits (Report SVA 2092, 1977). Only part of the total waste paper is recycled in paper-making industries although much more could be reused. Much of the cellulose in urban waste is either of such poor fibre quality or is so intimately mixed with non-cellulosics that it is not suitable for recovery and reuse for paper-making. Large amounts of cellulose-containing agricultural wastes and by-products are yearly produced as plant stems, straw, leaves, pulps and husks. Sugar cane bagasse, sugar beet vinasse, rice and wheat husks, corn cobs and husks and several other materials are generally brought to a central point in their processing cycle. The relative homogeneous materials may be a good source of raw material for further processing. When these wastes accumulate they form a disposal problem and rank as pollutants simply by their volume and lack of profitable use. In intensive livestock farming, animal wastes produced form an increasing disposal problem (SPOELSTRA, 1978). Therefore, methods have to be developed to store, transport and dispose of such wastes. Because of the high water content (up to 95%) and the presence of highly polluting substances, this kind of waste is less easily to handle than the relatively clean wastes like bagasse or straw.

Since wastes usually do not enter the economic or industrial cycle, it is often difficult to quote a price or value for them. Because they are of a rather low economic value the costs of handling, storage, and transport are important factors in processing. Depending on the kind of material, some wastes may be attributed a negative cost, perhaps equal to disposal costs.

This investigation was performed to study the possibilities of degrading cellulose-hemicellulose-lignin-complexes of pig faeces by microbial methods. The aim of the study was to collect useful information for processing piggery waste which is produced in intensive livestock farms in the Netherlands. The kind of plant cell wall residues which contain the cellulose-hemicellulose-lignin complexes present in pig wastes was studied, microorganisms capable to hydrolyse this material were selected and some aspects of the hydrolysing system of *Trichoderma viride* growing on these residues were investigated.

1.2. CELLULOSE-, HEMICELLULOSE-, AND LIGNIN-CONTAINING WASTES AS SUBSTRATE FOR ENZYMIC HYDROLYSIS

The utilization of plant cell wall polysaccharides is greatly simplified if the cellulose and hemicellulose are first hydrolysed to monosaccharides. These compounds can be used as a source of food consumable by man and animals or as a raw material to make solvents, plastics and other chemicals now made from petroleum. They can be converted microbially into single cell protein or they can be fermented to fuel such as methane or ethanol.

Two possibilities exist in selecting sources of plant cell walls for hydrolysing their polysaccharides. First, the special raising of crops for their cellulose and hemicellulose contents only, but as it is stated by BASSHAM (1975) all the land suitable for convential agriculture should be used for the world's food production. Secondly, one can use by-products and wastes from agricultural and industrial production. The use of timber and wood residues as a substrate is discussed by STONE (1976), that of agricultural residues by SLONEKER (1976) and that of solid wastes from food processing by COOPER (1976). Cellulose- and hemicellulose-containing by-products and wastes produced in the Netherlands in 1974 (animal wastes) and 1973 (other wastes) are listed in Table 1.1. Data are found in reports of the SVA (1977), CBS (1976) and are given by JANSE (1975).

Domestic refuse in the Netherlands is incinerated, composted or dumped. Dumping causes problems as is demonstrated by ROVERS and FARQUHAR (1973). Production of methane in dumped material covered with soil and used for plant growth may damage plants, probably by making the covering soil anaerobic (HOEKS, 1972). Under certain circumstances there may even be the danger of explosion. Percolation of rain water causes pollution of the environment. Actually, processes occurring in dumped refuse are not fully known. More severe pollution of the environment may occur when refuse is dumped in thicker layers. Composting of refuse is limited by the capacity of processing and deposit. Incineration yields thermal energy but causes air pollution and is only possible in highly populated areas where the total amount of refuse is enough to make incinerators remunerative. The principal cellulosic material of domestic refuse is paper; other cellulose- and hemicellulose-containing materials are residues of vegetables and fruits and garden wastes (SVA report, 1977). The greatest need of research when using domestic refuse for hydrolysis is in the area of refining. A

TABLE 1.1 Production of cellulose- and hemicellulose-containing by-products and wastes in the Netherlands, × 1000 tons.

By-product or waste	Total amount	Dry matter content %	Used as feed	Back to soil or to surface water
Urban wastes				
Domestic refuse	1,000	50	–	1,000
Sewage sludge	2,000	15	–	2,000
Agricultural by-products and wastes				
Leaves and tops of sugar beets	2,000	17	1,500	500
Foliage of beans and peas	55	20	55	
Stems and foliage of sprouts	25	20	25	
Foliage of potatoes	700	–	–	700
Vegetables and fruits overproduction	50	–	50	
Straw of grain	884	85	not known	600
Straw of grass seed production	65	85	not known	not known
Straw of peas and beans	11	85	11	
Animals wastes				
Poultry	1,200	35	–	1,200
Pigs	11,106	8.0	–	11,106
Calves for fattening	973	2.0	–	973
Cattle (excl. calves f.f.)	32,320	9.5	–	32,320
Industrial by-products and wastes				
Wet pulp of sugar beets	114	9	114	–
Dried pulp of sugar beets	297	90	297	–
Molasses	237	68	237	–
Other residues of beets	70	12	70	–
Wet grain of breweries	162	22	162	–
Malt sprouts	22	90	22	–
Pulp of apples	10	21	10	–
Potato pulp	500	112	500	–
Content of rumen of slaughtered cattle	35	12	–	35

use for the organic matter part in domestic refuse may be found in anaerobic digestion.

Sewage sludge, the sediment of urban sewage waste before purification, is often combined with settled activated sludge of waste water treatment and forms a pollution problem. Anaerobic digestion delivers gas that can be used as an energy source. In general, domestic waste provides a nutritionally balanced substrate for bacterial activity. HEUKELEKIAN (1957) gives the chemical composition of sewage sludge. Ether-soluble substances account for 34%, crude protein 27%, hemicellulose 3%, cellulose 4% and lignin 6%.

Agricultural wastes and by-products differ from domestic refuse in that they are almost entirely organic. As is shown in Table 1.1, most of the agricultural by-products are used as feed and are not true wastes. Those parts of the leaves and tops of sugar beets which cannot be used as feed are lost by mechanical harvest-

ing. Foliage of potatoes is poisonous and is left on the field; it might be a possible substrate for enzymic hydrolysis. Vegetables and fruits that are overproduced in agriculture are mostly sold to farmers and fed to animals. The amounts differ each year. Straw of cereals is partly used as roughage; this concerns especially barley and oats straw. However, straw is mostly needed as litter on farms. Straw may be a good substrate for enzymic hydrolysis, especially when such treatment makes it more digestible. Straw of grass seed production is burnt on the field, ploughed in or fed to cattle and horses.

Concluding, agricultural by-products are really by-products and no wastes. A disposal problem does not exist and the products actually form an essential part of cattle feeding. Import of such products is even necessary. Improving feed quality by enzymic treatment of the plant material would increase human food supply.

Animal wastes like manure are often considered as by-products of farming and are used as fertilizer. However, the need for increasing efficiency, particularly in livestock production, has led to intensification. The balance between crops and animals allowing recycling of the wastes as fertilizer may be disturbed and the simplest and most economical method of disposal of animal wastes through land spreading is not always possible. Therefore, methods must be developed to store, transport or break down excessive amounts of animal wastes. Problems are increasing as a result of the tendency to collect faeces and urine of pigs, calves and cattle as slurries. Poultry droppings are collected in a solid state. HARMON (1973) and ROBINSON (1971) described aerobic treatment of such wastes and HOBSON and SHAW (1971) discussed anaerobic digestion of piggery wastes. The cellulose content of droppings of poultry and pigs is about 15% and that of cattle about 25% (SMITH, 1973). The hemicellulose content was not reported by this author. Cellulose and hemicellulose as components of plant cell walls appeared to be hardly biodegradable in aerobic-treatment plants even after prolonged aeration as is described by ROBINSON (1971). So, more knowledge is needed under what circumstances this so-called fibre fraction, consisting of plant residues, is broken down by microorganisms. As can be seen from Table 1.1, animal wastes form the largest part of the cellulose-, hemicellulose- and lignin-containing wastes in the Netherlands. It may be expected that problems of disposal will increase by further intensification of agriculture and by increased tendency to collect droppings as slurries instead of solids.

All industrial by-products except the contents of rumen of slaughtered cattle, are used as feed in one way or another. Manufacturers are processing their by-products to make them available as roughages or feed additives. Import of dried beet pulp in 1973 amounted to 350,000 tons. It can be concluded that the industrial by-products are not readily available as source for cellulose and hemicellulose hydrolysis, except when such a process is improving their quality as animal feed. Domestic refuse and sewage sludge, which are the real wastes, and an increasing amount of farm slurries are the materials that can be considered as the most obvious sources of hemicellulose and cellulose for hydrolysis.

1.3. Structure and chemistry of the plant cell wall

The main groups of constituents of the plant cell wall are cellulose, hemicellulose and lignin. Normally, the polysaccharides of the plant cell wall are divided into the fibre polysaccharides and the matrix polysaccharides (Theander, 1977). The former compounds are largely crystalline and form microfibrils – mainly consisting of cellulose molecules – which are held together by hydrogen bonds and are surrounded by largely amorphous matrix polysaccharides, lignin and probably some protein. The matrix polysaccharides are usually divided into pectic substances and hemicelluloses. The hemicelluloses are very closely associated with cellulose in the secondary cell wall and are also much related to this compound in structure and conformation. The non-carbohydrate aromatic lignin is associated with polysaccharides and, at least with hemicelluloses, covalently linked to it. Therefore lignin hinders the enzymatic hydrolysis as well as the chemical extraction of the polysaccharides.

A good insight into the structure of the plant cell wall is given by Robards (1970). From the outside to the inside the plant cell wall consists of the middle lamella, the primary cell wall and the secondary cell wall. The middle lamella contains no cellulose but mainly pectic substances with some pentosan and glucosan polymers also present. In fact the middle lamella is not a component of the cell wall but is an intercellular layer generally present in plants. The primary cell wall is always deposited onto the middle lamella. It contains some unoriented microfibrils of cellulose but mostly non-cellulosic polysaccharides. During cell wall thickening highly oriented cellulose and hemicellulose are deposited to form the secondary wall. At the end of the thickening phase, formation of lignin becomes noticeable, beginning around the primary wall at the cell corners and extending from there into the secondary wall. When lignification is complete, the cell dies. The secondary cell wall is composed of a number of lamellae which are referred to as the S_1, S_2 and S_3 layers. These lamellae have microfibrils of cellulose which are packed closely together in a parallel manner and are oriented more precisely than the microfibrils in the primary cell wall.

The main structural polysaccharide in plants is cellulose. Cellulose is a long chain polymer built up of β-1,4-linked glucose units. The Haworth projection formula commonly used for carbohydrates, and the three-dimensional conformation formula are given in Fig. 1.1. These conformation formulae give an idea of the spatial arrangement of the glucose molecules and show the possibilities to form strong intermolecular and intramolecular hydrogen bonds. In the other common biological polymer of glucose, starch, glucose units are linked axially instead of equatorially which favours formation of helix structures and gives starch totally different properties compared to cellulose, although the chemical composition is the same as that of cellulose. Cellulose chains never branch, nor do they have side chains, although they may be laterally bound to other cellulose molecules or to hemicellulose molecules via the hydroxyl groups (Valent and Albersheim, 1974).

Preston (1952) has pointed out that there are regions in the cell wall, at least

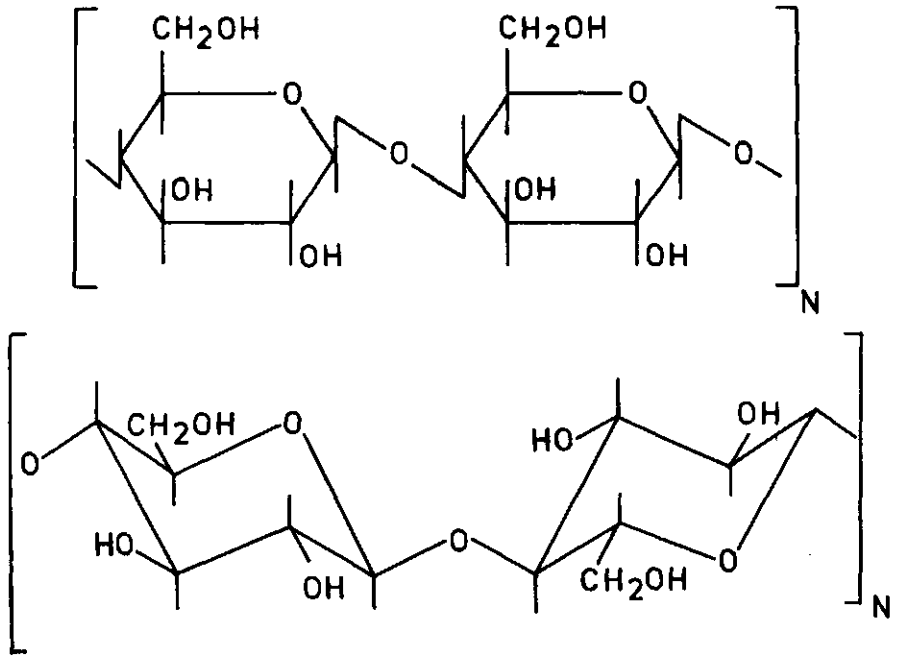

FIG. 1.1 The Haworth projection formula and the three-dimensional conformation formula of cellulose.

600 Å long and 35–75 Å wide, which have an ordered crystal lattice. These regions which consist of 35–100 cellulose molecules are known as micelles or elementary fibrils. The micelles are not separate structures, but restricted regions which have well-ordered cellulose molecules separated from other micelles by less-well-ordered molecules (paracrystalline cellulose). The micelles may become banded together to form microfibrils (ROBARDS, 1970). The microfibril is the basic morphological unit of the cell wall and determines for the greater part the behaviour and mechanical properties of the cell wall. Microscopic macrofibrils are formed by aggregations of microfibrils and may be up to 0.5 μm in diameter. The paracrystalline cellulose which surrounds the micelles has no true crystalline structure; it may account for 30–40 per cent of the total cellulose. The cell wall is composed of units of crystalline cellulose with spaces between the micelles and microfibrils of 10 Å and 100 Å, respectively. The size of cellulolytic enzymes is approximately 200 × 30 Å (WHITAKER, 1954) and thus it is not surprising that steric problems play a role in the enzymic breakdown of the cell wall. The incrusting materials which in addition to paracrystalline cellulose include hemicellulose and lignin are filling up the intermicellar and intermicrofibrillar spaces. Lignin gives in this way an effective resistance against degradation of cellulose by microorganisms by impeding penetration of hydrolytic enzymes into these spaces.

The structure of the plant cell wall with special reference to the hemicellulose

polysaccharides was studied by ALBERSHEIM et al. (1973), BAUER et al. (1973), KEEGSTRA et al. (1973) and WILDER (1973). A review is given by ALBERSHEIM (1975). The chemistry of the hemicellulose polysaccharides is much more complex than that of cellulose. In the hemicellulose fraction the polysaccharides consist of two or more sugars connected by several kinds of glycosidic linkages. Albersheim and his co-workers found that these polysaccharides consist of relatively small repeating sub-structures of which the largest has 10 sugar units (ALBERSHEIM, 1975). The formulae of some pentoses and hexoses which are common sugars in the hemicelluloses of plant cell walls are given in Fig. 1.2.

Albersheim and co-workers developed a model of the hemicellulose structure of a sycamore cell by using the technique of fragmented enzymic hydrolysis of the cell wall. They found that cellulose fibres are probably coated with a layer of xyloglucan. The glucose units of the xyloglucan lie parallel to the axis of the fibre and they are hydrogen-bonded to the fibre. At the reducing end of the xyloglucan molecule an arabinogalactan molecule is bonded by a glycosidic linkage. The arabinogalactan chains are running radially away from the cellulose fibre. A glycosidic linkage exists between the last galactose unit of the arabinogalactan chain and rhamnogalacturonan which lies parallel to the cellulose fibre and

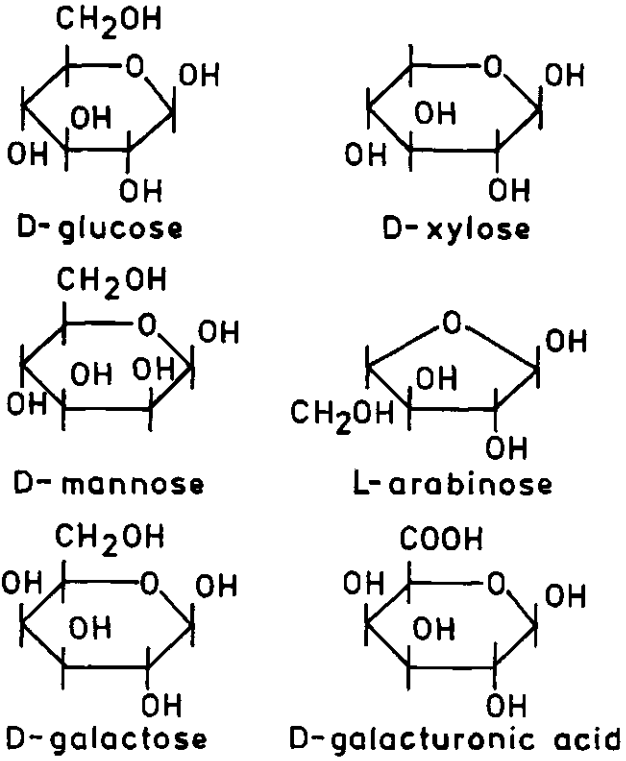

FIG. 1.2 Building stones of hemicellulose.

forms a more or less rigid matrix. In the cell wall also a protein fraction seems to have a structural function.

The sycamore belongs to the dicotyledons and it has been found that cell walls of dicotyledons have a closely related structure. The composition of the cell walls of monocotyledons like wheat, rice, sugar cane etc. is quite unlike that of the dicotyledons. Polysaccharides of a different composition are present which seem to have a similar function as xyloglucan, arabinogalactan and rhamnogalacturonan. Hemicellulose of grasses consists of xylans without any other constituents (THEANDER, 1977) and that of legumes of glucomannans. The primary cell walls of monocotyledons may nevertheless be constructed according to the same architectural principles as those of the dicotyledons.

Lignin is a component of many cell walls and varies in amount depending on plant species, kind of tissue, stage of maturity and nitrogen nutrition of the plants. Total amount in plant cell walls may be up to 20%. Generally, lignin is considered to be linked to the polysaccharide cell wall components although this has not been unequivocally proven (FREUDENBERG, 1968). Monographs on lignins were written by BRAUNS (1952), BRAUNS and BRAUNS (1960), PEARL (1967), FREUDENBERG and NEISH (1968) and SARKANEN and LUDWIG (1971). Lignin is considered to be an aromatic three-dimensional polymer of which the exact structure is not fully understood. In general, three aromatic alcohols are considered to be the building-stones of the lignin polymer, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Fig. 1.3; SARKANEN and LUDWIG, 1971). The relative quantities of these primary precursors in the plant cell wall depend on the plant species (FREUDENBERG, 1968). On the basis of the amounts of precursors it is possible to distinguish lignin of grasses, of soft and of hard woods.

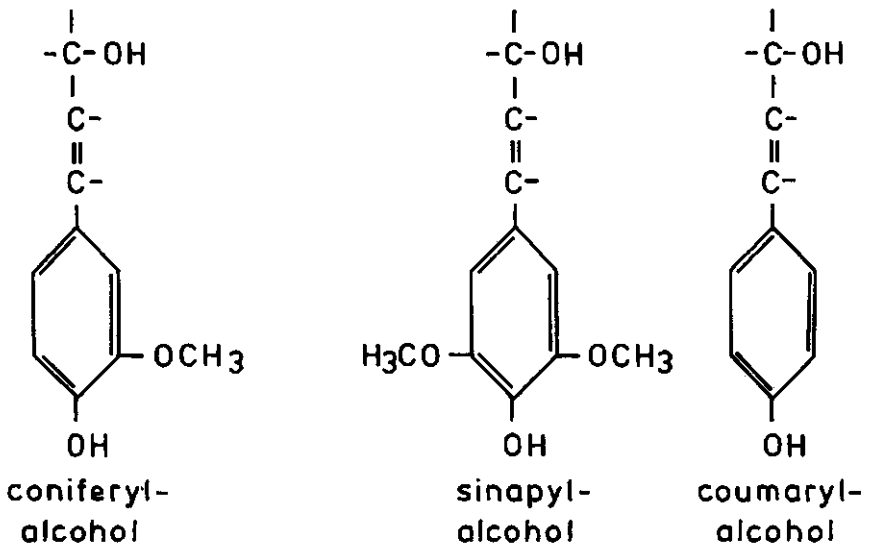

FIG. 1.3 Precursors of lignin.

The polymer is formed by an enzyme-initiated dehydrogenative polymerization of the primary precursors. The dehydrogenative polymerization provides the essential basis for understanding the peculiar structure of the lignin polymer. The need for a general structural principle is particularly urgent in view of the characterization of lignins. The isolation of representative lignin preparations presents formidable difficulties, and the chemical characterization of these preparations is no easy task because of the tendency of the material to undergo self-condensation reactions. Therefore, the structural representations proposed for lignin at the present state of knowledge remain speculative. FREUDENBERG (1964) made the first attempt to bring together the available information on the dehydrogenative polymerization of coniferyl alcohol with the combined analytical and reactivity data on spruce lignin. Modifications were introduced later and the altered formula is given in Fig. 1.4. (FREUDENBERG, 1968). It represents an average fragment of a larger lignin molecule. The formula gives a clear idea of the difficulties facing the investigator. Most of the monomeric units are linked by bonds of extraordinary stability. These include carbon-carbon linkages, either of the biphenyl type (9–10) or the alkyl-aryl type (17–18). The ether linkages are also quite resistant to hydrolysis. The structure of spruce lignin is probably

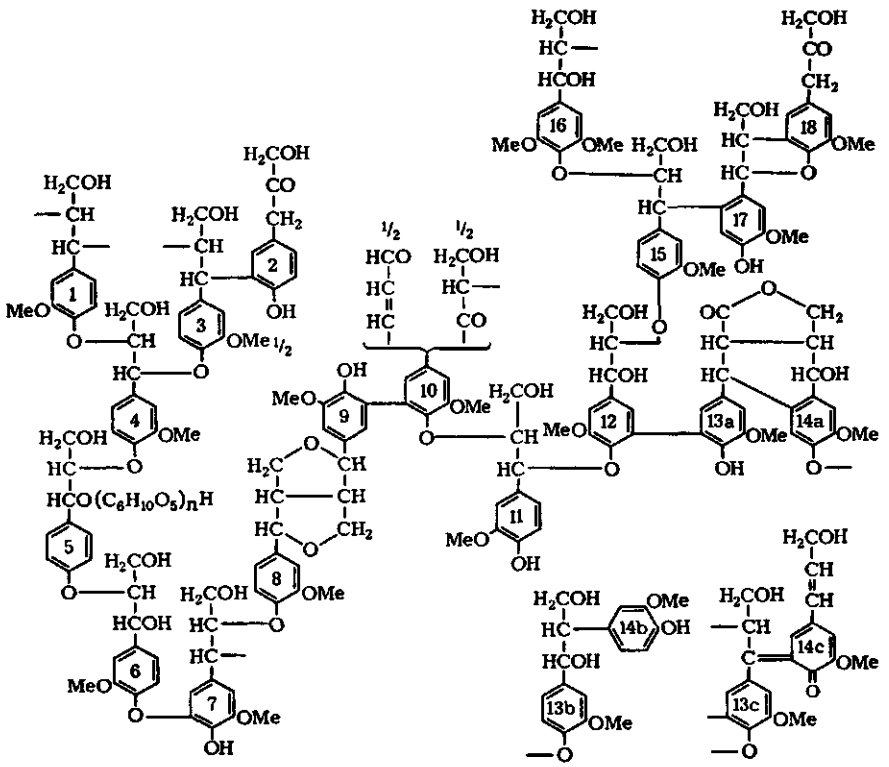

FIG. 1.4 Schematic model formula of spruce lignin (Freudenberg, 1968; with permission).

representative of gymnosperm wood lignins in general, and there is a good reason to believe that more or less analogous structures are present in all plant lignins (SARKANEN and LUDWIG, 1971).

As a cell wall component lignin does not merely act as an incrusting material but it also performs a function in the internal transport of water, nutrients and metabolites, gives rigidity to the cell wall and gives effective resistance against attacks by microorganisms by impeding penetration of destructive enzymes into the cell wall (SARKANEN, 1971).

1.4. MICROORGANISMS INVOLVED IN CELLULOSE, HEMICELLULOSE AND LIGNIN BREAKDOWN

1.4.1. *Fungi*

Basidiomycetes, ascomycetes and *Fungi Imperfecti* play an important role in the breakdown of wood and other plant materials (DICKINSON and PUGH, 1974). Factors affecting the cellulose degradation by fungi are discussed by REESE and DOWNING (1951). GREATHOUSE et al. (1951) and WOOD (1970) microscopically observed the penetration of hyphae into the cell luminae and subsequent digestion of layers of the cell wall. ANTHEUNISSE (1979) described degradation of coconut fibres by fungi. Observations of longitudinal sections on the coconut fibres showed thick hyphae in the centre of the fibres. In general, wood-rotting fungi are divided into white-rot, brown-rot, soft-rot and simultaneous-rot fungi.

White-rot fungi are described by COWLING (1961) and BECKER (1968). These basidiomycetes degrade lignin and hemicellulose preferentially while (white) cellulose fibres initially remain. The very slow decomposition of the material by white-rot fungi is mainly due to the slow diffusion of enzymes. After lignin and hemicellulose decomposition also cellulose degradation occurs (JURASEK et al., 1967; NORKRANS, 1967).

Brown rot is caused by basidiomycetes as described by BAILEY et al. (1968) and BECKER (1968). Cellulose is digested (JURASEK et al., 1967; NORKRANS, 1967) and perhaps hemicellulose (COWLING, 1961). Lignin remains, but not fully unaltered.

Soft rot of wood is caused by ascomycetes and *Fungi Imperfecti*. The polysaccharides of the cell wall are broken down but lignin remains stable (JURASEK et al., 1967). Diffusion of the extracellular enzymes is very slow and this seems to be the limiting step in breakdown of wood (BAILEY et al., 1968). A review concerning soft rot is given by LEVY (1965) and KAARIK (1974).

Simultaneous rot was studied by COWLING (1961) and JURASEK et al. (1967). Breakdown of cellulose, hemicellulose and lignin occurs simultaneously. In this case also the diffusion of enzymes appears to be the limiting factor.

Degradation of hemicellulose, cellulose and lignin by fungi occurs mostly on and in the soil. Reviews concerning decomposition of plants in soil are given by CHARPENTIER (1968) and DICKINSON and PUGH (1974). Although bacteria and actinomycetes play a role in cellulose hydrolysis fungi appear to be the most effective cellulolytic organisms (WINOGRADSKY, 1949; FERGUS, 1964; WOOD,

1968). Organisms with a high growth rate and a high cellulolytic activity belong to the genera *Aspergillus*, *Chaetomium*, *Fusarium*, *Myrothecium*, *Sporotrichum* and *Trichoderma*. Cotton fibres, a crystalline cellulosic material with a high degree of polymerisation, are readily degraded by species of the genera *Chaetomium*, *Fusarium*, *Myrothecium* and *Trichoderma* (BAILEY et al., 1968). Much work has been done on cellulose breakdown and production of microbial protein by species of the *Fungi Imperfecti*. MANDELS and REESE (1964) demonstrated high cellulolytic activity in *Trichoderma viride*. CODNER (1971) observed high pectinolytic and cellulolytic activity of *Trichoderma*, *Myrothecium* and *Chaetomium* spp. even on crystalline cotton cellulose.

UPDEGRAFF (1971) used *Myrothecium verrucaria* to produce microbial protein from waste paper. NORDSTRÖM (1974) studied the production of mycelium of *Aspergillus fumigatus* on bark. PEITERSEN (1975) used *Trichoderma viride* to produce microbial protein from barley straw. Production of sugar solutions from agricultural and urban cellulosic wastes by *Trichoderma* cellulase was studied by TOYAMA (1976).

CHRISTMAN and OGLESBY (1971) reported that lignin degradation is carried out effectively by fungi, especially basidiomycetes. However, due to the structure of the insoluble substrate, the plant cell wall, enzyme diffusion is very slow and so is degradation. GERRITS and BELS-KONING (1967) measured the degradation of lignin in compost by *Agaricus bisporus*. The amount of lignin remained unaltered until spawning but about 40% of the lignin was consumed after spawning during the next twelve weeks. Before degradation of lignin occurred, more than 50% of the cellulose and hemicellulose was utilized. The chemistry of lignin degradation by wood-destroying fungi has been described by KIRK (1975).

1.4.2. *Bacteria*

The cellulolytic rumen bacteria are perhaps the best studied anaerobic bacteria that are able to degrade cellulose. HALLIWELL (1957) showed that the mixed flora of bacteria and protozoa was able to degrade cotton fibres within three days. HUNGATE (1966) suggested that besides the free cellulolytic rumen bacteria also bacteria in protozoa are active in cellulose degradation. Most active species in the rumen appear to be *Ruminococcus flavefaciens* (KOCK and KISTNER, 1969), *Ruminococcus albus* (KOCK and KISTNER, 1969; VAN GIJLSWIJCK and LABUSCHAGUE, 1971) and *Bacteroides succinogenes* (HALLIWEL and BRYANT, 1963; DEHORITY and SCOTT, 1967).

Free-living cellulolytic bacteria have been isolated from soil and water; they belong to the genera *Cellulomonas*, *Cellvibrio*, *Sporocythophaga*, *Pseudomonas* and *Bacillus*. HARMSEN (1946) made the first systematic study of cellulolytic bacteria in soil. JURASEK et al. (1967) and NORKRANS (1967) stated that bacteria are slow in cellulose degradation and that contact between bacteria and substrate is necessary. VAN HOFSTEN (1975) gives a review concerning topological effects in microbial degradation of cellulose. BERG et al. (1968) isolated *Cellvibrio* strains from polluted water; they grew well on various cellulosic substrates but slowly on crystalline cotton cellulose. In aerobic enrichment

cultures with cellulose, VAN HOFSTEN et al. (1971) observed a synergistic relation between a cellulose-degrading *Sporocythophaga* sp. and a yellow-colony-forming Gram-negative bacterium which could utilize cellobiose as C-source. Similar observations were made by HARMSEN (1946). BERG et al. (1972a) observed good growth of *Cellvibrio fulvus* on several sugars and polysaccharides but not on highly substituted cellulose derivates e.g. carboxymethylcellulose. No growth was obtained with long cotton fibres but some growth occurred when the fibres were cut into small pieces. Lignin-free wood pulp was also degraded. The bacterium has cell-bound cellulase but some enzyme was found in the culture medium. Glucose repressed cellulase formation. Some electronmicroscopical observations concerning the attack of *Cellvibrio fulvus* and *Sporocytophaga myxococcoides* on cellulose were made by BERG et al. (1972b). *Cellvibrio fulvus* grew into the lumen of cellulose macrofibrils while the surface was not attacked. *Sporocytophaga myxococcoides* grew both in the lumen and on the surface of the fibril. The regular arrangement of the cells on the macrofibril was notable.

HAN et al. (1969, 1971) and SRINIVASAN (1969, 1975) studied cellulose degradation by *Cellulomonas uda*. These investigators demonstrated a synergism between *Cellulomonas uda* and *Alcaligenes faecalis*, a cellobiose-utilizing organism. When these bacteria were grown together, a five-fold increase in cell density and growth rate was observed compared to the growth of *Cellulomonas uda* alone.

CARTWRIGHT and HOLDOM (1973) isolated a strain of *Cellulomonas subalbus* from ground birch wood of which the cellulose was utilized by the bacterium.

Cellulolytic activity of a *Bacillus* sp. was described by FOGARTY and WARD (1972). The bacterium was isolated from wood and appeared to possess xylanase, amylase, pectinase and cellulase activity. OHTSUKI et al. (1976) measured cellulase activity in the culture medium of *Bacillus subtilis* var. *natta* and observed also a high xylanase activity. SUZUKI (1975) studied the cellulase formation in *Pseudomonas fluorescens* var. *cellulosa*. The results obtained suggested that cellulase in this bacterium is a constitutive enzyme, the formation of which is controlled by catabolite repression.

Lignolytic activity of soil bacteria was also studied. GOTTLIEB and PELCZAR in their review concluded (1951) that until that year no specific or identified bacterial species had been reliably associated with the natural degradation of lignin. Because of the impossibility to prepare an unalterated lignin, most research is done with low-molecular model substrates (KONETZKA et al., 1952). A flavobacterium which was capable to use α-conidendrin was isolated by SUNDMAN (1964). CARTWRIGHT and HOLSOM (1973) suggested that an *Arthrobacter* sp. isolated by these authors utilized the lignin residue left after the cellulose of wood was utilised by *Cellulomonas subalbus*. However, it is also possible that the organism grew on the residual carbohydrate associated with the lignin. The investigators concluded that bacteria play no major part in the degradation of lignin. CRAWFORD et al. (1973) studied the cleavage of arylglycerol-β-aryl ether bonds by *Pseudomonas acidovorans*. The bacterium was isolated from a lignin-rich environment. *Ps. acidovorans* dissimilated veratryl glycerol-β-(O-methoxyphenyl)

ether (CRAWFORD et al. 1975). However, no bacteria have been described that are able to use or degrade lignin.

Actinomycetes play an important role in the decomposition of cellulose in nature and they are considered to be the major cellulose decomposers in composting processes. However, relatively little is known about the cellulolytic activity of these organisms. KRAINSKY (1914) was the first to isolate two actinomycetes which were able to utilize cellulose as the carbon source. WAKSMAN (1919) isolated 27 actinomycetes able to grow very slowly on cellulose. Some *Micromonospora* spp. were isolated by JENSEN (1932) which were cellulolytic but many other isolated actinomycetes were not able to use cellulose as carbon source. In screening cellulolytic fungi, REESE et al. (1950) found an actinomycete that was able to use carboxymethylcellulose but unable to use cotton. The cellulase produced by *Streptomyces* Q.M.B. 814 was studied by REESE et al. (1959). The cellulase appeared to consist of a number of endoglucanases. FERGUS (1964) concluded that fungi were better cellulose decomposers than actinomycetes. ENGER and SLEEPER (1965) studied cellulase produced by *Streptomyces antibioticus*. The cellulase was acting at random on the cellulose molecule and produced glucose, cellobiose and oligosaccharides. MANDELS and WEBER (1968) isolated a *Streptomyces* sp. with little activity on cotton cellulose which was able to hydrolyse carboxymethylcellulose.

LOGINOVA et al. (1971) isolated a thermotolerant actinomycete which produced extracellular cellulase. STUTZENBERGER (1971) isolated a thermophilic actinomycete, identified as *Thermomonospora curvata*, from municipal refuse compost. Growth requirements for cellulase production and optimum assay conditions were determined by STUTZENBERGER (1972). CRAWFORD and MACCOY (1972) isolated a wide variety of thermophilic actinomycetes from soil. Only two species, *Thermomonospora fusca* and *Streptomyces thermodiastaticus* were able to grow on carboxymethylcellulose. BELLAMY (1974) claimed that thermophilic actinomycetes are probably the most effective organisms for microbial protein production from cellulosic wastes. CRAWFORD (1973) and CRAWFORD et al. (1974) described the production of microbial protein of good nutritional quality by growing the thermophilic strain of *Thermonospora fusca* on industrial paper waste. The ability to degrade lignocellulose at 55 °C was also investigated. Lignin content of pulps varied between 3 % and 18 %. *Thermonospora fusca* was found to degrade primarily the carbohydrate fraction of these substrates. Insignificant losses of lignin were observed. Increasing the lignin content of the pulps proportionally blocked carbohydrate utilization. It is thought that *T. fusca* plays a role in the decomposition of lignocellulose in nature; however, it is probably involved in carbohydrate degradation and not in lignin degradation.

LAMOT and VOETS (1976) isolated several actinomycetes from soil. The isolates were active against carboxymethylcellulose and some grew slowly on insoluble cellulose powder.

Actinomycetes are common in cellulose-rich environments. However, crystalline cellulose degradation occurs slowly and fungi appear to be much better decomposers of crystalline cellulose. CRAWFORD et al. (1973) observed degra-

dation of methoxylated benzoic acids by a *Nocardia* sp. isolated from a lignin-rich environment, but the degradation of lignin itself is not known among actinomycetes. GINNIVAN et al. (1977) studied the break-down of pig faeces by thermophilic actinomycetes.

1.5. ENZYMES INVOLVED IN THE HYDROLYSIS OF PLANT CELL WALLS

1.5.1. *General considerations*

Enzymes and their substrates are mostly water-soluble and consequently freely diffusible in the medium. In enzymic degradation of plant cell walls, however, the substrate is insoluble. For reactivity, enzymes and substrate must be surrounded by water molecules and the enzymes must be diffusible and extracellular. The external chains of insoluble polysaccharides of the plant cell wall are surrounded by water and have a certain degree of motility. In this way the amorphous polymers are accessible to enzymes but the crystalline fraction is not. The enzyme molecules are large (WHITAKER et al., 1954) and for diffusion sizable pores in the substrate are required. When the pores are too small the reaction is limited to the surfaces. Therefore, knowledge of the structure of insoluble substrates is of great importance for understanding the process of enzymic degradation. MCLAREN and PACKER (1970) reported hydrolysis of insoluble substrates by enzymes. Most of the work on enzymes which are involved in plant cell wall degradation is done with enzymes produced by fungi. NISIZAWA (1973) has written a review about cellulose hydrolysis.

1.5.2. *Enzymes for complex substrates*

Enzymic hydrolysis of polysaccharides is complex. Polysaccharidases have a high degree of specificity. Each enzyme has a site for only one kind of sugar in one configuration (α or β) and the monosaccharide must be linked to a particular carbon atom of the adjoining sugar unit. For each, glucan, mannan, galactan, xylan etc. there are specific hydrolysing enzymes. Homopolymers of mixed linkage, heteropolymers consisting of two or more different monosaccharides and branched polymers make hydrolysis even more complicated (REESE, 1975). The high degree of specificity of the hydrolysing enzymes is demonstrated by the fact that β-1,3 glucanase isolated by MOORE and STONE (1972) appeared to be inactive against β-1,3 xylan. The same observation was made by REESE and MANDELS (1963) with β-1,4 glucanase acting on β-1,4 xylan. Mixing and layering of polymers make hydrolysis also difficult. Several enzymes are required for degradation, and the underlying substrates are often slowly hydrolysed because enzyme diffusion is the limiting factor.

In wood degradation by fungi this difficulty is partly overcome by penetration of the organism into the complex substrate. Necessary enzymes are liberated at the hyphal tips producing holes (JURASEK et al., 1967).

1.5.3. *Enzymes involved in the hydrolysis of cellulose*

REESE et al. (1950,1952) showed that cellulase consists of a complex of enzymes

and they constructed the so-called C_1-C_x concept which is given below

$$\text{Crystalline cellulose} \xrightarrow[C_1]{} \text{Reactive cellulose} \xrightarrow[C_x]{} \text{Cellobiose} \xrightarrow[\beta\text{-glucosidase}]{} \text{Glucose}$$

Crystalline cellulose consists of several cellulose molecules which form ordered crystal lattices by interpolymeric H-bonds (elementary fibrils or micelles). The free cellulose molecules, i.e. reactive cellulose, are accessible to endo-enzymes (C_x).

C_1 appeared to be responsible for conversion of crystalline cellulose into a form accessible to the hydrolytic endo-enzymes C_x. The existence of C_1 was postulated by the fact that some organisms were able to degrade reactive (para-crystalline) cellulose but were unable to degrade crystalline cellulose. The C_x factor reflects several β-1,4 glucanases, at random acting enzymes, hydrolysing non-crystalline cellulose, soluble cellulose derivatives and β-1,4 oligomers of glucose. MANDELS and REESE (1957) demonstrated high cellulolytic activity of *T. viride*, that was highly increased by using mutants (MANDELS et al., 1971). ERIKSSON et al. (1968, 1974), HALLIWELL and RIAN (1971), PETTERSON et al. (1973), WOOD (1973), and MOO-YOUNG et al. (1977) demonstrated that culture filtrates of *Chrysosporium*, *Fusarium*, and *Penicillium* spp. and a basidiomycete contained high levels of C_1, although not as much that of *T. viride*. C_1 and C_x activity have been separated from some of these organisms and synergism has been shown between C_1 of one fungus and C_x of another.

The C_1-C_x concept dominated the thinking about cellulolytic enzyme systems for a long time. The development of better separation methods led to the preparation of purer components of the cellulase complex. This work, performed by ERIKSSON et al. (1968, 1974), PETTERSON et al. (1973), NISIZAWA (1973) and WOOD (1973), led to the concept that cellobiohydrolase, whose main function is to remove cellobiose units from the non-reducing end of the non-crystalline cellulose chains, is also able to convert crystalline cellulose into reactive cellulose (C_1 factor). REESE (1975) did not agree with this concept. He mentioned the work of STORVICK and KING (1960) who demonstrated the presence of cellobiohydrolase in cellulase of *Cellvibrio fulvus* but crystalline cellulose was not hydrolysed by this enzyme. REESE (1976) assumed that C_1 is an enzyme limited in its action to crystalline surfaces. He suggested the following modification of the mechanism proposed earlier.

$$\text{Crystalline cellulose} \xrightarrow[C_1]{} \text{Modified cellulose} \longrightarrow \begin{cases} \text{endo-glucanases } (C_x) \rightarrow G_3 + G_2 + G_1 \\ \text{Cellobiohydrolase} \longrightarrow G_2 \\ \text{Glucohydrolase} \longrightarrow G_1 \\ \text{Cellobiase} \longrightarrow G_1 \end{cases}$$

The C_1 factor, originally claimed to disrupt hydrogen bonds, is now believed to split covalent linkages and hydrogen bonds. The C_1 activity differs from the at random-acting endoglucanases in being active only upon crystalline cellulose.

Furthermore, it does not act on C.M.C. and has no ability to act on products of its own action, since it produces no soluble fragments from crystalline cellulose. C_x activity contains several at random acting endoglucanases capable of catalysing hydrolysis of the products of C_1 activity. The enzymes glucohydrolase and cellobiohydrolase are limited in their action to nonreducing chain ends.

1.5.4. *Hemicellulases*

Since for each kind of sugar linkage in polysaccharides specific hydrolytic enzymes are active, the number of hemicellulose-degrading enzymes will be enormous. However, in contrast to the cellulase enzyme system only little is known about other polysaccharidases.

LYR and NOVAK (1962) investigated ten species of ascomycetes, *Fungi imperfecti*, and basidiomycetes and observed simultaneous formation of cellulase, xylanase, mannanase and amylase on cellulose as carbon source. The course of the enzyme secretion demonstrated that all the enzymes were substrate-specific and were formed non-simultaneously. RITTER (1964) observed that *Mycorrhizas* in addition to cellulase produced xylanase, mannanase, amylase and pectinase when growing on cellulose. He considered xylanase and mannanase to be constitutive enzymes. REESE and SHIBATA (1965) stated that β-1,4 mannanases in fungi are inducible enzymes, as the yield of mannanase is 10–100-fold greater when the organism is grown on mannan instead of cellulose. All of the β-mannanases studied were endopolysaccharidases acting on long chains in a random manner and unable to act on mannotriose and mannobiose. Most microbial mannanases contained activity capable of removing galactose and glucose branches. ERIKSSON and WINELL (1968) found that a crude cellulase preparation of *Aspergillus oryzae*, contained cellulase, β-glucosidase, xylanase, xylosidase, mannanase and mannosidase. The molecular weight of the mannanase was determined to be 42,000 $\pm$ 2,000. ERIKSSON and RZEDOWSKI (1967) showed that cellulase and mannanase formation by *Chrysosporium lignorum*, which is identical with *Sporotrichum pulvurulentum*, was induced when cellulose was the C-source. Xylan did not appear to be an inducer of cellulase, mannanase and xylanase. ERIKSSON and GOODELL (1974) investigated mutants of *Polyporus adustus* lacking cellulase. Most of the mutants lacked mannanase and xylanase as well. In the wild type the level of cellulase, mannanase and xylanase was higher when the organism was growing in a medium containing cellulose than in a medium without cellulose. It is proposed that in *Polyporus adustus* the induction of this group of enzymes is under control of a single regulator gene. ERIKSSON (1975) observed the same phenomenon for *Sporotrichum pulvurulentum*. ZOUCHAVA et al. (1977) demonstrated that in cultivating some wood-rotting fungi, the α-mannosidase activity was about equal with mannan or cellulose as the carbon source. Mannanase activity of several wood-rotting fungi in media containing glucose, mannan or cellulose was equal, indicating that mannanases are constitutive enzymes. These authors also found no increase in mannanase activity by a several-fold transfer of the cultures to fresh mannan-containing media. It is not clear whether the

production of mannanase, xylanase and cellulase is enhanced by a specific substrate or by another inductor. From this review it can be concluded that all of the screened microorganisms producing cellulase formed also hemicellulases. The production of mannanase, xylanase and cellulase may depend on a common inductor (cellulose).

1.5.5. *The lignin-degrading enzymes*

Considering the size of lignin macromolecules, the predominant degrading enzymes must be extracellular. Enzyme studies on insoluble macromolecules of which the exact structure is unknown and which cannot be isolated in a pure state is no easy task. White-rot fungi are well-known for their ability to degrade wood lignin. The fungi of this type produce varying amounts of extracellular phenol-oxidizing enzymes of which peroxidase and laccase have been isolated. A good relationship exists between the production of these enzymes and the ability of the fungi to degrade lignin (KIRK and KELMAN, 1965). The brown-rot fungi, which leave the lignin essentially unaltered do not produce any detectable amount of phenol-oxidizing enzymes.

Numerous studies on the effect of white-rot fungi and their enzymes on lignin and lignin model compounds have been carried out and have led to different theories concerning the microbial degradation of lignin. The main theories have been reviewed by CHRISTMAN and OGLESBY (1971) and can be summarized as follows (GIERER, 1975).

Side chains in lignin units are oxidized at the α- or β-carbon atoms with formation of structures containing keto-groups and liberation of phenolic units: methyl-aryl ether bonds are also cleaved. β-Aryl ether linkages are hydrolysed and give alcoholic and phenolic derivatives. Fragmentation of lignin occurs by cleavage of alkyl-aryl-carbon-carbon bonds. In this type of reaction side chains are removed from the aromatic nuclei by oxidative coupling between radical intermediates of the phenoxyl and cyclohexadienonyl types. Intermediary *p*-quinoid structures and aldehydic or acidic fragments are formed. The enzymes catalyse the cleavage of the aromatic nuclei. After introducing the required hydroxylation pattern (formation of ortho- and paradiphenol structures) by demethylation or hydroxylation, the phenolic rings are cleaved to give aliphatic degradation products (usually carboxylic acids).

Separately or in combination, reactions of this kind should degrade lignin extensively. However, the pathway and mechanism of degradation of lignin is obscure and the results of many studies are contradictory and confusing. The specific enzymes involved in microbial degradation of lignin are not precisely known. It is commonly held that the degradation of lignin is oxidative because the composition of degraded lignin resembles that of humic acid. Degraded lignin as well as humic acid contains less methoxyl groups and more phenolic, hydroxyl, carboxyl and carbonyl groups. The extracellular laccase and peroxidase are believed to play an important role in these transformations. However, the significance of these enzymes in the process is uncertain.

2. MATERIALS AND METHODS

2.1. FUNGI EMPLOYED IN THIS STUDY

2.1.1. *Organisms derived from culture collections*

The following fungi were used in the present investigation. *Chaetomium globosum* CBS 139.38; *Myrothecium verrucaria* CBS 189.46; *Penicillium nigricans* collection Laboratory of Microbiology, Wageningen; *Trichoderma viride* QM 6a; *Trichoderma viride* QM 9123; *Trichoderma viride* QM 9414.

Trichoderma viride QM 9123 and QM 9414 are mutants of *Trichoderma viride* QM 6a (MANDELS et al., 1971), which sometimes is presented as *Trichoderma reesei.*

The organisms were maintained on malt agar and on cellulose agar slants at room temperature. Transfer of the stock cultures was performed every two months.

2.1.2. *Isolated cellulolytic fungi*

Isolation of cellulolytic fungi was performed by using the enrichment technique with a medium of the following composition (g/l): $(NH_4)_2SO_4$, 1.4; ureum, 0.3; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.3; soil extract, 250 ml; 0.1 M phosphate buffer, 750 ml; pH 5.5. The carbon source included (a) washed solids of pig faeces mainly consisting of plant cell wall residues, (b) Avicel (Koch Light) a crystalline cellulose powder, or (c) Indulin AT (Westvaco), a preparation of lignin.

One gram of soil, compost or manure was added as inoculum to 100 ml medium contained in 300 ml conical flasks and incubated on Gallenkamp orbital shakers at 28 °C and 200 rev/min. Under these conditions the oxygen transfer rate was found to be sufficient (SCHELLART, 1975) for maintaining the culture aerobic. No pH control was applied in this isolation procedure. After five days of incubation 2 ml of the cultures was transferred to fresh medium, a procedure repeated twice. Isolates of the dominant microflora were obtained after streaking a small amount of the enrichment culture on cellulose agar plates of the same composition as the enrichment medium. Isolated strains were maintained on malt agar and cellulose agar and transferred every two months.

2.2. MEDIA

The medium used for the enrichment of fungi is recorded in 2.1.2.

2.2.1. *Basal medium*

The basal medium applied in this study was the same as that used by MANDELS and WEBER (1969); it contained per 1 of tap water (g): KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; urea, 0.3; $MgSO_4.7H_2O$, 0.3; $CaCl_2.2H_2O$, 0.4 and one ml of

a solution of trace elements which contained per litre (mg): $FeSO_4.7H_2O$, 10; $MnSO_4.H_2O$, 5; $CuSO_4.5H_2O$, 50; $ZnSO_4.7H_2O$, 500; Na_2MoO_4, 5; H_3BO_3, 5 and $CoCl_2$, 5.

2.2.2. *Carbon sources*

Ten g of C-source per l of basal medium was added. Monosaccharides, disaccharides, all of them of analytical grade, and polysaccharides were used as C-compound. In some experiments also lignin (Indulin AT, Westvaco) and washed solids of pig faeces were applied as carbon source. In the case of solids of pig faeces 15 g dry weight per l of basal medium was used. The polysaccharides used included Avicel, a microcrystalline cellulose powder (Koch Light), sodium carboxymethylcellulose (pure B.P.C., Koch Light) xylan (Fluka AG); gum arabic (Sigma); galactomannan (Sigma) and Whatman no 1 filter paper.

Gas-chromatographic analyses of sugar components in hydrolysates of xylan, gum arabic and galactomannan showed that xylan contained 18% glucose, 42.5% mannose and 34.5% xylose, gum arabic 33% galactose, 35.5% arabinose and 15.5% rhamnose, and galactomannan 28% galactose and 59% mannose.

Solids from pig faeces were prepared as follows: fresh faeces were collected, separately from the urine, from castrated male pigs (Dutch Landrace or Yorkshire) held in metabolic cages. The faeces were diluted with the same volume of water and mixed thoroughly in a Waring blender during one minute. A volume of 0.05 M EDTA solution (pH 7.0) equal to the volume of dilute faeces was added and the slurry stirred for several minutes, followed by filtration through cheese cloth. Washing of the residue with the EDTA solution was repeated twice, followed by washing with 5% n-butanol solution (three times), 97% ethanol (three times) and acetone (three times). After washing with acetone the residual matter was dried in air at room temperature. By this procedure, water-soluble substances, proteins, fats, waxes, sandy material and bacteria were removed and a light yellow-brown-coloured straw-like preparation resulted. The washed solids were used (a) without further treatment, (b) ground or (c) delignified by boiling in NaOH (1.0 N) for 10 min. Grinding was performed in a Fritsch Pulverisette ball mill using a stainless steel beaker of 250 ml containing three balls (∅ 3 cm) or in a hammer mill with sieves of 10, 0.75, 0.30 and 0.08 mm pore diameter.

2.3. Growth conditions of cellulose-decomposing fungi

2.3.1. *Batch cultures in shaking flasks*

Organisms were usually grown in 300-ml conical flasks containing 100 ml of medium. The cultures were incubated in Gallenkamp orbital shakers at 28 °C and 200 rev/min. Control of pH was not applied in these experiments.

2.3.2. *Batch cultures in fermentors*

Experiments concerning the degradation of solids from pig faeces and the

production of cellulase, were carried out in Biotec fermentors of 3.2-l capacity, using working volumes of 3 l at a constant temperature of 29 °C. In these apparates the pH was recorded and controlled automatically with 1.0 N NaOH, using sterilizable glass electrodes. Development of foam was prevented by keeping the stirring speed below 250 rev/min and placing the air inlet above the liquid level. At an air flow of 2 l per litre of medium per minute the oxygen transfer rate was found to be adequate. To reduce evaporation from the fermentors, the air was moistened by passing it through stone spargers contained in flasks with water; hereafter it was sterilized by passing through miniature line filters (Microflow Ltd, Fleet, Hants, England). Samples of 100 ml of the cultures were taken with the aid of a glass siphon with a rather wide inside diameter (∅ 7 mm) to prevent clogging by mycelium and residual solids.

2.3.3. *Methods of inoculation*

Inocula of cellulose-decomposing fungi were prepared from 2–3 weeks old slant cultures. Spore suspensions were prepared in sterile deionized water. Ons ml of such a suspension, containing 10^6–10^7 spores, was used as inoculum per 100 ml of medium. In the experiment performed in the Biotec fermentors the inoculum was prepared from 3–4 days old mycelium grown in cellulose medium. In this case 20 ml of inoculum was used per litre of fresh medium. Inoculation by suspended mycelium avoided a prolonged lag phase that occurred when spores were used as inoculum.

2.4. Enzymic preparations

2.4.1. *Enzymic hydrolysis of washed solids of pig faeces*

Enzyme action of culture filtrates and of (industrial) cellulase preparations on solids of faeces was studied in 25-ml conical flasks containing 10 ml of Na-citrate buffer solution, 0.025 M, pH 4.8 and 200 mg of solids of faeces. An appropriate amount of enzyme solution (0.5–1 ml) was added. Commercial cellulase used was derived from *Aspergillus niger* (Sigma). The incubation temperature was 50 °C unless otherwise stated. Incubation was performed in a reciprocating water bath (140 r.p.m.) for 4 h. The reaction was stopped by cooling in ice. After cooling, the monosaccharides formed and the loss of weight of the insoluble material were estimated immediately.

2.4.2. *Determination of enzyme adsorption on substrate*

Culture filtrates (1.0 ml) with known cellulolytic activity were transferred to 10 ml of 0.025 M Na-citrate buffer (pH 4.8). After the addition of different amounts of substrate, followed by incubation for 30 min at 4.5 °C, the enzymic activity of the supernatant was determined. The temperature was kept at 4.5 °C to prevent hydrolysis of the solids.

2.4.3. *Analysis of cellulolytic activity of culture filtrates*

Culture filtrates of *T. viride* QM 9414 were evaporated at 30 °C to reduce the volume 10- to 20-fold. The concentrated filtrates were passed through a Sephadex G-15 column and the filtrate precipitated by the addition of $(NH_4)_2SO_4$. Precipitates were formed at 40, 60 and 80 % saturation. After passage through a Sephadex G-15 column, the 40 % $(NH_4)_2SO_4$ fraction was separated on a DEAE Sephadex column A-50 (Pharmacia, Uppsala, Sweden). The eluate was collected in fractions of 3 ml with the aid of a fraction collector.

The Sephadex G-15 column was eluted with 0.05 M Na phosphate buffer of pH 7.0 and the DEAE Sephadex column with an 0.05 M Tris-HCl buffer, pH 7.0. A 1.0 M NaCl gradient was used on the DEAE Sephadex column. Enzyme activity was estimated after all of the treatments.

2.5. Analytical methods

2.5.1. *Determination of dry weight*

Residues of batch cultures were harvested by centrifuging 100 ml of the culture, washing twice with deionized water and drying at 103 °C until constant weight.

2.5.2. *Determination of ash*

Determination of the ash content was performed by heating the oven-dry samples at 550 °C for 6 hours. After cooling, the samples were dried at 103 °C until constant weight.

2.5.3. *Determination of hemicellulose*

One gram of washed solids of faeces or 2 g of fresh faeces was supplied with 50 ml of 1.0 N H_2SO_4 and transferred to a glass tube which was sealed and heated for 6 hours at 100 °C, unless otherwise stated. During hydrolysis the tube was shaken frequently. After cooling, the slurry was centrifuged. The supernatant was used for carbohydrate analysis. The amount of hemicelluloses was estimated by measuring the reducing sugars in the hydrolysate. If necessary, the hydrolysates were neutralized by adding solid $Ba(OH)_2$. Precipitated $BaSO_4$ was removed by centrifugation.

2.5.4. *Determination of cellulose*

After washing with acetone and air drying, the residue of the hemicellulose determination was transferred to a tube which was supplied with 2 ml of 72 % sulphuric acid and left at 30 °C for 1 h in order to solubilize the cellulose. Subsequently, 50 ml of water was added, the glass tube sealed and the contents hydrolysed completely at 100 °C within 6 hours. After cooling, the slurry was spun down and the supernatant analysed for glucose which resulted from the hydrolysis of cellulose.

2.5.5. *Determination of lignin*

The residue of the cellulose hydrolysis was washed twice with deionized water and dried at 103 °C until constant weight. The dried samples were subsequently heated at 550 °C for 6 hours whereupon the ash content was measured. Oven-dry sample minus ash content was defined as lignin.

2.5.6. *Determination of reducing sugars*

Reducing sugars were determined according to the method of Somogyi and Nelson (SOMOGYI, 1952), as described by HODGE and HOFREITER (1962), and expressed as glucose equivalents.

2.5.7. *Determination of total hexoses*

Total hexoses were estimated by the anthrone method as described by HODGE and HOFREITER (1962), using glucose as standard.

2.5.8. *Determination of glucose*

Glucose was measured enzymatically according to the specific reaction with D-glucose-oxidase (AB KABI, Stockholm) according to FALES (1963). As standard glucose was used.

2.5.9. *Determination of uronic acids*

Hexuronic acids were determined by the carbazole method of BITTER and MUIR (1962) with glucuronic acid as standard.

2.5.10. *Gas liquid chromatographic analysis of monosaccharides*

Individual hexoses and pentoses in neutralized hydrolysates were separated as alditol-acetate derivatives by using the technique of gas liquid chromatography. Conversion of hexoses and pentoses into alditol-acetate derivatives was performed according to ZEVENHUIZEN (1973). First, meso-inositol was used as internal standard to quantify this method (SLONEKER, 1971). Later on, quantification was performed by separately measuring the glucose concentration in neutralized hydrolysates by specific reaction with D-glucose-oxidase according to FALES (1963). Sugars were identified by comparing retention times of unknown peaks in the GLC with those of known reference sugars. Alditol-acetate derivates were separated by the method of LÖNNGREN and PILOTTI (1971) by using a Becker Unigraph-F type 407 gas chromatograph, equipped with a flame-ionization detector. A stainless steel column (200 cm × 4 mm) containing 3 % of OV-225 on Chromosorb W-HP (100–120 mesh) was used, while nitrogen was the carrier gas (30 ml min^{-1}). The column temperature was 200 °C.

2.5.11. *Determination of soluble protein*

Soluble protein was measured by using the method of LOWRY et al. (1951). Crystalline serum albumin served as standard.

2.5.12. *Determination of the endoglucanase activity*

Endoglucanase activity (C_x enzymes) was determined viscosimetrically according to PEITERSEN (1975a). A sodium carboxymethylcellulose solution was prepared by stirring 2.5 g CMC (Koch-Light) in 1-l of a 0.05 M Na-citrate buffer (pH 4.8). The assay was carried out using an Ostwald viscosimeter at 25 C. The enzyme activity was measured as follows: 9 ml of the CMC solution was incubated with 1 ml (diluted) sample at 25 °C for 5 min. The efflux time, t, was measured and the activity expressed relative to the efflux time for the CMC solution, t_o

$$1\ C_x \text{ unit} = \frac{t_o - t}{t_o} \cdot \frac{1}{a}$$

where a is the size of the sample in millilitres. This gives a linear relationship between C_x and a for $t > {}^2/_3\ t_o$.

2.5.13. *Determination of the exoglucanase activity*

Exoglucanase activity was measured by incubating 0.5 ml of (diluted) enzyme solution or culture filtrate with 1 ml of a 2.5 g CMC solution in 1 litre of 0.05 M Na-citrate buffer (pH 4.8) at 40 °C for 30 min. Liberated monosaccharides were determined by measuring their reducing power. Exoglucanase activity was expressed as glucose ($\mu g\ ml^{-1} h^{-1}$).

2.5.14. *Determination of the filter-paper activity*

The cellulolytic activity of the supernatant of the cultures was measured by the filter-paper assay of MANDELS et al. (1971), using the modifications of GRIFFIN et al. (1974). According to this assay, 100 mg of coiled Whatman no 1 filter paper was placed in a glass tube. One ml of 0.05 M Na-citrate buffer solution (pH 4.8) was added and subsequently 0.5 ml of enzyme solution or of supernatant. Incubation was performed at 50 °C for 30 min. Liberated reducing sugars were measured according to the method of SOMOGYI (1952). The amount of sugars, measured as mg glucose produced in this test, is called the filter-paper activity (FPA). The measurement of the cellulase activity is complex and there is no absolute unit as can be measured for a single enzyme acting on a soluble substrate. The unit value will depend on the nature and concentration of the substrate chosen. The filter-paper assay is not perfect but it is simple, reproducible and quantitative and it predicts enzyme action under practical saccharification conditions.

2.5.15. *Determination of hemicellulase activity*

Hemicellulase activity was measured by incubating 0.5 ml of (diluted) enzyme solution or culture filtrate with 1 ml of a suspension of 5 g xylan (pure, Fluka) in 1 litre of 0.05 M Na-citrate buffer (pH 4.8) at 40 °C during 30 min. Reducing power produced after incubation was measured with glucose as standard. Hemicellulase activity was expressed as glucose produced in $\mu g\ ml^{-1} h^{-1}$.

3. NATURE AND COMPOSITION OF THE CELLULOSE – HEMICELLULOSE-LIGNIN COMPLEXES IN PIG FAECES

3.1. INTRODUCTION

Pig faeces form an increasing pollution problem in pig fattening. Owing to the lack of agricultural land for using the animal wastes as fertilizer, disposal in the vicinity of the production units is difficult and it is necessary to look for other possibilities to utilize or destroy these wastes. In all treatment methods the residues of the plant cell wall appear to be more or less resistant against biodegradation (ROBINSON, 1971). For that reason an investigation was performed concerning the microbial degradation of cellulose-, hemicellulose-, and lignin-containing material of pig faeces.

To begin with, botanical origin and chemical composition of plant cell wall residues were investigated. The plant cell wall material found in the faeces of pig originates from the feed. In the Netherlands, and in many other countries with intensive fattening of pigs, the animals are fed with mixed feed until a slaughter weight of 100 kg is reached. Pigs of this weight hardly digest plant cell wall material (DAMMERS, 1964). The composition of the mixed feed used for fattening the pigs, which supplied the faeces for the present investigation, is given in Table 3.1. The composition of mixed feed may show large variations depending on the manufacturer.

During grinding of the ingredients, the temperature may rise up to 60 °C which renders the plant cell wall material more resistant to enzymic degradation. This was found to be true of the hydrolysis of cellulose-containing material by cellulase (GHOSE and KOSTICK, 1969). Soybeanmeal is a by-product which is left after oil extraction of soybeans. This ingredient is heated to 100 °C to destroy an antitrypsin factor. The crude fibre content (see below) of soybeanmeal is about

TABLE 3.1. Composition of mixed feed for intensive fattening of pigs.

Ingredient	Percentage
Wheat meal	20.0
Maize	18.5
Barley	15.0
Milocorn	10.0
Soybeanmeal	20.0
Maize glutenmeal	5.0
Wheat middlings	4.5
Lucerne (Alfalfa meal)	2.0
Soybean oil	1.0
Vitamines and minerals	4.0

7%. Maize glutenmeal, a by-product of the starch industry, has been heated at 100 °C during the production process. The crude fibre content of this material is 10%. Wheat middlings, produced from ground wheat, contain about 10% crude fibre. Alfalfa is added to mixed feed as artificially dried plant material. The crude fibre content of dried alfalfa is estimated at 20–30 %.

Mixed feed usually contains about 3–6 % crude fibre of which 25 % is digestible. However, as was demonstrated by VAN SOEST (1964), the crude fibre fraction of feed or faeces is not identical with the amount of plant cell wall residues. The determination of this fraction is based upon successive extractions with acid and alkali. In this procedure 80 % of the hemicellulose and 50–90 % of the lignin is removed, while the cellulose output is 50–80 % (VAN SOEST, 1973). Thus, the crude fibre determination of feed is not a good measure of the amount of plant cell wall residues in faeces.

Data from literature on the composition of pig faeces are scarce, contradictory, and mostly presented as fertilizer capacity. CONRAD and MAYROSE (1971) stated that the conversion of feed in intensive fattening of pigs was about 84 % of the dry matter while 16 % was lost with the faeces. KOLENBRANDER and de la LANDE CREMER (1967) gave a digestibility of 83 %. According to these authors total production of faeces depends on the amount of feed taken in, the composition of the feed and the age of the animals. TIETJEN and VETTER (1972) estimated the production of faeces to be 2–5 kg per 100 kg of pig a day. Dry matter and organic matter content of faeces depended on the kind of feed, water intake, age of the animal and way of farming. DALE (1972) and MINET and SMITH (1975) found 20–30 % dry matter in pig faeces, with 80 % of the dry matter being organic. KOLENBRANDER en de la LANDE CREMER (1967) found 23 % dry matter of which 70 % was organic matter and 30 % ash. HOBSON and SHAW (1971) reported an ash content of 4–8 % in the dry matter fraction of faeces. These investigators found 10 % hemicellulose, 10 % cellulose and 5 % lignin in the dry matter. Determinations were made by them using the acid and neutral detergent method of VAN SOEST (1965) which gives a better estimation of cell wall residues than the crude fibre determination does. ROBINSON (1971) found 16.5 % crude fibre in swine faeces, and SMITH (1973) 15 % cellulose in the dry matter of faeces of pigs for fattening. Because of differences in the methods of determination, in composition of the feed, in age and race of the pigs, and in the way of farming, the given data are hardly comparable.

In this chapter the results are given of a microscopic investigation of the tissues of cell wall residues and the results of a chemical analysis of fresh faeces and of three preparations of solids of pig faeces.

3.2. MICROSCOPIC EXAMINATION OF PLANT CELL WALL RESIDUES OF PIG FAECES

As reported before the cell wall of higher plants in general consists of an intercellular middle lamella, a primary cell wall and, depending on tissue and

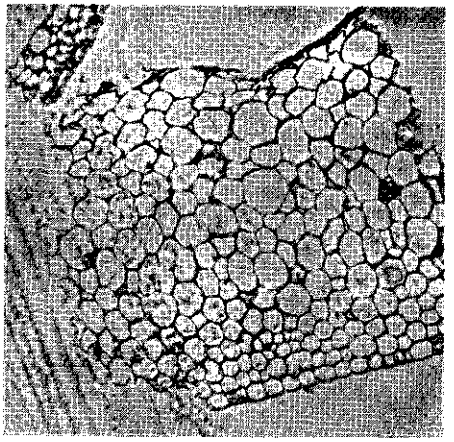

FIG. 3.1 Transverse section of parenchyma cells of a dicotyledon (190 ×).

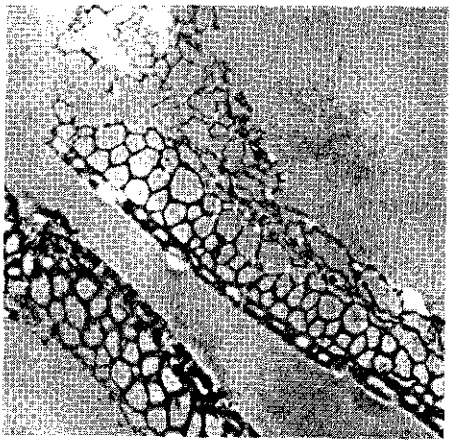

FIG. 3.2 Transverse section of stem tissue of a monocotyledon (270 ×).

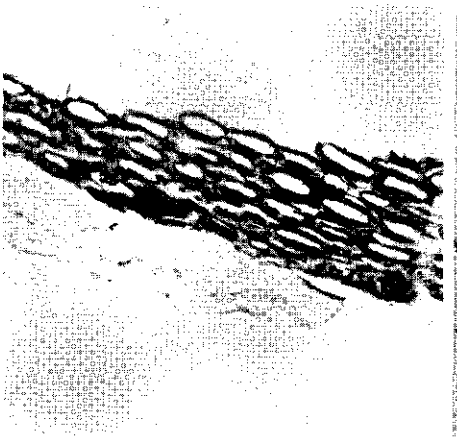

FIG. 3.3 Collenchyma (270 ×).

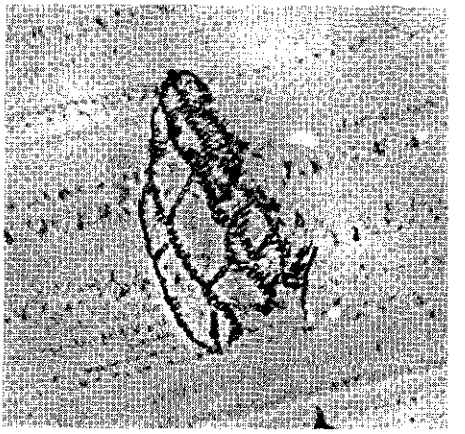

FIG. 3.4 Pericarp cells with thickened walls (270 ×).

maturity, a secondary cell wall which is divided into S_1, S_2 and S_3 layers. ROBARDS (1970) gave an extensive description of the structure of the plant cell wall.

In the present investigation phase-contrast microscopy was performed to detect the kind of plant tissue from which the plant cell wall residues of the faeces originated. Figures 3.1, 3.2, 3.3 and 3.4 show tissue particles derived from solids of faeces. Fig. 3.1 represents a transverse section of parenchyma cells from a dicotyledon. Parenchyma cells are often isodiametric, having thin and often only primary cell walls. Cellulose of parenchyma has a low degree of polymerisation and a crystallinity of 40%; the hemicellulose content may be up to 50% (ROBARDS, 1970). Fig. 3.2 gives a transverse section of stem tissue of a

monocotyledon. The left upper side shows a vascular bundle. Parenchyma and collenchyma are visible. The cell walls of collenchyma are thicker particularly with cellulose and lignin at the corners. Collenchyma cells also tend to be more elongated than parenchyma cells. A transverse section of collenchyma with heavily thickened cell walls existing of cellulose and lignin is shown in Fig.3.3. Fig. 3.4. shows a transverse section of the first layer of mesocarp of wheat with thickened cell walls. This kind of tissue is characterized by the presence of

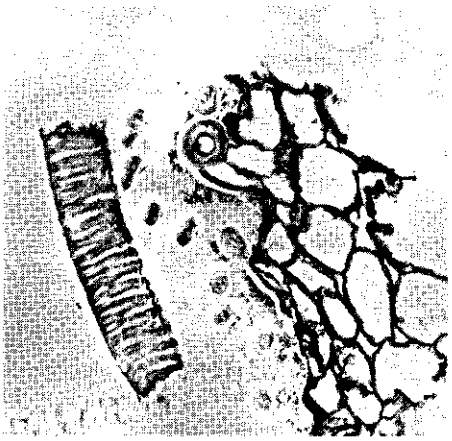

FIG. 3.5 Transverse section of the spermoderm of soy bean seed from left to right: palisade cells, beaker cells and parenchyma (270 ×).

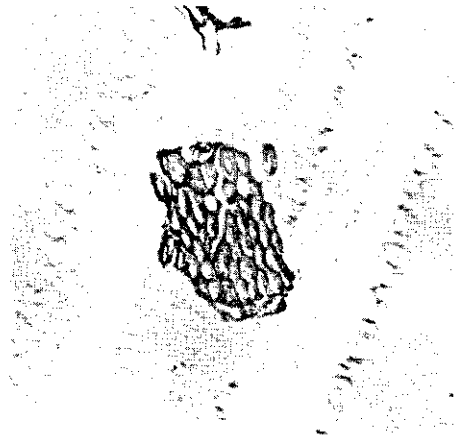

FIG. 3.6 Surface view of the palisade cells of the spermoderm of soy bean (270 ×).

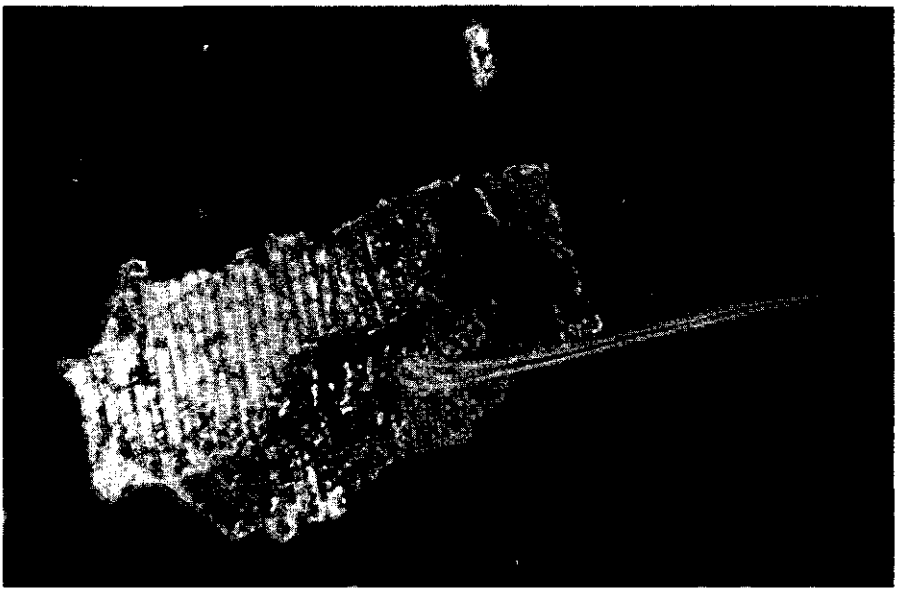

FIG. 3.7 Crossing layer and hair of wheat bran (80 ×).

secondary cell walls with a high quantity of cellulose, hemicellulose and lignin.

Fig. 3.5 shows a transverse section of the spermoderm of soybean seed. Palisade cells, beaker cells and parenchyma are visible. The cell wall of palisade cells and subepidermis (beaker cells) are thickened, having a secondary cell wall with cellulose and lignin. Fig. 3.6 shows a surface view of the palisade cells of soya with heavily thickened secondary cell walls consisting of both cellulose and lignin. Fig. 3.7 shows the crossing layer with a hair of wheat bran. A transverse section of typical palisade parenchyma of a leaf is given in Fig. 3.8.

Figs. 3.9, 3.10 and 3.11 show tissue particles of caryopsis i.e. the pericarp,

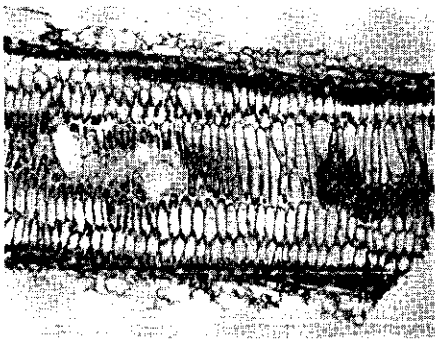

FIG. 3.8 Palisade parenchyma of leaf (120 ×).

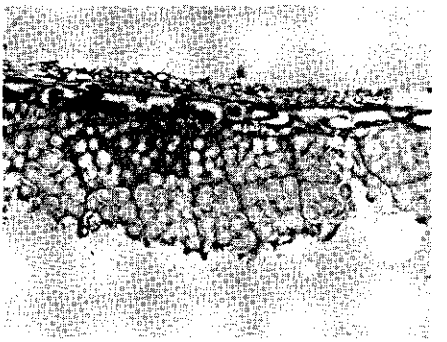

FIG. 3.9 Endosperm with starch or aleuron granules (270 ×).

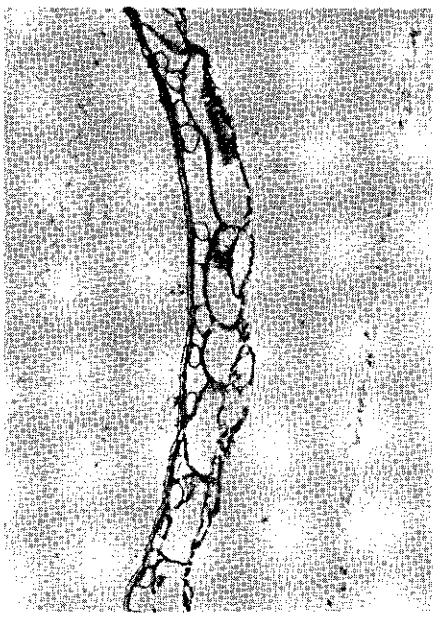

FIG. 3.10 Seed coat (left) and pericarp of a cereal (270 ×).

FIG. 3.11 Crossing layers in the seed of a cereal (270 ×).

consisting of a single cell layer grown together with the seed coat (bran) (Fig. 3.10). The bran shows in transverse section two crossing layers (Fig. 3.11). Fig. 3.9 shows the endosperm with starch or aleuron granules. The upper cell layer of the endosperm usually contains the aleuron granules.

Concluding, in the residues of plant cell wall material in faeces all common plant tissues are recognizable originating from monocotyledons as well as dicotyledons. Different kinds of parenchyma, collenchyma and sclerenchyma are seen of which the last two are probably the main sources of cellulose-, hemicellulose- lignin complexes. Although the samples for microscopic examinations were taken at random, no details can be given about the quantitative distribution of the different kinds of tissue in pig faeces. By phase-contrast microscopy degradation of plant cell walls due to the enzymic activity of the digestive tract of the animal could not be detected.

3.3. Composition of freshly voided faeces and three samples of solids

3.3.1. *Dry matter and ash contents*

The dry matter content of freshly voided faeces may differ from day to day and from animal to animal. In faeces from five healthy pigs it varied from 20 to 25 % and the ash content of the dry material from 18–20 %. The faeces used in this study were derived from animals which were held in metabolic cages. This means that contamination of feed and faeces with sand or dust was prevented. In practice, the ash content may vary widely due to, among other external influences, the housing conditions of the animals.

The dry-matter content of washed and air-dried solids of faeces was usually 93 ± 2 %. The ash content as percentage of the dry matter of three washed samples was 2.8, 6.6 and 3.3 %, respectively. The reduction of the ash content of

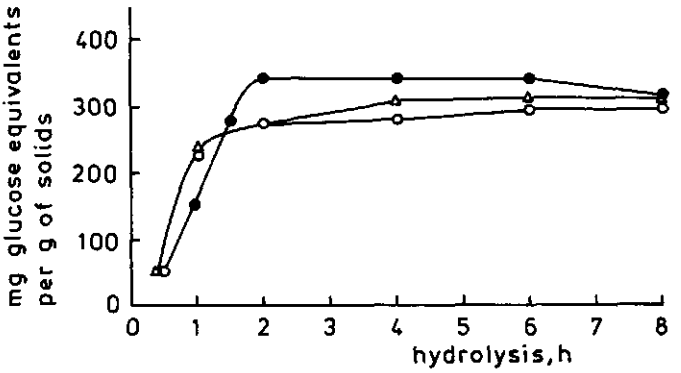

FIG. 3.12 Hydrolysis of the solids of faeces. Reducing sugars expressed as mg glucose equivalents per g of solids hydrolysed by 1.0 N (●——●), 1.5 N (△——△) and 2.0 N (○——○) H_2SO_4.

washed solids compared to that of freshly voided faeces is explained by the fact that in addition to water-soluble substances and bacteria most of the sand is washed away.

3.3.2. *Nitrogen content*

The nitrogen of freshly voided faeces originates from microbial protein and undigested feed protein and, so-called metabolic faecal protein. In pig faeces nitrogen accounted for about 5 % of the dry matter; in 3 washed samples of solids it amounted to 0.7, 1.2 and 0.7 %, respectively. This decrease was due to the washing out of bacteria and of soluble proteinaceous fractions. Nitrogen may be a component of the cell walls of plants and it may be present as protein in the residues of grains.

3.3.3. *Hemicellulose*

Hydrolysis with dilute mineral acid is a well-known method for hydrolysing non-cellulosic polysaccharides (ADAMS, 1965). Hydrolysis is a function of acid concentration, time and temperature of reaction. Fig. 3.12 shows the relation between time of hydrolysis of the solids of pig faeces (sample 1) and amount of reducing power produced, measured as glucose equivalents, at three different H_2SO_4 concentrations. Hydrolysis with 1.0 N H_2SO_4 for two hours gave a maximum sugar yield of 340 mg glucose equivalents per g of dry matter of washed solids. Hydrolysis under the same conditions but with 1.5 or 2.0 N H_2SO_4 yielded lower amounts of monosaccharides. This discrepancy was due to chemical degradation of the monosaccharides formed. Degradation of these sugars was slightest at a low H_2SO_4 concentration. Prolonged hydrolysis with 1.0 N H_2SO_4 did not render more reducing power available; a decrease was not observed either. Under these circumstances there was obviously equilibrium between monosaccharides produced by hydrolysis and monosaccharides degraded by the acid. Hydrolysis with 1.5 and 2.0 N H_2SO_4 resulted in a slight increase of monosaccharide production following the first two hours of incubation, apparently because hydrolysis of hemicellulose was somewhat more affected by the higher activity than the simultaneously occurring degradation of the produced monosaccharides. It was suggested that the most acid-labile fraction of the produced monosaccharides was degraded in an earlier stage than in the experiment with 1.0 N H_2SO_4.

To confirm these observations, glucose, arabinose and xylose were heated at 100 °C in 2.0 N H_2SO_4 under the same circumstances as hydrolysis of the polysaccharides occurred. After 6 hours of boiling, 3 % of glucose, 12 % of arabinose and 14 % of xylose were degraded. As no monosaccharides were present at the beginning of the hydrolysis of plant cell material, it was impossible to calculate a conversion factor for the hydrolysis of hemicellulose. Xylose is probably the most sensitive monosaccharide but xylan is more resistant against sulphuric acid hydrolysis than araban. Therefore, it is possible that the arabinose-containing fraction of the hemicellulose is hydrolysed first followed by the xylose-containing fraction. After prolonged acid hydrolysis also some

TABLE 3.2. Monosaccharides obtained from faecal solids during hydrolysis with H_2SO_4 at 100 C.

Treatment	Monosaccharides, mg per g of dry solids			
	Arabinose	Xylose	Galactose	Glucose
1 N H_2SO_4, 2 h	205	124	10	1
1 N H_2SO_4, 6 h	129	173	21	17
2 N H_2SO_4, 2 h	106	145	15	4
2 N H_2SO_4, 6 h	121	145	18	16

glucose originating from cellulose may be liberated. The results of analyses of monosaccharides in hydrolysates of washed solids from pig faeces made by gas liquid chromatography are given in Table 3.2.

From these data it can be seen that the highest amount of arabinose was obtained after hydrolysis with 1 N H_2SO_4 for 2 h, the highest amount of xylose after 6 h hydrolysis with 1 N H_2SO_4. Due to the heterogeneous composition of plant cell walls, and the differences in sensitivity of the polysaccharides to hydrolysis, analysis of the hemicellulose fraction in a one step procedure is difficult to achieve. The best results are obtained by hydrolysis during varying periods of time, followed by a quantitative analysis of the monosaccharides formed.

3.3.4. *Cellulose*

Crystalline cellulose is solubilized by treatment with 72% sulphuric acid at 30 °C. After hydrolysis of the hemicellulose fraction, the residue of washed solids was subjected to the action of 72% sulphuric acid.

Fig. 3.13 shows the amounts of anthrone-positive material (hexose) and of residue, which consisted of lignin and ash, in relation to time of treatment. After only one hour all hexose-containing material was in solution and the residue became constant in weight. However, after a solubilizing time of one hour, only 70 mg of free glucose was measured with glucose oxidase, indicating that the major part of the cellulose fraction was present in solution as oligosaccharides. As is shown in Fig. 3.14 prolonged hydrolysis with 1.0 N H_2SO_4 of concentrated-acid-treated solids was necessary to free all of the glucose as monosaccharides. The difference between the values measured with the anthrone method and those measured with glucose oxidase were due to anthrone-positive material which was not present as glucose. In gas-liquid-chromatographic analyses of the hydrolysate, hemicellulose sugars could be detected.

3.3.5. *Lignin*

The lignin content of the solids estimated in the residue of the cellulose determination appeared to be constant already after 1 hour of hydrolysis at 30 °C (Fig. 3.13). Lignin determined in this way is called Klason lignin (SARKANEN and LUDWIG, 1971). Klason lignin measured in the presence of protein, as it is the case in freshly voided faeces, is contaminated with degradation products of

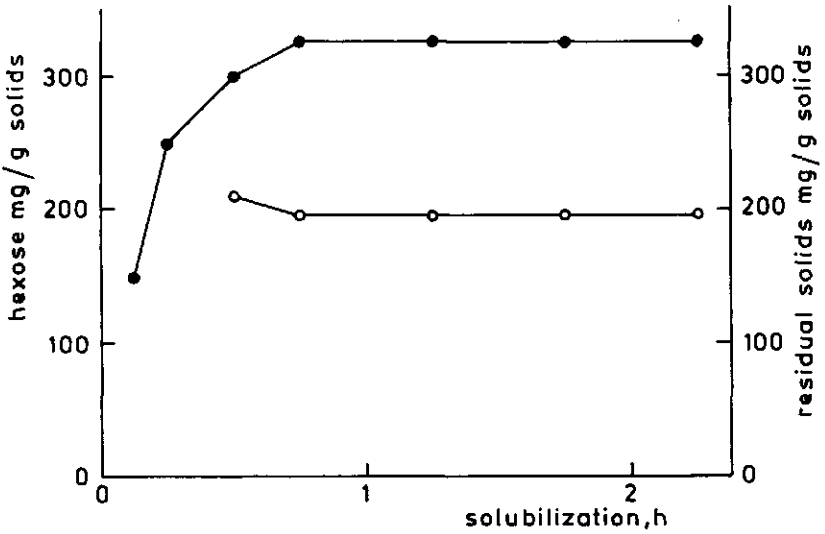

FIG. 3.13 Hydrolysis of the solids of faeces. Hexose expressed as mg glucose per g of solids (●——●) and mg residual matter (lignin + ash) per g of solids (○——○) after solubilizing the residue of the hemicellulose determination with 72% H_2SO_4.

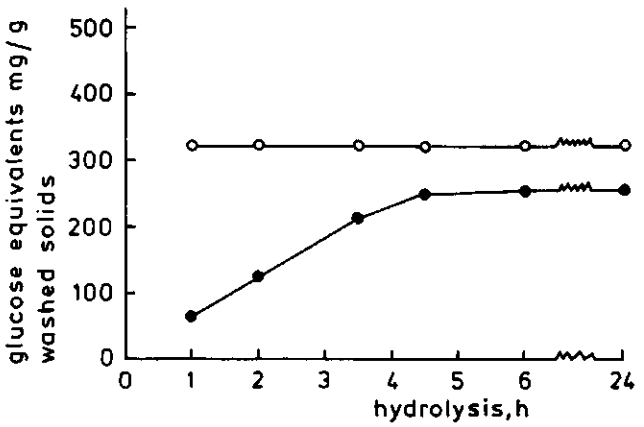

FIG. 3.14 Hydrolysis of the solids of faeces. Hexose (○——○) and glucose (●——●) per g of solids estimated after hydrolysis with 1.0 N H_2SO_4 of solubilized solids.

TABLE 3.3. Composition of freshly voided faeces and of three samples of washed solids.

	Freshly voided faeces	Sample I	Sample II	Sample III
Dry matter	20–25%	93%	93%	93%
and in % of dry matter				
Ash	18–20	2.8	6.6	3.3
Nitrogen	5	0.7	1.2	0.7
Lignin	24	16	19	17
Anhydroglucose	12	24	25	24
Anhydroxylose	7.5	16	15	20
Anhydroarabinose	5	18	18	19
Other sugars	1.5	3	3	3
Uronic acids	2	5	5	n.d.

protein. Therefore, the values found for lignin in analysis of freshly voided faeces were much too high.

3.3.6. *The composition of freshly voided pig faeces and washed solids*

The results of the previously described analyses are summarized in Table 3.3. The washed solids sample number I was prepared of the freshly voided faeces used for analysis. Samples II and III originated from other samples of faeces.

Klason lignin in freshly voided faeces was found to be 24 %. When the faeces were first treated with the neutral detergent solution of Van Soest (VAN SOEST, 1964) only 8 % lignin was found. Proteins are extracted by this method. Monosaccharides measured are given as anhydroglucose, -xylose and -arabinose because the sugars occur as polysaccharides in the original material.

3.4 *Concluding remarks*

The dry matter of freshly voided faeces from pigs consisted for about 35–40 % of insoluble residues of plant material. The remaining part, soluble material, sandy material and bacteria, were washed out in preparing the washed solids. The dry matter of these washed solids consisted for about 60–65% of polysaccharides, which were hydrolysable. Thus, approximately 25 % of the dry matter of faeces of pigs is a possible substrate for micro-organisms which can utilize the polysaccharides of the plant cell wall. This is in agreement with the data found by HOBSON and SHAW (1971). These investigators used the method of Van Soest which gives more reliable results than the crude fibre determination does; the former method is used by many other investigators.

The lignin content of the washed solids makes pretreatment of the raw material probably necessary because of its inhibitory effect on biodegradation of polysaccharides. The amount of lignin of 16–19 % in washed solids is relatively low compared to the amount of polysaccharides present, i.e. 60–65 %. Lignin itself is slowly degraded by micro-organisms giving low yields of biomass. The carbohydrate fraction of the solids appears to be the most suited substrate for micro-organisms, although it seems to be enclosed in a lignin matrix which has to be disrupted to enable a ready degradation of the cellulose and hemicellulose.

4. ISOLATION OF CELLULOLYTIC MICROORGANISMS AND MICROBIAL DEGRADATION OF SOLIDS OF PIG FAECES

4.1. INTRODUCTION

Media used for the isolation of cellulolytic microorganisms are in general composed of mineral salts with cellulose or cellulose derivatives as carbon and energy source. The techniques and media used are described by HARMSEN (1946), WINOGRADSKY (1949), CHARPENTIER (1968) and EGGINS and LLOYD (1968). The C-source usually applied is ground filter paper, cellulose powder, carboxymethylcellulose or cellobiose. Many problems are involved in the isolation of pure cultures of cellulolytic microorganisms. When using agar plates, cellulose degradation is sometimes inhibited by the presence of other metabolizable saccharides (CHARPENTIER, 1968). Recognition of cellulose degradation in the case of cell-bound cellulase is difficult for lack of clear zones in the agar plates. Little is known about the importance of synergism in cellulose degradation and isolation of microorganisms living in association is difficult (BEVERS and VERACHERT, 1974). RIVIÈRE (1961) described methods in isolating *Cytophaga* and *Vibrio* species by using antibiotics for eliminating overgrowing cellulose-degrading organisms. Identification of all of the organisms responsible for cellulose degradation, for example in soil, is no easy task. However, isolation of some fast-cellulose-decomposing organisms is relatively easy. EGGINS et al. (1968) observed dominant growth of *Trichoderma viride* and *Fusarium* spp. on a mineral medium with filter paper as C-source and soil as inoculum after twelve hours of incubation.

One of the problems in maintaining cellulolytic microorganisms in stock culture is the loss of cellulolytic activity. BEVERS and VERACHTERT (1974) reported methods to maintain cellulolytic organisms generally used by several investigators.

Analysis of the residues of plant cell walls in pig faeces showed the presence of 65% polysaccharides (Chapter 3). The presence of about 18% lignin and the ultrastructure of the polysaccharides may hinder total utilization of the material by cellulolytic microorganisms. To gain some information on the possibility of utilizing this material as a C-source, some isolated cellulolytic fungi and some fungi which are known to be good cellulose decomposers were screened for their capacity to utilize polysaccharides of washed solids of pig faeces.

4.2. RESULTS

4.2.1. *Isolation of cellulolytic microorganisms*

Isolation of cellulolytic microorganisms was performed as described in Chap-

ter 2. During isolation, fungi appeared as the predominant organisms on the cellulose medium as well as on the medium containing washed solids of faeces as the C-source. Under these isolation conditions no organism was isolated which was able to use lignin (Indulin AT) as a C-source.

About 40 fungal strains and 6 bacterial strains were isolated. All of the bacteria were Gram-negative rods. Although vibrio-like organisms were visible in the enrichment culture, isolation of these organisms failed. Four of the isolated bacteria lost their cellulolytic activity after storage for a few weeks on agar slopes. The other two appeared to be much slower cellulose decomposers as compared with the fungi. They formed yellow, smooth colonies on agar plates and probably belong to the genus *Cellulomonas*.

Of the forty isolated fungi, nine were selected for their fast growth on cellulose. Strains C 191, C 192 and C 193 were derived from compost, C 320, C 321, C 322 from soil and Mh 4, Mh 7 and Mh 8 from manure. Strains C 191, C 320 and Mh 8 lost their cellulase activity a few months after isolation. Strain C 193 was identified as a basidiomycete, C 321 as *Humicola grisea*, Mh 7 as *Humicola fuscoatra* while identification of strain Mh 4 failed by lack of sexual reproduction. Identification was performed by the Centraal Bureau voor Schimmelcultures. Among the other 31 strains were *Sporotrichum*, *Fusarium* and *Aspergillus* spp. but these organisms decomposed cellulose very slowly.

4.2.2.1. Growth of cellulolytic fungi on different C-sources

Table 4.1 contains the results of an experiment on the growth of different fungi on the monosaccharides D-xylose, L-arabinose, D-mannose and D-glucose and the dissaccharide lactose. Xylose, arabinose, mannose and glucose are considered to form the larger part of the monosaccharides liberated on hydrolysis of plant cell wall polysaccharides. Lactose, a dissacharide consisting of glucose and galactose linked together by β-1,4 bonds, might be a cellulase inducer. Some fungi hardly used arabinose and lactose as C-source. MANDELS (1975), inoculating with a spore suspension, observed a lag phase of 80 h when growing *Trichoderma viride* on lactose or L-arabinose and observed also a slower growth rate with these sugars. Except for strain C 191, all of the fungi tested readily used glucose, xylose and mannose as C-source.

The results of growth experiments with different polysaccharides are given in Table 4.2. Carboxymethylcellulose and gum arabic in concentrations of 10 g/l make the growth medium very viscous. Therefore, when these compounds were supplied, the medium was diluted four times with a 0.05 M phosphate buffer solution (pH 5.0). However, the medium with 2.5 g gum arabic per l was still very viscous and the generally poor growth may have been due to the low oxygen transfer rate resulting from the high viscosity. Strain C 191 appeared no longer to be able to use cellulose as carbon and energy source. The strains C 193 (the basidiomycete), and Mh 4, and *Penicillium nigricans*, *Myrothecium verrucaria* and *Trichoderma viride* QM 9414 utilized the polysaccharides xylan, gum arabic, mannan, CMC and filter paper. These fungi also readily used xylose, arabinose, mannose and glucose, the most important sugar building stones of the polysac-

TABLE 4.1. Growth[1] of cellulolytic fungi on mono- and disaccharides using mycelium cultivated on glucose as inoculum[2] [3])

Fungus	Carbon source				
	D-xylose	L-arabinose	D-mannose	D-glucose	Lactose
C 191	±	−	−	+ + +	+
C 192	+ + +	±	+ + +	+ + +	±
C 193 (basidiomycete)	+ + +	+ + +	+ + +	+ + +	±
C 320	+ + +	±	+ + +	+ + +	+ + +
C 321 *Humicola grisea*	+ +	−	+ +	+ +	−
C 322	+ + +	±	+ +	+ +	±
Mh 4	+ +	+ +	+ + +	+ +	±
Mh 7 *Humicola fuscoatra*	+ +	±	+ + +	+ + +	±
Mh 8	+ +	+	+ + +	+ + +	±
Chaetomium globosum	+ + +	±	+ + +	+ + +	±
Penicillium nigricans	+ + +	+ + +	+ + +	+ + +	+ ±
Myrothecium verrucaria	+ + +	+ + +	+ + +	+ + +	±
Trichoderma viride QM 6a	+ + +	+ +	+ + +	+ + +	+ +
Trichoderma viride QM 9123	+ + +	+ +	+ + +	+ + +	+ +
Trichoderma viride QM 9414	+ + +	+	+ + +	+ + +	+ +

TABLE 4.2. Growth [1] of cellulolytic fungi on soluble polysaccharides using mycelium cultivated on cellulose as inoculum[2] [3]).

Fungus	Carbon source					
	Xylan	Gum arabic	Mannan	C.M.C.	Filter paper	Lignin
C 191	±	−	±	−	−	−
C 192	+	±	+ +	+ + +	+	−
C 193 (basidiomycete)	+ +	±	+ + +	+ + +	+	−
C 320	+	±	+ + +	+	+	−
C 321 *H. grisea*	±	−	+ +	+ + +	−	−
C 322	+	±	+	+ + +	−	−
Mh 4	+ + +	+ +	+ + +	+ +	+	−
Mh 7 *H. fuscoatra*	+ +	±	+ + +	+ + +	+	−
Mh 8	+ +	+ +	+ + +	+ + +	+	−
C. globosum	+	±	±	+ + +	+	−
P. nigricans	+ + +	±	+ + +	+ + +	+	−
M. verrucaria	+	±	+ + +	+	+	−
T. viride QM 6a	+	±	+	+ + +	+	−
T. viride QM 9123	+	±	+ +	+ + +	+	−
T. viride QM 9414	+	±	+ +	+ + +	+	−

[1]) − No visible growth after 96 h of incubation; ± slight growth after 96 h; + maximum growth after 96 h; + + maximum growth after 73 h; + + + maximum growth after 48 h.
Filter paper: − no digestion of paper after 7 days; + paper digested after 7 days.
[2]) All reported values are averages of at least five experiments.
[3]) For basal medium see Chapter 2; Temp. 28°C.

charides of the plant cell wall (Table 4.1). Therefore, these five fungi were selected for comparing their capacities to use the solids of pig faeces as carbon and energy sources.

4.2.2.2. Utilization of washed solids from pig faeces as C- and energy source by five selected fungi

These experiments were performed as described in Chapter 2. Instead of 15 g solids per l culture medium only 5 g was added to study the degradation of the lignin-polysaccharides complexes. Table 4.3 shows the weights of the mixtures of residual solids of faeces and produced mycelium after different periods of incubation.

No strain was able to break down the solids of faeces to such an extent that less than 3.5 g/l residual solids plus mycelium was left.

Table 4.4 gives the amounts of nitrogen in the mixtures of solids and mycelium. Theoretically the amount of solids which is not utilised may be expressed by the following formula:

$$W_r = W_t - 100.\frac{N_r}{k} \qquad (1)$$

where

W_r = weight of residual solids (g) at time t

W_t = weight of the mixture of solids and mycelium (g) at time t

N_r = nitrogen (g) in the mixture of solids and mycelium

k = percentage of nitrogen in mycelium

The nitrogen content of the mycelium is dependent on the type of fungus, C-source, C/N ratio, pH, aeration of the nutrient medium and temperature (SCHELLART, 1976). RHODES et al. (1961) determined the nitrogen content of a large number of fungal strains, grown in pure culture with glucose as C-source and found the highest N-content (9 %) in a strain of *Trichoderma*. SOLOMONS (1973) reported a fungal N-content of 11.8 % but did not mention the name of the fungus. When the nitrogen contents of mycelia of the strains used in this experiment are assumed to be about equal, it can be seen that *Penicillium*, *Myrothecium* and *Trichoderma* are the best decomposers of solids of pig faeces (cf Table 4.5).

The data of Table 4.5 show that the maximum decrease of solids was reached within five days of incubation. Strain C 193 formed a relatively high amount of mycelium within three days of incubation with solids but after a longer period cell lysis probably occurred as is demonstrated by the low values of assimilated nitrogen (Table 4.4). It may be concluded that in the described experiments approximately 40 % of the washed solids of pig faeces was utilized as carbon and energy source by the fungi tested.

To make a comparison of sugars produced in excess and sugars consumed by

TABLE 4.3. Weights of the mixtures of residual solids of pig faeces and mycelium expressed as g per l culture liquid after different periods of incubation in a medium with washed solids as C-source[1]) [2])

Fungus	Days			
	3	5	7	9
C 193 (basidiomycete)	4.1	3.9	3.6	3.5
Mh 4	4.3	3.8	3.7	3.7
P. nigricans	4.3	3.8	3.8	3.7
M. verrucaria	4.0	3.5	3.5	3.5
T. viride QM 9414	4.2	3.7	3.6	3.7
Uninoculated medium	5.0	5.0	5.0	5.0

[1]) All reported values are averages of at least five experiments.
[2]) For basal medium see Chapter 2; temperature 28°C.

various cellulolytic fungi growing in solids-containing nutrient medium, residual sugar in the culture liquid was estimated. Sugars assimilated were calculated from the data of Table 4.5 assuming that 50% of the consumed polysaccharides were assimilated.

From the results obtained (Table 4.6) it can be seen that the amounts of reducing sugars produced in excess by fungi and accumulating in the culture filtrates differed widely for different fungi. By far the largest amounts were produced by *T. viride* strain QM 9414 which produced high amounts of reducing sugars already after three days of incubation. With prolonged incubation time the amount of soluble sugar rose considerably.

Trichoderma QM 9414 used in these experiments was selected for its high level of free cellulase produced in the culture medium and not for its growth rate (MANDELS et al., 1971). The four other fungi tested seemed to use the products of enzymic action immediately and almost no accumulation of reducing sugars

TABLE 4.4. Nitrogen expressed as mg per g of insoluble material (solids + mycelium) after different periods of incubation in a medium with washed solids as C-source[1]) [2])

Fungus	Days			
	3	5	7	9
C 193 (basidiomycete)	15.9	15.4	9.7	2.9
Mh 4	3.5	11.8	10.0	7.0
P. nigricans	17.4	18.4	18.4	16.2
M. verrucaria	12.5	14.3	12.9	12.5
T. viride QM 9414	11.9	17.5	16.7	13.5
Uninoculated medium	0	0	0	0

[1]) All reported values are averages of at least five experiments.
[2]) For basal medium see Chapter 2; temp. 28°C.

TABLE 4.5. Residual solids of pig faeces in g per l culture liquid after different periods of incubation in a medium with 5 g/l washed solids as C-source.

Fungus	Days			
	3	5	7	9
C 193	3.3	3.2	3.2	3.4
Mh 4	4.1	3.3	3.3	3.4
P. nigricans	3.4	3.0	3.0	3.0
M. verrucaria	3.4	2.9	3.0	3.0
T. viride QM 9414	3.6	2.9	2.9	3.1

TABLE 4.6. Soluble reducing sugar (mg/l) produced and acid-hydrolysable polysaccharides (mg/l) assimilated during growth on a medium with solids of pig faeces as C-source.

Fungus	Days							
	3		5		7		9	
	Soluble	ass.	soluble	ass.	soluble	ass.	soluble	ass.
C 193	1	850	4	900	10	900	18	800
Mh 4	0	450	6	850	7	850	9	800
P. nigricans	3	800	28	1000	25	1000	21	1000
M. verrucaria	9	800	28	1050	19	1000	10	1000
T. viride QM 9414	54	700	70	1050	88	1050	106	950

TABLE 4.7. Lowry protein in culture filtrates of fungi growing on solids of pig faeces expressed as µg serum albumin per ml.

Fungus	Days			
	3	5	7	9
C 193	40	40	100	170
Mh 4	50	50	70	170
P. nigricans	250	240	300	350
M. verrucaria	310	290	250	210
T. viride QM 9414	180	300	410	550

occurred in the medium. So far no explanation can be given of the high sugar concentration in the culture medium of *T. viride* when growing on solids from pig faeces. As shown earlier, the organism readily responds to the component sugars of cellulose when supplied in the free state.

To gain some information about the protein-containing soluble cellulolytic enzymes produced, soluble Lowry protein was estimated in the various culture filtrates on cellulose medium (Table 4.7).

The high levels of soluble protein produced by *T. viride* explain why this organism was selected for cellulolytic activity. *P. nigricans* and *M. verrucaria* produced also considerable amounts of soluble protein especially in the first day

of growth. Strain Mh 4 produced only one fifth of the amount of soluble protein produced by *Trichoderma*. The protein measured in culture filtrates of strain C 193 after prolonged incubation originated probably from lysis.

4.3 Conclusions and discussion

Fungi appeared to be the predominant cellulolytic organisms that were isolated by the described procedure. No strain capable of using Indulin AT (lignin) as a carbon and energy source was isolated. Indulin AT may differ from natural lignin by modifications that took place during preparation and such modifications may influence biodegradability. The five selected strains, which all grew well on various mono- and polysaccharide substrates, utilized washed solids of pig faeces for growth. Residual mixtures of solids of faeces + mycelium left after growth did not differ very much, but assimilated nitrogen in mycelium or in soluble protein appeared to be the highest in *Penicillium*, *Myrothecium* and *Trichoderma*.

No selected strain was able to degrade completely the washed solids of pig faeces. *T. viride* appeared to produce active cellulase and therefore this strain was chosen for further experiments. Under the growth conditions as described above only 40 % of the washed solids was degraded. Lignin and polysaccharides protected by lignin against degradation were probably left. It might be possible by physical, chemical or enzymic methods to disrupt the lignin matrix, eliminating its protecting action. The aim of further experiments was to make a larger part of the washed solids available to *T. viride*.

5. GROWTH OF *TRICHODERMA VIRIDE* ON WASHED SOLIDS OF PIG FAECES

5.1. INTRODUCTION

The solids fraction of pig faeces consists of cellulose, hemicellulose and lignin (Chapter 3) and can serve as energy source for cellulolytic microorganisms (Chapter 4). As was reported in Chapter 1, several microorganisms have the ability to grow on cellulose and cellulose-containing materials. *T. viride* appeared to be most suitable in producing cellulase with the ability of effective hydrolysis of native cellulose as was demonstrated by MANDELS and WEBER (1969). HAN and ANDERSON (1975) studied *T. viride* in order to improve the feed quality of rye-grass straw. They observed a three-fold increase of crude protein content. GRIFFIN et al. (1974) investigated cellulase production and growth of *T. viride* in feedlot waste. The fungus utilized two thirds of the carbohydrates of this material producing cellulase with properties comparable to commercial preparations. The fermented waste which was essentially odour-free contained all of the original nitrogen but had 24% less organic matter. PEITERSEN (1975b) studied cellulase and protein production by *T. viride* growing on NaOH-treated barley straw. Production of cellulase and microbial protein was investigated at straw concentrations of 1–2%. Maximum enzyme yields were reached after 4–10 days. The protein content of the product was 21–26% and up to 70% of the straw was utilized. The yield constants were calculated to be 0.40–0.56, i.e. of the same order as those which can be obtained by growing the fungus on glucose.

T. viride grows readily on a carbon source and nutrient salts (MANDELS and WEBER, 1969). Cellulolytic enzymes of this fungus are produced when the organism is grown on cellulose, sophorose, lactose or cellobiose. NISIZAWA et al. (1972) reported that glucose strongly represses enzyme production in the presence of cellulose or other inducers. High yields of cellulolytic enzymes are obtained when the fungus is grown on pure cellulose or on complex cellulosic materials (MANDELS and STERNBERG, 1976). MANDELS and REESE (1964) suggested that cellobiose is the true inducer in a medium supplied with cellulose, but that yields of cellulolytic enzymes are low when *Trichoderma* is grown on cellobiose because the synthesis of these enzymes is repressed and/or inactivated by the rapid hydrolytic degradation of the sugar to glucose. When the fungus is growing on cellulose, cellobiose is slowly released so that repression and inactivation normally do not occur. However, in the investigation of MANDELS and STERNBERG (1976) slow addition of cellobiose to a *Trichoderma* culture did never increase yields of cellulolytic enzymes and the question is still open whether cellobiose is in fact the inducer or whether the true inducer is produced during growth.

MANDELS et al. (1974) pointed out that conditions for maximum production of cellulolytic enzymes are not the same as conditions for optimum growth. Growth

was more rapid at 30 °C but enzyme yields were higher at 25 °–28 °C. Addition of peptone at one-tenth the cellulose level stimulated growth and enzyme production. When the pH was adjusted at 4.8, it was found that the less the pH was allowed to fall below this value the less enzyme was produced although growth was stimulated. A possible explanation of this effect is that at pH values close to the optimum of growth of the fungus (4.8), cellulolytic enzymes are more efficient in releasing soluble sugar which in turn will lead to catabolite repression of the enzymes.

A cellulose content of 0.75 % has been reported to be the optimum concentration for the production of cellulolytic enzymes by *T. viride* (MANDELS and WEBER, 1969). However, these experiments were carried out in shaking flasks without pH control. As was shown by STERNBERG (1976), uncontrolled pH may fall to 2.5 in a medium containing 2 % cellulose. Under such acid conditions growth of *T. viride* is extremely slow (BROWN et al., 1975) and cellulolytic enzymes are inactivated. By preventing the pH from dropping below 3.0, *T. viride* can be cultured successfully in a medium containing 2 % cellulose.

5.2. RESULTS

5.2.1. *Growth of* T. viride *on glucose, xylose, arabinose and cellobiose*

The larger part of the solids of pig's faeces consist of polymers of glucose, xylose and arabinose (Chapter 3). When *T. viride* grows on these solids, the extracellular enzymes of the fungus hydrolyse the polysaccharides and produce glucose, cellobiose, xylose and arabinose. As is shown in Fig. 5.1, glucose, xylose and cellobiose are consumed at the same rate by the fungus, but arabinose is consumed slower. Acidification of the medium due to the consumption of NH_4^+ is directly related to the rate of sugar consumption and to the growth rate. After the cessation of growth, the pH rises again.

When *T. viride* spores are inoculated into glucose medium there is a lag phase of 10–20 hours during which germination of the spores occurs and growth of the

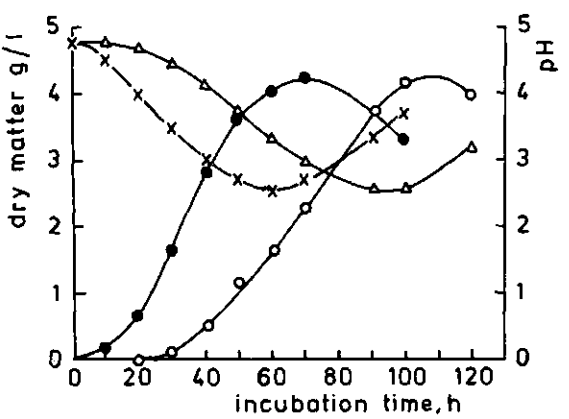

FIG. 5.1 Growth of *T. viride* in shaking flasks on basal medium with different carbon sources (10 g/l). Spore inoculum, temp. 29°C. ●——● growth on either D-glucose, D-xylose or cellobiose, ○——○ growth on L-arabinose, x——x pH of the culture liquid with either glucose, xylose or cellobiose, △——△ pH with arabinose.

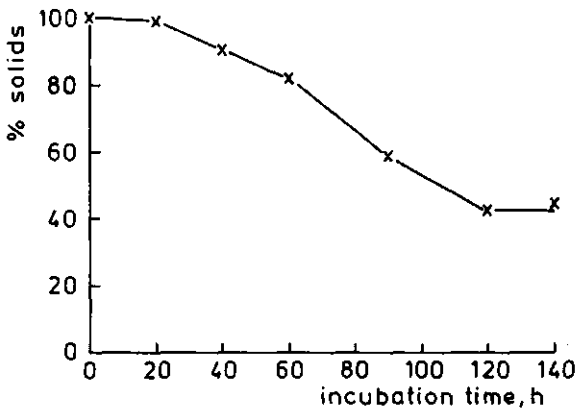

FIG. 5.2 Residual solids (cellulose + mycelium) expressed as percentage of initial substrate in the culture medium of *T. viride* growing on Avicel (10 g/l). Mycelial inoculum, temp. 28°C.

fungus initiates. Hereafter growth proceeds fast yielding about 0.4 g of mycelium per g of glucose. Maximum yield is obtained after 60–70 h where upon lysis occurs. The rise of pH after cessation of growth is possibly due to the appearance of protein degradation products in the medium following cell lysis.

5.2.2. *Growth of* T. viride *and production of cellulolytic enzymes on Avicel*

T. viride was cultivated in a Biotec fermentor under controlled conditions concerning pH, air flow and stirring rate as described in Chapter 2. Avicel, a microcrystalline cellulose powder, was added as carbon source and production of protein and cellulolytic enzymes were followed during growth. Fig. 5.2 gives the course of insoluble residue (residual cellulose + mycelium) during growth. At t = 0, ten g of cellulose powder was present in the medium. When the fungus developed and consumed the cellulose, the original insoluble cellulose was partly replaced by biomass. After 120 h of incubation the insoluble solids present consisted of a mixture of unfermented cellulose and mycelium. No attempt was

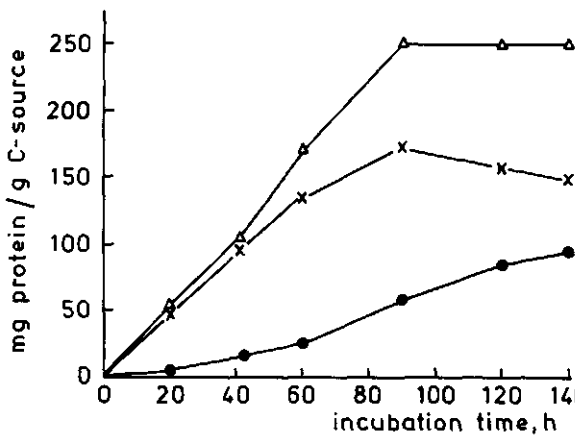

FIG. 5.3 Protein in mg/g of carbon source supplied, produced by *T. viride* during growth on Avicel (10 g/l). Mycelial inoculum, temp. 28°C. ●——● soluble Lowry protein, x——x insoluble crude protein, △——△ total protein calculated from insoluble + Lowry protein.

made to estimate the amount of mycelium separately but as was visible by microscopic examination, only little cellulose was left.

After 90 h of incubation the amount of crude protein contained in the residual solids (cellulose + mycelium) amounted to 150 mg, calculated per g of initially added C-source (Fig. 5.3). Total crude protein on Avicel amounted to about 250 mg per g of added cellulose (Fig. 5.3) which is in good agreement with the values given by MANDELS and STERNBERG (1976). It is difficult to estimate mycelial weight from the protein values. PEITERSEN (1975a) stated that the mycelium of *T. viride* grown on cellulose consists of 50 % crude protein. RHODES et al. (1961) determined the nitrogen content of a large number of fungi and found the highest N-content (9 %) in a strain of the genus *Trichoderma*. However, it is often difficult to make an exact comparison of the composition of several fungal species by using literature data because of the variation in growth conditions. Protein content of mycelium depends among other things on the carbon source. When the carbon compound is hard to metabolize, the amount of protein in the biomass is decreased. Under suitable growth conditions 50 % of the original C-source might be converted into mycelium.

Utilization of an insoluble carbon source like cellulose requires extracellular enzymes for hydrolysing the polymer. It is unknown if the amount of cellulolytic enzymes in the culture liquid is correlated with the amount of soluble nitrogen and the ratio insoluble/soluble nitrogen. As can be seen from Fig. 5.3, almost 20 % of the total crude protein was present in the soluble form after 90 h of incubation. At that time growth ceased but soluble protein still increased. The latter material is hardly collectable in contrast to the insoluble mycelial protein which is easily separated from the culture medium by filtering. In the case of solids of faeces the ratio insoluble/soluble nitrogen was considerably lower (Figs. 5.6 and 5.9). As to the enzymic proteins in the culture medium MANDELS et al. (1971) found that a solution with a filter paper activity (FPA; 2.5.14) of 4 contained 1.0 mg of protein per ml which is in good agreement with the values given in Fig. 5.4.

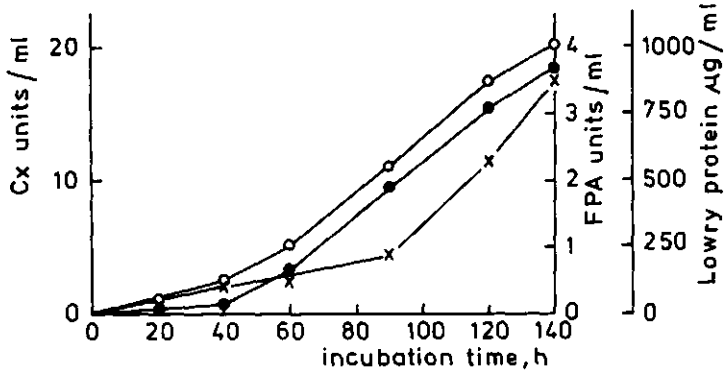

FIG. 5.4 Cellulolytic activities and soluble protein (µg/ml) in the culture medium of *T. viride* growing on Avicel (10 g/l). Mycelial inoculum, temp. 28 °C. x——x C_x units, ●——● FPA units, ○——○ Lowry protein.

The endoglucanase activity (C_x; 2.5.13) and the filter paper activity are produced approximately concomitantly. The larger part of the enzymic activity is set free into the culture medium after 90 h of incubation. Prior to this time the enzymic activity apparently is bound to the mycelium and the substrate.

5.2.3. *Growth of* T. viride *and production of cellulolytic enzymes on ground solids of pig faeces*

T. viride was grown in a Biotec fermentor on washed pig faeces. The washing procedure is described in Chapter 2. After washing, the faeces were ground by a hammer mill to pass a 0.2 mm sieve which made them much easier to utilize by the fungus. Instead of 10 g of cellulose per l, 15 g of solids of pig faeces was added as the carbon source to the basal medium. In Chapter 3 it was demonstrated that the solids of faeces consisted for 65 % of polysaccharides. *T. viride* is unable to use lignin as the C-source so that all of the carbon must be derived from the polysaccharide fraction of the solids. Fig. 5.5 gives the course of residual solids during incubation. Comparison of these values with the data of Fig. 5.2 shows two remarkable facts. First, growth of *T. viride* on solids of pig faeces started earlier than that on pure cellulose. This might have been due to the presence of some easier hydrolysable constituents of the solids like hemicellulose and non-crystalline cellulose. Secondly, growth on solids of faeces decreased already after 40 h of incubation when the carbon source seemed to be exhausted. It is a well-known fact that lignin can interfere with the hydrolysis of other cell wall constituents and this might have been the reason why growth decreased after 40 h of incubation.

In Fig. 5.6 the amounts of crude protein produced by *T. viride* during growth on ground solids are plotted. Protein was calculated per g 'acid-hydrolysable' carbon source, which means per gram of hemicellulose and cellulose contained in the solids added to the basal medium. Only 100 mg of crude protein was formed of which the larger part (60 %) was in the soluble form. Fig. 5.7 gives C_x units and FPA units produced in the culture medium by *T. viride* when the fungus was

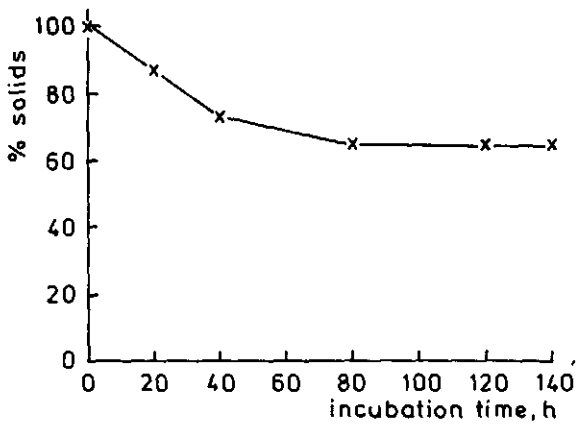

FIG. 5.5 Residual solids + mycelium as % of initial substrate in culture medium of *T. viride* growing on solids of faeces (15 g/l). Mycelial inoculum, temp. 28°C.

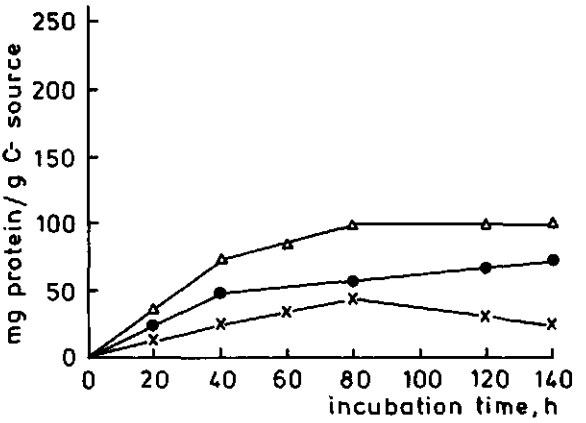

FIG. 5.6 Protein (mg per g cellulose + hemicellulose present in the solids supplied) produced by *T. viride* during growth on solids of faeces (15 g/l). Mycelial inoculum, temp. 28°C. x——x insoluble crude protein, ●——● soluble Lowry protein, △——△ total protein calculated from insoluble + Lowry protein.

grown on ground solids. Final activities were only 30% of the activities reached when Avicel was the C-source.

From the results obtained it is concluded that washed and ground solids of pig faeces represent a poor carbon source for *T. viride*. This is probably due to the presence of lignin which to a certain degree protects cellulose against a ready degradation by cellulolytic enzymes.

5.2.4. *Growth of* T. viride *and production of cellulolytic enzymes on NaOH-treated solids*

In order to investigate the influence of lignin on hydrolysing pig faeces by *T. viride*, washed and ground solids were treated with 0.25 N NaOH. As described by STONE et al. (1965), HAN and CALLIHAN (1974) and PEITERSEN (1975b), alkali treatment of cellulosic materials improves enzymic hydrolysis. Some hemicellulose and a larger part of the lignin is dissolved. The effect of alkali treatment

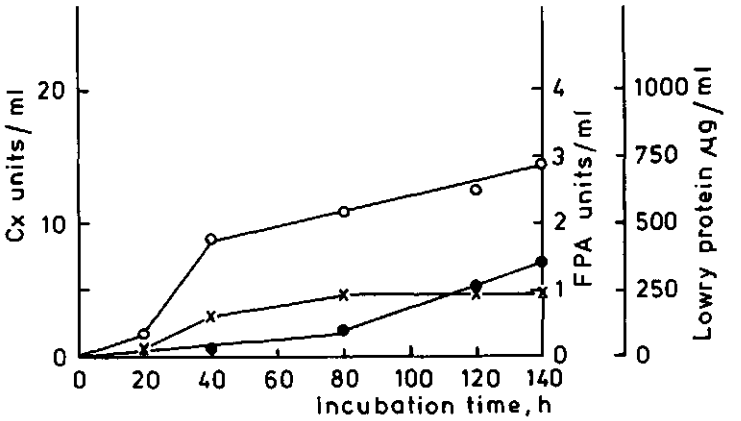

FIG. 5.7 Cellulolytic activities and soluble protein (μg/ml) in the culture medium of *T. viride* growing on solids of faeces (15 g/l). Mycelial inoculum, temp. 28°C. x——x C_x units, ●——● FPA units, ○——○ Lowry protein.

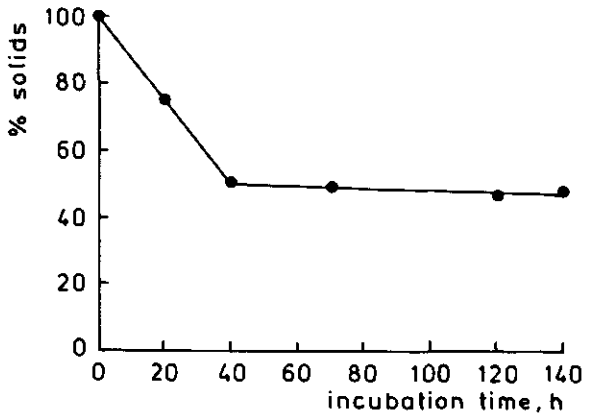

FIG. 5.8 Residual solids + mycelium expressed as percentage of substrate initially added to the culture medium of *T. viride* growing on NaOH-treated solids of faeces (10 g/l). Mycelial inoculum, temp. 28°C.

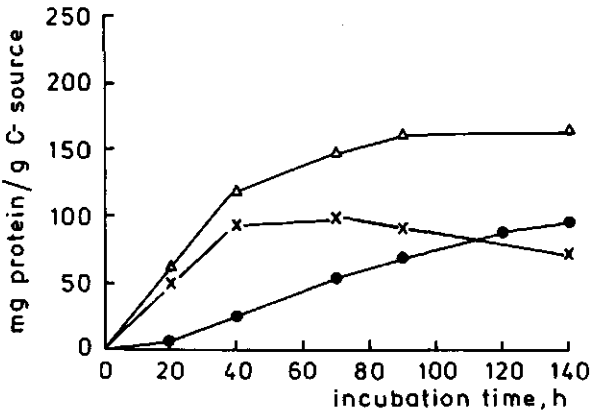

FIG. 5.9 Protein (mg/g of carbon source supplied) produced by *T. viride* during growth on NaOH-treated solids of faeces (10 g/l). Mycelial inoculum, temp. 28°C. ●——● soluble Lowry protein, x——x insoluble crude protein, △——△ total protein calculated from insoluble + Lowry protein.

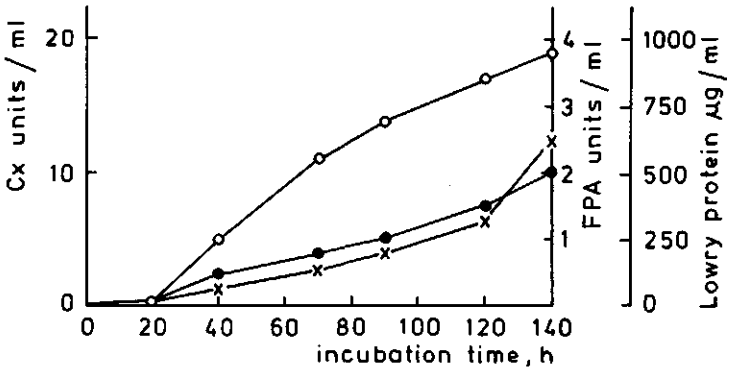

FIG. 5.10 Cellulolytic activities and soluble protein (µg/ml) in the culture medium of *T. viride* growing on NaOH-treated solids of faeces (10 g/l). Mycelial inoculum, temp. 28°C. x——x C_x units, ●——● FPA units, ○——○ Lowry protein.

depends on the kind and age of the plant material. In general, 1–4 % solutions of NaOH or NH_4OH are used at temperatures of 100 °C. In this study 0.25 N NaOH and a boiling time of 10 min. were applied. After boiling and cooling, the solids were collected by spinning down and washing until they were free of NaOH. Ten g of the modified product was added as carbon source per l of basal medium. As is shown in Fig. 5.8, up to 50 % of the original solids disappeared within the first 40 hours of incubation with *T. viride*. Growth was faster as compared to that with pure cellulose (cf Fig. 5.2) which was probably due to the presence of easily hydrolysable polysaccharides. Fig. 5.9 shows the total crude protein formed by *Trichoderma* which reached a maximum of 160 mg per g of initially added carbon source after 80 h.

Protein production on NaOH-treated solids proceeded similarly to that on Avicel during the first 40 h. The ratio soluble protein/insoluble protein was increasing with decreasing availability of the polysaccharides in the substrate, as can be seen from Figs. 5.3, 5.6 and 5.9. Avicel is probably the most suitable carbon source, whereas only a relatively small part of ground solids of pig faeces was readily hydrolysed. NaOH treatment improved the hydrolysis of the solids. However, the solids are only partly accessible to the enzymes and, thus, to hydrolysis. As is shown in Fig. 5.10, the cellulolytic activity of the culture liquid of *T. viride* grown onNaOH-treated solids is intermediate between the activities of the spent medium when Avicel and untreated ground solids were used as the carbon source (Figs. 5.4 and 5.7). This may have been due to quantitative or qualitative differences of the enzymes involved.

5.3. Conclusion

T. viride metabolizes glucose, xylose and arabinose but growth on solids of pig faeces is poor. The fungus produces extracellular cellulolytic enzymes to convert the insoluble carbon source into metabolizable monosaccharides. This extracellular protein is hard to collect, due to its soluble state. The ratio soluble/insoluble protein was found to be lowest with Avicel as the carbon source and highest with untreated ground solids. During the growth of *T. viride* on solids of faeces mycelium is mixed with the residual carbon source. After completion of growth of this fungus the remaining part of the faeces solids consists of hardly hydrolysable material. Mycelium grown on solids and mixed with their residues is probably suitable as protein source for animal feedstuff. This conception is in agreement with the results of Peitersen (1975b) who cultivated *Trichoderma* on NaOH-treated straw and in preliminary feeding studies fed the produced protein to rats. The protein contained no toxic compound, but due to the high content of lignin it was not fully utilized.

The mycelium was growing on and between the particles of the substrate and separating the two was not possible. Pretreatment of the faeces solids with NaOH was not sufficient to produce a carbon source for *T. viride* which was of the same quality as that of Avicel. Grounding proved to be insufficient as

pretreatment. The percentage of fungus protein in solids after 90 h of incubation appeared to be 30 % when Avicel was the C-source, 20 % when NaOH-treated solids of faeces were used and only 10 % when ground solids of faeces were the carbon source in the culture medium.

6. HYDROLYSIS OF PIG FAECES BY CELLULASE FROM *TRICHODERMA VIRIDE*

6.1. INTRODUCTION

As was demonstrated in Chapter 5, production of microbial protein from solids of pig faeces is not very rewarding. A process which makes it possible to dissolve the hydrolysable part of the substrate seems to be more attractive. The use of solids of faeces as substrate for the production of microbial protein is simplified if first the polysaccharides of the solids are hydrolysed to monosaccharides. Separation of the unhydrolysable part of the solids can be performed by filtration when the hydrolysable polysaccharides are converted into monosaccharides. The sugar solution obtained this way can be used for microbial protein production but also for chemical or microbial production of ethanol and other useful chemicals. Hydrolysis of the polysaccharides can be performed by acid or enzymic action. Conversion of cellulose into glucose by enzymic methods was

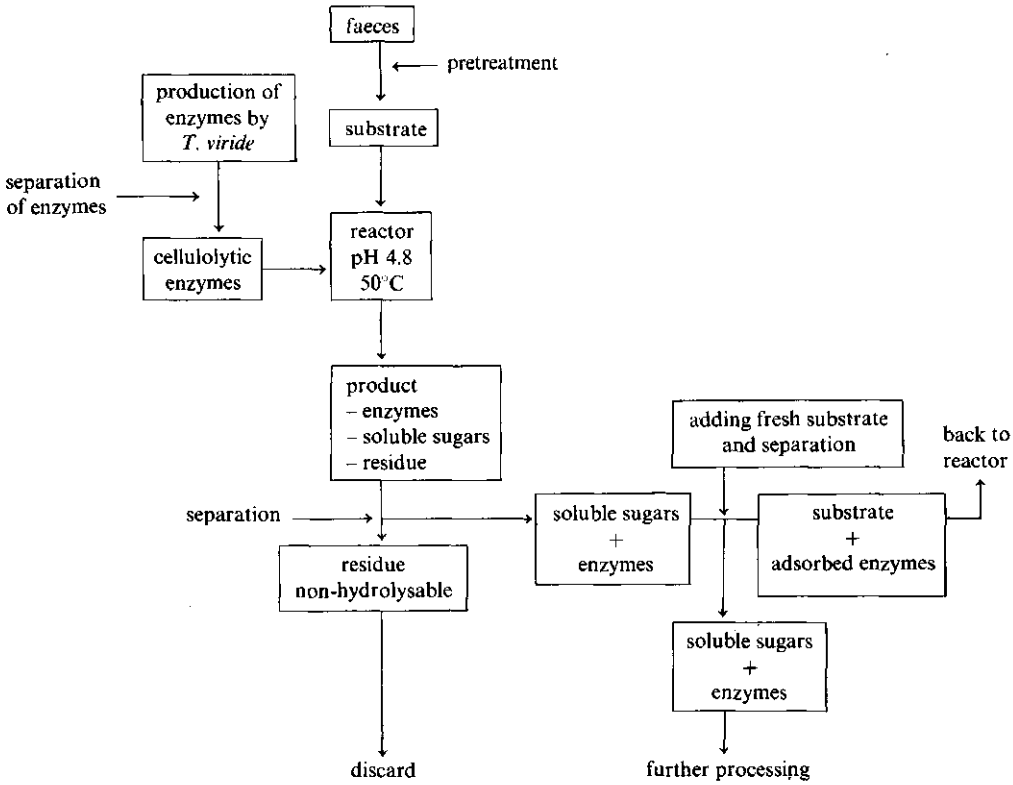

FIG. 6.1. Outline of the conversion of polysaccharides of faeces into soluble sugars.

extensively studied by GHOSE and KOSTICK (1960), GHOSE (1969), MANDELS and KOSTIC (1973) and REESE (1976). Enzymic hydrolysis has some advantages as compared to acid hydrolysis. When using acid, corrosion-free apparatus are necessary. The crystalline structure of cellulose needs high temperatures and high acid concentrations for hydrolysis; under these conditions sugar decomposition is inevitable. Enzymic hydrolysis proceeds optimally at 50 °C, at atmospheric pressure and at a pH of 4.8; no losses of liberated sugars occur. An outline of the process is given in Fig. 6.1.

A necessary step in the process as given in Fig. 6.1 is the production of an enzyme solution of sufficient strenght to hydrolyse the solids of the faeces. Production of cellulolytic enzymes by fungi, especially *T. viride* has extensively been studied during the last ten years. Recent review articles were written by MANDELS (1975), WOOD (1975), STERNBERG, (1976) and NYSTROM and ALLEN (1976). Cellulase of *T. viride* is by far the best cellulolytic enzyme system to convert insoluble crystalline cellulose into soluble saccharides. *Trichoderma* cellulase contains high levels of C_1 and C_x activities which are needed to hydrolyse crystalline cellulose.

ANDREN, ERICKSON and MEDEIROS (1976) described the results of hydrolysis of cellulose-containing waste materials. In that study an enzyme solution was used which was obtained by growing *T. viride* on pure cellulose. The enzyme solution was needed to hydrolyse materials which contained hemicellulose in addition to cellulose. Cellulase consists of inducible enzymes (MANDELS and REESE, 1960) and the same is true of hemicellulases (Chapter 1). Therefore, in the experiments described in the present study, the enzymes were produced by growing *T. viride* in a medium which contained as carbon source solids of faeces ground to pass a 0.08 mm sieve.

The investigations described in this chapter included the influence of incubation time, concentration of enzyme solution and concentration of substrate on the hydrolysis by *T. viride* cellulase of solids of pig faeces.

In hydrolysing faeces, adsorption of cellulolytic enzymes on the substrate is necessary. HUANG (1976) reported the adsorption of 50% of the protein of an enzyme solution on cellulosic substrates within 15 minutes. The adsorbed protein was slowly set free into the solution when monosaccharides were produced. REESE (1976) stated that 90% of the cellulolytic enzymes were adsorbed on pure cellulose and liberated upon hydrolysis of the polymer. Therefore, it is possible to re-use the enzymes in the proposed process by re-adsorption of the used enzymes to fresh substrate.

In this chapter also experiments are described concerning the composition of the enzyme complex produced by *T. viride* when growing on the solids of faeces. As is mentioned by MANDELS and STERNBERG (1976), cellulase is a very stable enzyme. Spent culture solutions can be kept indefinitely under refrigeration and little activity is lost during prolonged digestion at 50 °C. Culture filtrates can be rapidly concentrated by the use of ultrafiltration membranes, vacuum evaporation or precipitation by 66% acetone with no loss of activity.

Hydrolysis of cellulose-containing materials is difficult due to the insolubility

of the cellulose, the high degree of crystallinity and the presence of lignin that restricts access of the enzyme to the glycosidic bonds. Therefore, it is necessary to pretreat the cellulose (MANDELS et al., 1974). Physical and chemical pretreatments for enhancing cellulose saccharification are extensively described by MILLET, BAKER and SATTER (1975). Chemical pretreatment to remove lignin, swelling the cellulose with acid or alkali, or dissolving and regenerating the polymer increase its susceptibility to enzymic attack. Physical pretreatment such as shredding or grinding does not remove lignin or swell cellulose, but it reduces particle size and therefore increases available surfaces. The most successful grinding treatment is ball milling (MANDELS et al., 1974)) which reduces crystallinity as wel as particle size. GHOSE (1969) and GHOSE and KOSTICK (1969) heated the cellulose at 200 °C before grinding, but such a treatment has to be avoided with complex cellulose because of possible reactions with impurities.

6.2. RESULTS

6.2.1. *Production of cellulolytic enzymes*

Degradation of cellulose-containing materials proceeds by the action of cellulolytic enzymes. As is shown in Chapters 4 and 5, *T. viride* appeared to be one of the best organisms to produce enzymes that are able to degrade cellulose and cellulose-containing material.

Many investigators suggest that cellulolytic enzymes are inducible by the substrate (Chapter 1). Therefore, it has advantage to produce cellulolytic enzymes needed in the process presented in Fig. 6.1 on solids of faeces.

Fig. 6.2 shows the production of cellulolytic enzymes and the course of pH of the nutrient solution during growth of *T. viride* in mineral medium using 0.08 mm ground solids of faeces as carbon source. The fungus produced acid when consuming carbohydrate with NH_4^+ as the nitrogen source (Chapter 5). The

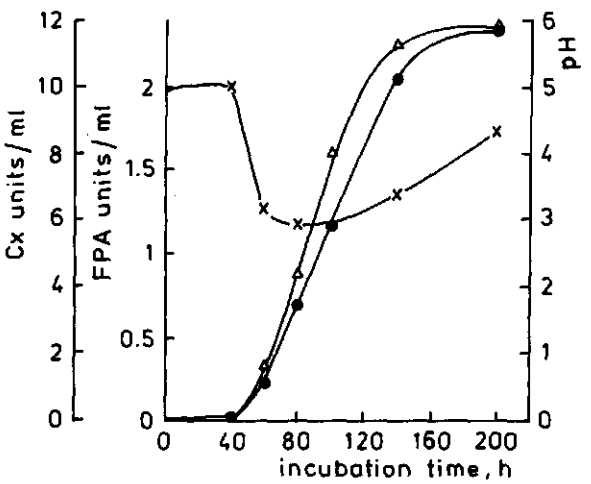

FIG. 6.2 Production of cellulolytic enzymes and course of pH during growth of *T. viride* on 0.08 mm ground solids of pig faeces in a Biotec fermentor Mycelial inoculum, temp. 28°C. x——x pH; △——△ C_x units; ●——● FPA units.

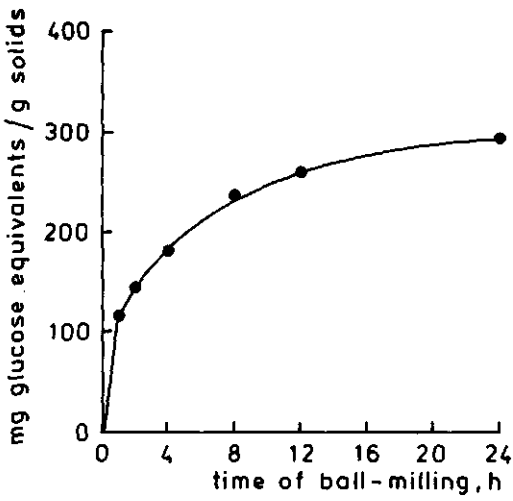

FIG. 6.3 Released reducing sugars expressed as glucose equivalents in mg per g of solids of pig faeces, ball-milled for different periods and hydrolysed at 50°C for 24 h with culture liquid of *T. viride* with an FPA of 1.2 units/ml.

rate of acid production was related to the rate of carbohydrate consumption and to the rate of production of cellulolytic enzymes. A significant feature of the pH pattern was that as soon as the carbohydrate was consumed the pH rose and the enzyme production decreased. Rise of the pH occurred probably by the secretion of products of proteolysis.

The amount of cellulolytic enzymes excreted by *T. viride* growing on basal medium with NaOH treated solids (Chapter 5) or with 0.08 mm ground solids applied in these experiments did not differ very much. In both cases 10–12 C_x units/ml and 2 FPA units/ml were measured, while 1 – 1.2 g of soluble protein per l of culture liquid was measured (Fig. 6.2).

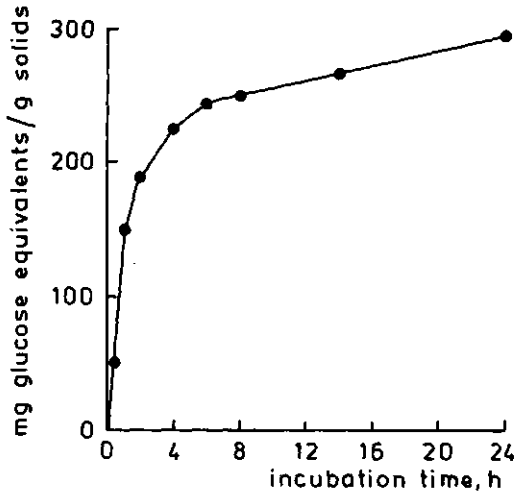

FIG. 6.4 Released reducing sugars expressed as glucose equivalents in mg per g of 24 h ball-milled solids of faeces, incubated at 50°C with spent culture liquid of *T. viride* with an FPA of 1.2 units/ml for different periods.

TABLE 6.1. Reducing sugars produced per g of hammer-milled solids after incubation of the solids in a *T. viride* cellulase solution of 1.2 FPA/ml at 50°C for 24 h.

Sieve	Reducing sugar in mg/g of solids
1 mm	58
0.2 mm	68
0.08 mm	145

6.2.2. *Hydrolysis of solids of faeces with culture liquid of* T. viride

To study the effect of grinding, the solids were ball-milled for different periods before incubating them with culture liquid. Fig. 6.3 shows the production of reducing sugars by incubating 20 g of solids of pig faeces per l of spent culture solution of *T. viride* at 50 °C for 24 h. The cellulolytic activity of the culture filtrate equalled 1.2 FPA units/ml. Ball milling reduced particle size and cristallinity of the cellulose. Grinding with a hammer mill only reduced particle size but it is technologically a more suited method. As can be seen from Fig. 6.3 and Table 6.1 the amount of reducing sugars produced is higher when ball milling was applied as pretreatment.

The influence of ball milling on the structure of the cellulose, hemicellulose and lignin complex was confirmed by the observation that particle size of 0.08 mm hammer-milled solids was smaller than that of the solids ground for 6 h in a ball mill, but degradation of the ball-milled particles was better.

Reducing sugars produced from solids of faeces by treatment with spent culture liquid of *T. viride* are not present as monosaccharides as can be concluded from the comparison of the decrease of insoluble dry matter (Table 6.2) with the data shown in Fig. 6.3 and Table 6.1. Pretreatment of 24 h ball milling followed by enzymic hydrolysis caused the solubilization of 74 % of the solids which is equal to the loss of weight when acid hydrolysis of the polysaccharides was applied (Chapter 3). Determination of glucose with glucose oxidase after enzymic hydrolysis for 24 h of the ball-milled solids showed that all of the cellulose present in the solids was converted into glucose. This means that hemicellulose was converted into soluble oligosaccharides and only to some extent into monosaccharides.

TABLE 6.2. Decrease of solids weight upon hydrolysis of the ground solids by *Trichoderma* cellulase of 1.2 FPA/ml at 50°C for 24 h.

Mill	Decrease in solids weight (% of original value)
Hammer mill	
1 mm	9.7
0.08 mm	25.3
Ball mill	
30 min	17.7
24 h	73.7

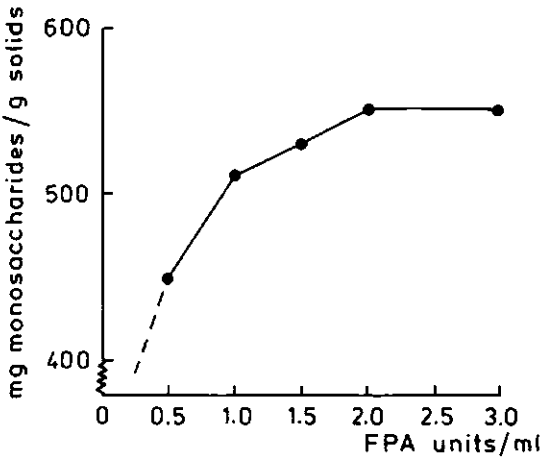

FIG. 6.5 Monosaccharides released by hydrolysing 24 h ball-milled solids of faeces (20 g/l) with culture liquid of *T. viride* of different FPA at 50°C for 4 h followed by hydrolysis of disaccharides and oligosaccharides with 1 NH_2SO_4.

6.2.3. *Influence of time of hydrolysis and enzyme activity*

In order to investigate the influence of time of hydrolysis, 24 h ball-milled solids (20 g/l) were incubated for different periods with culture liquid of *T. viride* with an FP activity of 1.2 unit per ml. The larger part of the reducing sugar was produced within the first two hours of incubation (Fig. 6.4).

Fig. 6.5 shows the amounts of monosaccharides, set free from 20 g solids after incubation with spent culture solutions with different activities of cellulase followed by hydrolysis of the reaction product with 1.0 N H_2SO_4 at 100°C. Different concentrations of cellulolytic enzymes were obtained by vacuum evaporation. Culture liquid with 1.0 FPA unit/ml appeared sufficient to hydrolyse 80% of the polysaccharides of the solids. The effect of a more concentrated enzyme solution is very small. An FP activity of 0.5 units per g substrate seems to be optimal for the hydrolysis of solids of faeces.

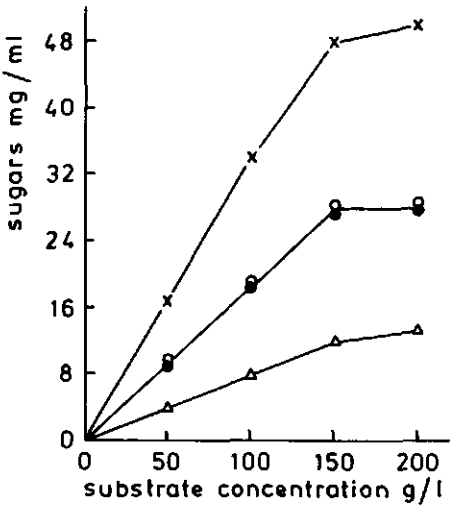

FIG. 6.6 Sugars in the incubation medium after hydrolysis of 4 h at 50°C. The incubation liquid contained different amounts of 24 h ball-milled solids in spent nutrient solution of *T. viride*. △——△ free glucose, ○——○ total glucose, ●——● reducing mono- and oligosaccharides and x——x total reducing monosaccharides.

6.2.4. *Influence of the substrate concentration*

In order to obtain information about the optimum substrate concentration for hydrolysis of solids of pig faeces with cellulolytic enzymes of *T. viride*, different amounts of 24 h ball-milled solids were incubated with solutions of cellulolytic enzymes of such an activity that per g of substrate 0.5 FPA unit is present. Glucose and reducing sugars were measured before and after hydrolysis with 1.0 N H_2SO_4.

As is shown in Fig. 6.6, a substrate concentration of 150 g/l appeared to be optimal. The suspension of the solids at higher concentrations is too viscous as a result of which stirring is difficult. At this substrate concentration, the enzyme activity of the solution should equal 7.5 FPA units/ml. This enzyme concentration is readily attained by vacuum distillation of the culture filtrate at 35 °C. After hydrolysis for 4 h under the conditions described 80 % of the polysaccharides of the solids is converted into oligosaccharides and monosaccharides. Fifty percent of the sugars appeared to be in solution as monosaccharides.

6.2.5. *Adsorption of cellulolytic enzymes to the substrate*

Culture filtrate of *T. viride*, grown without pH control on mineral medium with solids of faeces as carbon source, was incubated for 30 min at different concentrations of the substrate (Chapter 2.4.2). To reduce enzymic hydrolysis, incubation was performed at 4 °C. The influence of temperature on adsorption of macromolecules to an insoluble substrate is low and equilibration is reached usually within 10–20 min (KRUYT and OVERBEEK, 1969).

Fig. 6.7 shows the course of adsorption of cellulolytic enzymes to solids of faeces. It will be seen that 65 % of the FPA was adsorbed and 80 % of the C_x activity at a substrate concentration of 20 g of solids of faeces per litre. These results are in good agreement with the data given by HUANG (1976) and REESE (1976). Adsorption of C_x activity slightly increased at higher substrate concentrations viz. up to 90 %.

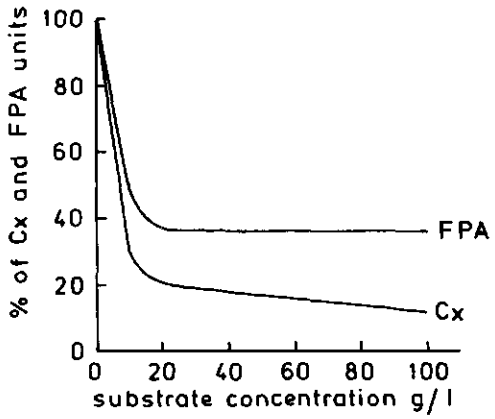

FIG. 6.7 Adsorption of cellulolytic enzymes by solids of faeces. After incubation at 4.5 °C for 30 min the solids were removed by centrifugation and enzyme activities of the supernatant measured.

Cellulolytic enzymes applied for degradation of solids of faeces according to the process suggested in Fig. 6.1 are first adsorbed to the substrate. At 50 °C and pH 4.8 polysaccharides are hydrolysed and set free into the culture liquid. When the accessible polysaccharides are hydrolysed the adsorption places are lost and theoretically all the cellulolytic enzymes will return into the soluble state. A similar behaviour of the cellulolytic enzymes was observed in cultures of *T. viride* when growth of the organism was completed and cellulose was exhausted (Chapter 5 and Fig. 6.2). No investigations were performed to confirm this theory. Therefore, a small part of the cellulolytic enzymes might be bound to the unhydrolysable solids after hydrolysis of the polysaccharides.

When hydrolysis is complete the reaction mixture can be separated by centrifuging into insoluble residues and supernatant which contains soluble sugars and enzymes. The purpose of the described process is to produce a sugar solution for further processing. The soluble enzymes in the liquid can be separated by re-adsorption to fresh substrate. According to the data of Fig. 6.7, 65% of the FP activity and 80 % of the C_x activity in the liquid can be re-used.

6.2.6. *Analysis and characterization of enzymic activities produced by* T. viride *on solids of faeces*

Characteristics of *Trichoderma* cellulase can only be determined when purification of the cellulolytic enzymes of the culture filtrate is accomplished. According to WOOD (1971) good results are obtained by fractionating the enzymes on

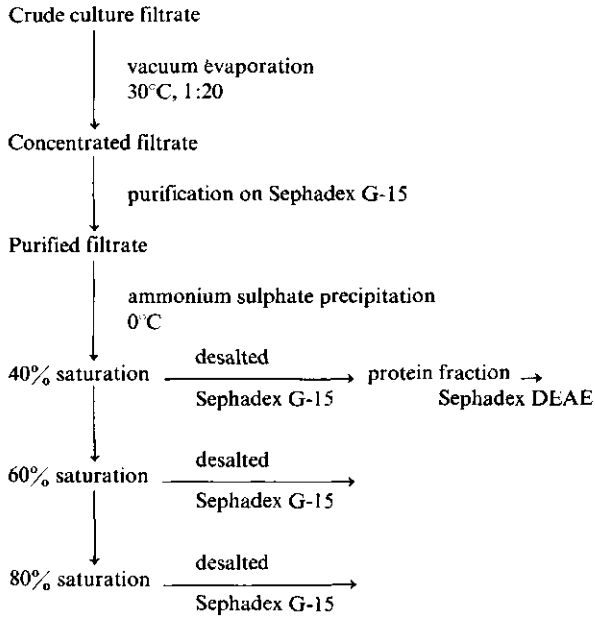

FIG. 6.8. Outline of purification of *Trichoderma* cellulase.

Sephadex columns and concentrating them by acetone precipitation, vacuum evaporation or $(NH_4)_2SO_4$ precipitation. In the present investigation culture filtrate of *Trichoderma* was concentrated and purified as outlined in Fig. 6.8. The relative amounts of enzyme after each step are given in Table 6.3. During evaporation, when the filtrate was concentrated more than ten times, a precipitate was formed. This precipitate probably contained a considerable amount of hemicellulase of which only 13 % was left in solution after concentration. No precipitate was formed at 20 % saturation with ammonium sulphate; most of the enzymic activity was found in the precipitate formed after 60 % saturation with ammonium sulphate. The latter fraction was analysed by using DEAE-Sephadex. The protein distribution in the eluate is given in Fig. 6.9 and the distribution of exo- and endoglucanase (C_x) activities in Figs 6.10 and 6.11, respectively. Protein peaks A and C of Fig. 6.9 contained β-glucosidase and endo- and exoglucanase. Peak B contained none of the tested enzyme activities. Endoglucanase activity was also found in fraction 40 which contained a very small amount of protein. Peak D of Fig. 6.9 corresponded with endoglucanase activity whilst peak E appeared to have no enzymic activity. HUANG (1976) studying cellulase of *T. koningii*, also observed the presence of enzymic activity in fractions of DEAE-Sephadex eluate with a very low level of protein. Hemicellulase was found in the first fractions of the eluate (Fig. 6.12). The low hemicellulase activity in protein peak C was probably due to the presence of β-1,4 bound glucose in the xylan sample used. Determination of hemicellulase is discutable because of the unknown but probably varying structure of hemicellulose in plant cell walls. The substrate xylan chosen for hemicellulase determination is only partly suitable for the hemicellulase present in the mixture of enzymes of *T. viride*. As long as the chemical structure of hemicellulose in solids of faeces is unknown it is impossible to use specific substrates for determining enzymic activities.

TABLE 6.3. Relative amounts of enzyme derived from culture filtrate of *T. viride* purified according to the outline of Fig. 6.8.

	Endo-glucanase C_x	Exo-glucanase	Filter paper activity	Hemi-cellulase	Protein (Lowry)	Reducing sugars
Culture filtrate	100	100	100	100	100	100
Concentrated filtrate	37	56	83	13	93	83
Purified filtrate	43	84	63	20	51	20
40% $(NH_4)_2SO_4$ precipitate	24	47	28	12	19	7
60% $(NH_4)_2SO_4$ precipitate	1	1	1	3	2	1
80% $(NH_4)_2SO_4$	0	0	0	0	1	1

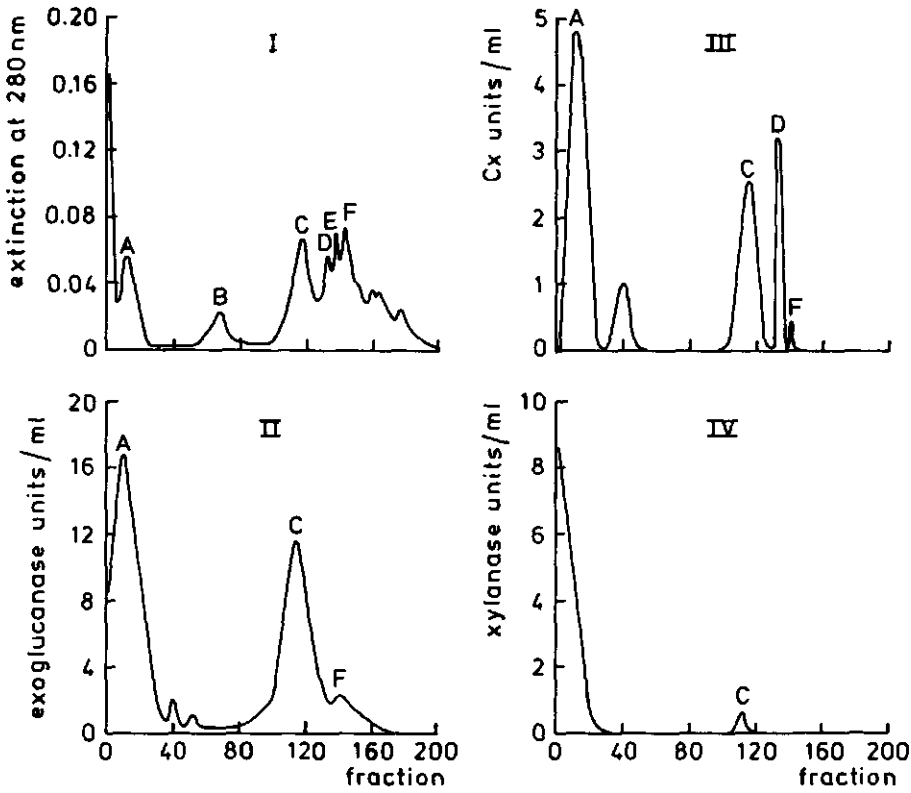

FIG. 6.9 (I) Protein in spent culture liquid of *T. viride* estimated by measuring extinction at 280 nm of the purified culture liquid.
FIG. 6.10 (II) Exoglucanase activity of culture liquid of *T. viride*.
FIG. 6.11 (III) C_x activity of culture liquid of *T. viride*.
FIG. 6.12 (IV) Hemicellulase activity of culture liquid of *T. viride*.

6.3. DISCUSSION AND CONCLUSIONS

T. viride grown on the solids of faeces pretreated by grinding produces adequate amounts of cellulolytic enzymes to hydrolyses the polysaccharides of this substrate. The filter paper activity of the enzyme complex is somewhat lower than the filter paper activity found by MANDELS and WEBER (1967) when crystalline cellulose (Avicel) was used as the carbon source. Filter paper activity of the enzyme complex is a measure of the ability of the enzymes to degrade crystalline cellulose. Many investigators mention the inducibility of the cellulolytic enzymes (Chapter 1). Therefore, it might by concluded that crystallinity of the cellulose in solids of faeces is somewhat lower than the crystallinity of cellulose of Avicel. This suggestion was confirmed by preliminary results of the determination of crystallinity of solids by X-ray diffraction.

T. viride produces also considerable amounts of hemicellulase that converts all non-cellulose polysaccharides into soluble oligo- and monosaccharides. Determination and quantification of hemicellulase is difficult because of unknown substrate characteristics.

The fact that pretreatment of the solids of faeces is necessary for complete hydrolysis and that grinding to small particles is sufficient to that purpose demonstrates the influence of steric hindering of enzyme action by lignin and other structure characteristics of the solids. The intermicrofibrillar spaces of the cellulose fibres are 100 Å in diameter. WHITAKER (1954) estimated cellulolytic enzymes to be 200 × 30 Å. Therefore, pretreatment which causes increase of surface enhances degradation of the solids. Grinding is easy but technologically expensive when ball milling or hammer milling to 0.08 mm is necessary.

Suspensions up to 150 g solids per litre are readily hydrolysable when the substrate is made accessible to cellulolytic enzymes by any treatment. Under the described circumstances up to 70 % of the solids are hydrolysed into sugars. The produced sugar solution can be separated and used for further processing.

A solution of cellulolytic enzymes to degrade solids of pig faeces can be produced by growing *T. viride* on basal medium with solids as carbon source. The cellulolytic enzymes can be collected by centrifuging the culture liquid followed by acetone precipitation or vacuum evaporation. After hydrolysis of the polymers of the solids of faeces the enzymes are set free in the sugar solution and can be re-used by adsorption to fresh substrate. Up to 65% of the enzymes is released and is present in the incubation liquid, i.e. is recycled this way.

SUMMARY

The purpose of this investigation was to study the microbiological degradation of the cellulose-hemicellulose-lignin complexes of the faeces of pigs. Cellulose, hemicellulose and lignin are components of the cell wall of plants and residues of plant material occur in large quantities in faeces and other organic waste material. The development of the intensive livestock farming is leading to the production of large quantities of manure that cannot always be disposed of in the usual way by lack of agricultural land. To prevent deterioration of the environment, other ways of handling the manure should be sought. Priority should be given to those procedures according to which the insoluble organic components of the faeces are decomposed by microorganisms and the energy produced utilized by man. Examples of such procedures are the aerobic break-down of the organic residues with production of large amounts of biomass that can be utilized as feed additive (single-cell protein). Anaerobic treatment of manure may lead to the production of methane. In the present investigation attention was centred on the aerobic break-down of the undigested organic residues of pig faeces.

Chapter 1 contains a general introduction and gives a review of literature pertaining to waste material with cellulose, hemicellulose and lignin as main components. In the Netherlands most of the organic waste material occurs as manure, sewage sludge and domestic wastes.

The chemistry and structure of the plant cell wall were discussed. The presence of lignin strengthens the structure of plant tissue but seriously hampers the break-down of other cell wall components like cellulose and hemicellulose which are closely linked to the lignin molecules.

Fungi play an important part in the degradation of cellulose-hemicellulose-lignin complexes of plant residues. Some fungi are able to decompose lignin, others utilize cellulose and hemicellulose, leaving lignin untouched. Cellulolytic bacteria occur in considerable numbers in nature, but break-down of lignin by bacteria is hardly reported in the literature.

Extra-cellular, substrate-specific enzymes produced by microorganisms are involved in the break-down of plant residues. Several enzymes play a part in the degradation of the cellulose-hemicellulose-lignin complexes. Those involved in the decomposition of cellulose are known under the collective name of cellulase. Hemicellulose is a group name for several plant cell wall heteropolysaccharides which are degraded by substrate-specific enzymes. Little is known of enzymes involved in lignin break-down.

In Chapter 2 a survey is given of materials and methods used in this investigation. A method was described for the collection of insoluble residues of plant material from pig faeces. For practical reasons, this preparation was used in the experiments to be described.

Chapter 3 gives the results of microscopic and chemical analyses of undigested plant residues of pig faeces. These residues were derived from plant material, mainly ground seeds and fruits, supplied as mixed feed to the animals. Various types of plant tissue (parenchyma, collenchyma and sclerenchyma) were observed showing that considerable amounts of cellulose-, hemicellulose-, and lignin-containing cell walls had passed more or less undamaged the digestive track of the pigs (Figs. 3.1-3.11).

The chemical analysis of pig faeces showed that 30–40 % of the dried matter consisted of insoluble plant residues containing 65–70 % polysaccharides (cellulose and hemicellulose) and nearly 30 % lignin. In order to use these plant residues as carbon source for fungi, pretreatment of the material is necessary as the high lignin content hampers the ready utilization of the cellulose and hemicellulose by the cellulolytic fungi.

Chapter 4 described the isolation of cellulolytic microorganisms. Mainly fungi were isolated and a number of them together with some fungi obtained from culture collections were tested for their ability to utilize as carbon source: (a) monosaccharides that occur in the polysaccharides of plants (b) some model polysaccharides related to plant cell walls (c) lignin and (d) undigested plant residues from pig faeces. The results of these experiments are shown in Tables 4.1, 4.2 and 4.3. None of the fungi tested was able to decompose lignin. When the undigested plant residues of pig faeces had been supplied as carbon source only 30–40 % of the material was utilized. This was mainly due to the protection of the polysaccharides against fungal attack by the lignin. In order to improve the availability of the polysaccharides to the fungi, breaking or removal of the lignin matrix of the plant tissue is required.

Of the fungi tested for growth on residues of faeces, *Penicillium nigricans*, *Myrothecium verrucaria* and *Trichoderma viride* QM 9419 gave the best results. As the *Trichoderma* strain produced the highest amounts of cellulolytic enzymes, this organism was chosen for further research.

In Chapter 5 experiments were described concerning the growth of *Trichoderma viride* on (a) crystalline cellulose (Avicel), (b) undigested plant residues from pig faeces, and (c) plant residues from faeces treated with alkali to dissolve and remove lignin. Crystalline cellulose was the best carbon source. It was almost completely utilized by *T. viride* giving about 250 mg of protein per g of cellulose consumed. The undigested carbon compounds of pig faeces were utilized for about 49 % and the alkali-treated residues for 60 %.

The amount of cellulolytic enzymes in the culture liquid of *T. viride*, calculated per unit of soluble protein, was with cellulose as carbon source three times as high, and with alkali-treated residues twice as high as with untreated plant residues from pig faeces. Spent culture solutions of *T. viride* supplied with pure cellulose or undigested residues of pig faeces contained relatively large amounts of soluble protein. An unknown proportion of this protein represented enzymes, presumably mainly cellulolytic enzymes.

The production of biomass from the undigested residues of pig faeces by use of

Trichoderma viride ('single-cell protein') to prepare mixed feed is for the following reasons no appropriate procedure: (a) *T. viride* only partly utilizes the polymers of the undigested residues of pig faeces, (b) the fungal protein is mixed with part of the faeces constituents which could not be metabolized by the fungus and which are also resistant to digestion by the animal owing to the protection of cellulose and hemicellulose against break-down by the presence of lignin, (c) the soluble protein of the culture solution of the fungus is difficult to collect.

Chapter 6. A procedure was proposed (Fig 6.1) according to which the insoluble residues of pig faeces are submitted to a solution of cellulolytic enzymes. The hydrolysis of the polysaccharides (at 50°C and pH 4.8) proceeds in a relatively short time giving a solution of sugars which can be used for further purposes. A suitable enzyme solution can be obtained by growing *T. viride* on residues of faeces. The enzyme solution of the spent nutrient solution of the fungus can be easily concentrated by acetone precipitation or by vacuum evaporation. The substrate for the enzymic procedure, the undigested residues of faeces, are adequately degraded when a pretreatment has been applied (grinding to a particle size of 0.08 mm).

When the proposed procedure is applied, 150 g of insoluble residues per l of enzyme solution can be hydrolysed; 70 % of the residues is converted into soluble sugars within 4 h. The cellulolytic enzymes are initially adsorbed to the insoluble substrate but are released when hydrolysis proceeds; they can be separated from the undissolved material together with the dissolved sugars. Separation of sugars and enzymes can be achieved by mixing with new substrate which adsorbs the enzymes. After separation of the sugar solution the substrate with enzymes are transferred to the reactor. In this way part of the enzymes can be re-used.

In Chapter 6 also the results are given of the purification and separation of a number of enzymes involved in the degradation of the cellulose-, hemicellulose-, lignin complex (Fig. 6.8). The mixture of enzymes, excreted by *T. viride* when the organism was growing on undigested plant residues of pig faeces, was separated into several protein fractions each with its own enzyme activity with respect to different substrates, i.e. endoglucanase, exoglucanase and hemicellulase activity (Figs. 6.9–6.12).

Degradation of undigested components of pig faeces in view of recycling of the faeces is no simple procedure. Microbial (enzymic) break-down is possible if a suitable pretreatment is applied. The economical applicability of the results obtained in this investigation will depend on: (a) the occurrence of excessive amounts of manure, (b) disposal problems, particularly in connection with the environment, (c) alternative methods for processing excessive amounts of manure.

SAMENVATTING

Doel van het beschreven onderzoek was de microbiologische afbraak te bestuderen van cellulose-hemicellulose-lignine-complexen van varkensmest. Cellulose, hemicellulose en lignine zijn bestanddelen van de celwand van planten; restanten van plantaardig materiaal komen in grote hoeveelheden voor in mest en ander afval van organische oorsprong. De ontwikkeling van de intensieve veehouderij heeft tot gevolg dat grote hoeveelheden mest geproduceerd worden die moeilijk volgens de gebruikelijke methode, d.w.z. benutting als meststof, verwerkt kunnen worden.

Om verontreiniging van het milieu te voorkomen zijn andere verwerkingsmethoden noodzakelijk. Van belang hierbij zijn de processen volgens welke de in mest voorkomende onoplosbare restanten van plantaardige oorsprong worden afgebroken door micro-organismen onder benutting voor de mens van de vrijkomende energie. Voorbeelden hiervan zijn de aërobe processen waarbij men tracht zo veel mogelijk celmateriaal van de desbetreffende micro-organismen te verkrijgen om dat als veevoer te gebruiken (zgn. single-cell protein). Als anaërobe tegenhanger kan de methaanproduktie worden genoemd. In het hier te bespreken onderzoek is in het bijzonder aandacht geschonken aan de aërobe afbraak van onverteerde afvalstoffen van plantaardige oorsprong in varkensmest.

Hoofdstuk 1 geeft een algemene inleiding en een literatuuroverzicht van verschillende soorten afval met als bestanddelen cellulose, hemicellulose en lignine. Aangetoond werd dat in Nederland de grootste hoeveelheden afval voorkomen als dierlijke mest, rioolslib en huishoudelijk afval.

De chemie en structuur van de plantecelwand werden besproken. Lignine blijkt een matrix-structuur in de plantecelwand te vormen die de afbraak van andere celwandcomponenten (cellulose, hemicellulose) door enzymen bemoeilijkt. De chemische structuur van lignine is zodanig dat enzymatische afbraak hiervan waarschijnlijk zeer langzaam verloopt.

Micro-organismen die een rol kunnen spelen bij de aërobe afbraak van cellulose- hemicellulose- lignine-complexen zijn met name de schimmels. Sommige schimmels kunnen lignine afbreken; andere kunnen cellulose en hemicellulose van de genoemde complexen benutten zonder dat de lignine wordt aangetast. Celllulolytische bacteriën zijn bekend en komen in de natuur in aanzienlijke aantallen voor maar lignine-afbraak door bacteriën wordt in de literatuur nauwelijks vermeld.

De enzymen die een rol spelen bij de afbraak van onoplosbaar plantaardig materiaal dienen extracellulair te zijn. Deze enzymen zijn substraat-specifiek. Verwacht mag worden dat verscheidene enzymen een rol spelen bij de afbraak van cellulose- hemicellulose-lignine-complexen. De enzymen die een rol spelen bij de afbraak van cellulose worden meestal samengevat onder de term cellulase.

Hemicellulose, een groepsnaam voor heteropolysacchariden van de plantecelwand, wordt enzymatisch afgebroken door substraat-specifieke enzymen. Enzymen die een rol spelen bij de afbraak van lignine zijn niet bekend, hoewel verondersteld wordt dat enzymen die fenol oxyderen hierbij een rol spelen.

In Hoofdstuk 2 werd een opsomming gegeven van de in het onderzoek gebruikte materialen en methoden. Een werkwijze werd beschreven om de onoplosbare restanten van plantaardige oorsprong uit varkensmest te wassen. Om praktische redenen is het onderzoek met het op de beschreven wijze verkregen preparaat uitgevoerd.

Hoofdstuk 3 geeft de resultaten van een microscopische en chemische analyse van de door het dier niet verteerde onoplosbare restanten van plantaardige oorsprong in varkensmest. Deze restanten waren afkomstig van het aan de dieren verstrekte mengvoeder. In de mest waren restanten terug te vinden van de aan het mengvoer toegevoegde ingrediënten van plantaardige oorsprong. Parenchym, collenchym en sclerenchym bleken aanwezig te zijn (Fign. 3.1–3.11). Kwantitatieve gegevens betreffende de verdeling van de verschillende soorten weefsels van plantaardige oorsprong konden uit het onderzoek niet verkregen worden. Hoewel in microscopische preparaten beschadigingen en afbraak van het materiaal zichtbaar waren, kon dit niet éénduidig toegeschreven worden aan de passage door het maag-darmkanaal van het dier.

De chemische analyse van varkensmest en van daaruit verkregen onoplosbare restanten van plantaardige oorsprong toonde aan dat de droge stof van varkensmest voor circa 30–40 % uit dit onoplosbaar materiaal bestond. Deze residuen bevatten 65–70 % polysacchariden (cellulose en hemicellulose) en bijna 20 % lignine. Het hoge gehalte aan lignine maakt een voorbehandeling van het materiaal noodzakelijk omdat lignine de microbiologische afbraak van de polysacchariden belemmert.

Hoofdstuk 4 beschrijft de isolatie van cellulolytische micro-organismen. Onder de beschreven proefomstandigheden bleken schimmels de dominante organismen te zijn. Negen geïsoleerde schimmels en zes schimmels afkomstig van bestaande collecties werden getoetst op hun vermogen te groeien op (a) monosacchariden die voorkomen in de polysacchariden van planten, (b) enkele model polysacchariden verwant aan plantecelwanden, (c) lignine en (d) uit mest gewassen onverteerde residuen (Tabellen 4.1, 4.2 en 4.3). Geen der schimmels bleek in staat lignine te benutten als C-bron. Bij toediening van onverteerde mestbestanddelen als koolstofbron aan schimmels bleek circa 30–40 % benut te worden, hoewel de koolstofbron limiterend was. Geconcludeerd werd dat deze onvoldoende benutting toegeschreven moet worden aan de structuur van het materiaal waarbij lignine de voornaamste rol speelt. Het verbreken van de beschermende ligninematrix was dan ook een eerste eis voor het verkrijgen van een betere benutting van de polysacchariden door de micro-organismen.

Penicillium nigricans, *Myrothecium verrucaria* en *Trichoderma viride* QM 9419

bleken de beste groei op het genoemde materiaal te vertonen. De hier genoemde *Trichoderma*-stam produceerde de grootste hoeveelheid cellulolytische enzymen en daarom is het onderzoek met dit organisme voortgezet.

In Hoofdstuk 5 zijn experimenten beschreven betreffende de groei van *T. viride* op (a) kristallijne cellulose (Avicel), (b) onverteerde planteresten uit mest en (c) met loog behandelde mestbestanddelen. Van de onderzochte verbindingen bleek kristallijne cellulose de beste koolstofbron te zijn. Het werd vrijwel volledig door *T. viride* benut onder de vorming van ± 250 mg eiwit per gram verbruikte cellulose. De onoplosbare bestanddelen van varkensmest werden voor circa 40 % benut, de met loog behandelde residuen voor 60 %. De voorbehandeling met loog tast de beschermende ligninematrix aan waardoor cellulose en hemicellulose beter worden benut door de schimmel.

De in de cultuurvloeistof aanwezige cellulase berekend per eenheid oplosbaar eiwit was bij gebruik van cellulose als C-bron drie keer zo hoog, en bij met loog behandelde mestbestanddelen twee keer zo hoog als met onbehandelde bestanddelen van varkensmest. Bij het kweken van *T. viride* in voedingsoplossingen met cellulose en met de genoemde mestbestanddelen werden opvallend hoge gehalten aan oplosbaar eiwit in de gebruikte oplossing gevonden. Een niet nader bepaald gedeelte hiervan was enzym-eiwit.

Het produceren van biomassa uit bestanddelen van varkensmest met behulp van schimmels (zgn. single-cell protein) voor de bereiding van veevoer is bij toepassing van de beschreven werkwijze om de volgende redenen niet zinvol. (a) *T. viride* benut de onbehandelde bestanddelen van mest slechts gedeeltelijk, (b) het schimmeleiwit is vermengd met niet door de schimmel benutte residuen die slecht verteerbaar zijn voor het dier, (c) het buiten de biomassa aanwezige oplosbare eiwit is moeilijk af te scheiden.

In hoofdstuk 6 werd een proces voorgesteld (Fig. 6.1) waarbij de onoplosbare mestbestanddelen bij 50 °C en pH 4,8 behandeld worden met een oplossing van cellulolytische enzymen. Doel van dit proces is in relatief korte tijd de hydrolyse van de aanwezige polysacchariden te laten verlopen en een oplossing van suikers te produceren die benut kan worden als grondstof voor verdere verwerking. Een geschikte enzymoplossing kan verkregen worden door *T. viride* te laten groeien op onoplosbare mestbestanddelen. De enzymoplossing kan op eenvoudige wijze worden geconcentreerd door precipitatie met aceton of door indamping onder vacuum. Het substraat voor de enzymbehandeling, de onverteerde mestbestanddelen, wordt in voldoende mate afgebroken wanneer een voorbehandeling is toegepast. Malen tot een deeltjesgrootte van 0,08 mm blijkt noodzakelijk en dit kan technologisch moeilijk te realiseren zijn.

Wanneer volgens het voorgestelde proces te werk gegaan wordt kan 150 g mestbestanddelen per liter gehydrolyseerd worden. Een hogere concentratie heeft tot gevolg dat de suspensie te viskeus wordt en dat mengen moeilijker is. De afbraak verloopt dan niet optimaal. Onder de beschreven omstandigheden wordt in 4 uur 70 % van de mestbestanddelen omgezet in oplosbare suikers. De

cellulolytische enzymen die eerst worden geadsorbeerd aan het te hydrolyseren substraat komen bij voortschrijdende hydrolyse weer vrij in de incubatie-vloeistof en worden uiteindelijk samen met de opgeloste suikers afgescheiden. Deze enzymen kunnen uit de suikeroplossing teruggewonnen worden door ze te laten adsorberen aan vers substraat. Na scheiding van substraat met geadsorbeerde enzymen van de opgeloste suikers kan het substraat met enzymen teruggebracht worden in de reactor. Op deze wijze is het mogelijk een gedeelte van de cellulolytische enzymen opnieuw te benutten.

Tenslotte zijn in hoofdstuk 6 de resultaten beschreven van de scheiding en zuivering van een aantal enzymen die betrokken zijn bij de afbraak van het cellulose-hemicellulose-lignine complex (Fig. 6.8). Het mengsel van enzymen dat door *T. viride* werd uitgescheiden bij groei op onverteerde plantenresten in varkensmest kan worden gescheiden in een aantal eiwitfracties ieder met een eigen enzymactiviteit t.o.v. verscheidene substraten, i.c. endoglucanase-, exoglucanase- en hemicellulase-aktiviteit (Fig. 6.9–6.12).

Afbraak van de onverteerde bestanddelen van varkensmest met als oogmerk recycling van de mest is niet eenvoudig. Microbiële (enzymatische) afbraak is mogelijk indien een geschikte voorbehandeling wordt toegepast. De (economische) toepasbaarheid van de verkregen resultaten zal afhangen van (a) de mate waarin overschotten aan mest voorkomen, (b) de hierbij optredende afzetproblemen en de daarmee samenhangende milieuproblematiek en (c) mogelijke alternatieve verwerkingsmethoden.

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