# FUNGAL DELIGNIFICATION OF LIGNOCELLULOSES : PHYSIOLOGICAL ASPECTS AND ENHANCEMENT OF RUMEN FERMENTATION.

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Dedicated

To all those who for circumstances of life

were not privileged to have a formal education

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ABSTRACT

This project was primarily concerned with clarifying the extent to which fungal delignification in solid substrate fermentations could be optimised and applied to enhancing the nutritional value of high-fibre lignocellulosic ruminant feeds. Ten fungi were assessed for their ability to grow on media containing cell-wall related phenolic compounds and polysaccharides. Five strains were selected as being particularly active in secreting enzymes depolymerising native insoluble crystalline celluloses and lignins. The key cell wall phenolic ferulic acid was shown to exhibit differential patterns of inhibition of saccharification of cell wall carbohydrates with differential effects on cellulose depolymerisation, endoglucanase and B-glucosidase production. Two strains were shown to secrete phenol oxidases, which were presumed to be laccases, and three produced lignin oxidases, assayed as ability to decolourise polymeric dyes in surface agar culture.

Solid substrate fermentation of hay and barley straw with lignocellulose-degrading fungi (*Pleurotus sajo-caju*, *Chaetomium cellulolyticum* and *Trichoderma harzianum*) gave increases in acid detergent lignin except with *Coriolus versicolor* or *Phanerochaete chrysosporium*. *P. sajo-caju*, *C. versicolor* and *P. chrysosporium* reduced the lignin content of spruce and birch sawdust but had no marked effect on that of rice bran. All the five fungi reduced polysaccharide contents of lignocelluloses, but depletion

was not at a rate directly related to period of fermentation. Fermentations with mixed cultures of three lignolytic fungi (*C. versicolor*, *P. sajo-caju* and *P. chrysosporium*) led to greater reduction in lignin content than did with monocultures. Although synergistic attack was observed on total insoluble polysaccharides and hemicelluloses, this was not found with depolymerisation of celluloses. Accumulation of total solubles, both carbohydrates and phenolics, was also markedly higher with mixed than with monocultures.

Five lignocelluloses were evaluated as feeds in a simulated rumen model system (RUSITEC). The lignocelluloses were supplied prior to and following fungal delignifications. Fungal pretreatment of spruce sawdust enhanced feed digestibility and daily carbon dioxide production; with untreated sawdust production of total gas and volatile acids, and bacterial population decreased with incubation in the rumen. Addition of chitin and D(+) glucosamine, major components of fungal cell walls, had no adverse effect on digestibility, production of acetate or fermentation gases except when concentrations reached >30% chitin and >1% glucosamine.

The influence of cell-wall related phenolic compounds and a toxic fungal metabolite, aflatoxin, on fibre digestion and the output of rumen fermentation end products was studied. Addition of the phenolics or fungal metabolite led to varied effects on bacterial populations, volatile acid and gas ($CH_4$ & $CO_2$) evolution.

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## ABBREVIATIONS

| | |
|---|---|
| $a_W$ | water activity |
| ADF | Acid detergent fibre |
| ADL | Acid detergent lignin |
| $^oC$ | degrees centigrade |
| CMC | Carboxymethylcellulose |
| CMCase | Carboxymethylcellulase |
| cm | centimetre |
| CP | Cellulosic polysaccharide |
| DNS | Dinitrosalicylic acid |
| EDTA | Ethylenediamine tetracaetic acid |
| FID | Flame ionisation detector |
| GLC | Gas liquid chromatograph |
| GC | Gas chromatograph |
| HWD | Hot wire detector |
| h | hour |
| $gl^{-1}$ | gram per litre |
| X g | Centrifugal force |
| g | gram |
| mg | milligram |
| Kg | Kilogram |
| l | litre |
| mm | millimetre |
| ml | millilitre |
| mMol | Millimole |
| min | minute |
| M | molar ( moles per litre ) |

| | |
|---|---|
| M.C. | moisture content |
| NDF | Neutral detergent fibre |
| MASTERTEK (supplied by Four -F nutrition North Yorkshire) | Mixture of polyvalent and divalent ions |
| NCP | Non cellulosic polysaccharide (largely hemicellulose) |
| NSP | Non starch (soluble) polysaccharide |
| nm | nanometre |
| OD | Optical absorbance units |
| ppm | parts per million |
| r.p.m. | revolutions per minute |
| RUSITEC | Rumen simulation technique |
| RBB | Remazol Brilliant Blue |
| SSF | Solid substrate (state) fermentation |
| sec | seconds |
| umol | micromole |
| UV | ultraviolet |
| ul | microlitre |
| VFA | Volatile fatty acid |
| w/w | ratio weight/weight |
| w/v | ratio weight/volume |

## COMMUNICATIONS TO LEARNED SOCIETIES

1. **ASIEGBU, F.O ; PATERSON, A. AND SMITH, J.E.** (1990). The effects of lignin model compounds on growth, biomass production and lignocellulose degrading enzyme systems of some wood degrading fungi. Paper presented at the 116th meeting of Society for General Microbiology at the University of Warwick, UK, 9 - 12th April. Conference Abstract Proceedings P.66

2. **ASIEGBU, F.O.; MORRISON, I.M. ; PATERSON, A. AND SMITH, J.E.** (1990). Use of rumen simulation technique for analysis of fungal fermentations of lignocellulosic materials. Paper presented at fourth International Mycological congress, Regensburg, Germany (FRG) 28th Aug. - 3rd September. Abstract vol.4, 220.

3. **ASIEGBU, F.O. ; PATERSON, A. AND SMITH, J.E.** (1991). Application of dual/ multiple lignolytic fungal strains in lignin degradation studies. Paper presented at the first International Conference of UK Biotechnology, 24 - 27th September at the University of Leeds, UK. Abstract vol.1 , F1.

# INTRODUCTION

## 1.1 Preamble

Utilisation of urban, industrial, municipal and agricultural wastes as energy and feed sources has been widely studied (Alexander, 1974; Zadrazil, 1980; Fan *et al.*, 1981a &b; Reid, 1985; Paterson, 1989; Coughlan, 1990). Han (1978) reported that agricultural wastes amount to over two hundred million tons annually in the United States; about 111 - 952 metric tons of crop residues are produced annually in Africa and Asia (Ryu, 1989). Moreover, U.S annual production of agroindustrial wastes is over two billion tons constituting a major potential pollution risk (Reddy & Erdman, 1977). The largest reservoir of renewable organic matter is the lignocellulosic materials, estimated at several billion tons annually (Sarbolouki & Moacanin, 1980; Knapp, 1985; Ryu, 1989).

The conversion of such quantities of organic matter(Table 1.1a) into useful products would essentially double agricultural productivity and world food supply (Sloneker, 1976; Nicolini *et al.*, 1987). Unfortunately, the chemical and physical properties of the polymeric components of these wastes limit their bioconversion and biodegradation (Wood, 1985; Paterson, 1989). Numerous methods have been studied to enhance exploitation of energy stored in plant cells (Table 1.1b), including anaerobic digestion for biogas

TABLE 1.1a ANNUAL PRODUCTION OF CROP RESIDUES IN THE WORLD

| CROP RESIDUES | WORLD TOTAL | % | AFRICA | NORTH AMERICA | SOUTH AMERICA | ASIA | EUROPE | USSR | OCEANIA |
|---|---|---|---|---|---|---|---|---|---|
| Corn stover & cobs | 376 | 18 | 18 | 180 | 29 | 83 | 50 | 11 | 0.4 |
| Rice hulls & straws | 545 | 26 | 10 | 10 | 17 | 503 | 2 | 1 | 3 |
| Sugar cane, baggasse & tops | 300 | 14 | 22 | 58 | 99 | 111 | 0.2 | - | 10 |
| Wheat Straws | 522 | 25 | 9 | 96 | 17 | 176 | 129 | 76 | 19 |
| Others | 327 | 17 | 52 | 45 | 24 | 79 | 79.8 | 45 | 4.6 |
| Total | 2070 | 100 | 111 | 389 | 186 | 952 | 261 | 133 | 37 |
| World production(%) | | | 5.4 | 19 | 9 | 46 | 12.6 | 6.4 | 1.8 |

All production figures in $10^6$ metric tons.

From : Ryu (1989).

Table 1. 1b Existing and potential applications for bioconversion of lignocelluloses into products ( Wood, 1985 ).

| Products | Substrates | Technology available | Technology profitable | Constraints |
|---|---|---|---|---|
| Mushroom | Composted : straw sawdust whole wood | yes | yes | -Consumer resistance to greater consumption |
| Fuels or food Single cell protein | Solid substrate straw wood | no | no | -product cost. -uniformity of scale up. -rate of treatment. |
| N-fixation: Soil conditioner. Biological control. | Straw | no | ? | -scale up to farm level. -treatment cost |
| Pulp. | Wood Straw | yes | no | -product quality -product cost |
| Chemicals eg.ethanol glucose, butanediol | Wood Straw food by product | yes | no | -other feedstocks -nature of feedstocks |
| Enzymes: cellulases hemicellulases | Wood Straw sawdust | yes | no | product cost with liquid fermentation sources. |
| Fuel or food | hydrolysed straw sawdust wood | yes | no | -price of pretreatment -cost of product. |

production (Ranade *et al.*, 1987), acid and enzymic hydrolysis followed by fermentation to a variety of chemicals (Flickinger & Tsao, 1978; Rivers & Emert, 1988), and direct fermentation of waste lignocellulose to microbial protein or chemicals (Chahal, 1982). Moreover, biological processes have been developed for pulping of bast fibres with either fungi or pectinolytic bacteria (Yoshihara & Kobayashi, 1981; Kirk & Reid, 1990), treatment of wood pulping liquors to defoam and deodourise (Eaton*et al.*, 1980; Jurasek, 1990), waste treatment (Kirk & Chang, 1981) and enhancement of rumen digestibility of feeds (Reid & Seifert, 1982; Al-Ani & Smith, 1988; Yadav, 1988). It has been calculated that increasing the digestibility of lignocellulosic fibres could provide enough feed to double the global cattle population and beef production (Viesturs *et al.*, 1981; Reid, 1989a).

The use of biological pretreatment techniques, as alternatives to existing delignification methods, for nutritional enhancement of lignocelluloses, is economically attractive, low in energy requirement and particularly suited to the technologies of developing countries but has received limited attention in recent years. Microbes grow and degrade polymers ranging from natural lignocelluloses to synthetic plastic hydrocarbons. Such abilities can be used in production of novel foods and feeds (Sanni, 1981; Solomons, 1986). Many fungi have been shown to attack lignins.

The phenol-oxidase secreting white rot fungi have been widely studied while actinomycetes and eubacteria, brown rot and soft rot fungi can at least modify lignins (Crawford & Crawford, 1984; McCarthy, 1987; McCarthy & Broda, 1984 ; McCarthy *et al*, 1986). Moreover, most studies have been carried out using submerged batch culture or semisolid fermentations. Recently solid substrate (SSF) has been shown to be better suited than submerged fermentations in effecting lignin breakdown (Lonsane *et al* , 1985; Hesseltine, 1987). However, such technologies have limitations such as poor heat transfer and problems in scale up (Smith, 1985).

Acceptance of processes for upgrading of forage depend largely on product performance in the rumen or in *in vitro* simulation. Previously assessment of digestibility was carried out by feeding trials on ruminants and monogastrics which required much space, long periods (months & years) and large quantities of materials, and were expensive. Recently, techniques using crude cellulase enzymes to monitor *in vitro* digestibility have been used more widely; such methods have constraints as data obtained may not always give a full assessment of microbial activity in the rumen. The Rumen Simulation Technique (RUSITEC), if properly operated, yields reliable values for rumen volatile acid output and gas production similar to those observed in animals (Hobson, 1971; Dinsdale *et al*, 1978; Czerkawski & Breckenridge 1979a & b; Zadrazil &

Brunnert, 1981; Reid & Seifert, 1982; Balasubramanya *et al.*, 1987). However, "RUSITEC", like most simulation systems, also has its limitations.

Enhancing accessibility of plant polysaccharides to rumen enzymes, through delignification in fungal fermentations, could contribute significantly to increasing digestion of ruminant feeds. Current technologies are not feasible in developing countries and are very polluting. Initial studies in this laboratory using mutant strains of the white rot *Phanerochaete chrysosporium*, defective in cellulolytic activity, did not result in improvements in digestibility of bagasse (Al - Ani, 1985). However, earlier reports indicated that lignin depolymerisation can be enhanced substantially through strain improvement techniques (Buswell *et al.*, 1984; Johnsrud & Eriksson, 1985). Solid substrate fermentations using wild-type fungi appear currently to have the greatest promise for development.

## 1.2 LIGNOCELLULOSES

Lignocelluloses can compete with petroleum and coal as primary sources of organic compounds for fuels and chemicals (Smith *et al.*, 1987). Three major polymeric constituents of lignocelluloses are recognised - lignins, celluloses and non-cellulosic polysaccharides or hemicelluloses. Selective delignification or bio-utilisation of lignocelluloses have been widely

studied as supplements to conventional chemical feedstocks (Philips & Humphrey, 1983; Rolz, 1984; Wood, 1985; Harking, 1986; Wayman & Parekh, 1990). However, physical and chemical barriers constitute problems in commercial exploitation.

Barriers to efficient bioconversion of lignocelluloses have been summarised by Wood (1985) as :

- crystallinity of the major part of cellulose fraction
- lignin encrustation of cellulose limits the rate of hydrolysis
- surface area of cellulose crystals is rate-limiting in attack
- differential recalcitrances of the major polymers
- pore size in cell wall structures restricts enzyme movement
- degree of polymerisation of major polymers
- presence/toxicity of low molecular weight compounds
- low moisture contents
- morphological heterogeneity of cell walls at molecular levels

The composition of wood lignocelluloses varies between species and within individual plants, since, for instance in aspen wood, chemical constituents differs significantly in relation to site, climate, age and other factors (Wayman & Parekh, 1990). Thus analyses of wood composition should be treated with caution.

Determination of wood composition was initiated with the discovery by Peter Klason as described by Effland (1977) that wood polysaccharides dissolve readily at room temperature in 12M(72%) $H_2SO_4$, leaving a residue of darkened but recoverable lignin. Sugar composition of woods can be estimated by gas chromatographic analysis of acid hydrolysates. Recent improvements to lignocellulose analyses have been reported by a number of authors (Van Soest, 1963a & b, 1965; Englyst & Cummings, 1988). However such summative analyses ignore the frequently significant proportions of other components which are important in individual lignocellulosic materials.

## 1.3 LIGNINS

Lignins form a significant proportion of dead plant remains and their degradation by micro - organisms is of ecological importance as this process is central to the carbon - oxygen cycle that yields atmospheric carbon dioxide. Partially degraded lignocellulosic materials accumulate in temperate soils as humic and fulvic substances or humus and these can be converted to peat which may be transformed to lignite or coal (Knapp, 1985). Humus influences the structure, aeration and moisture-holding properties of soils and acts as an ion exchanger that is able to store and release nutrients to plants and trees (Ander & Eriksson, 1978).

Lignin is widely, though not universally, distributed throughout the plant kingdom. It has been identified in primitive plants, such as club mosses and ferns, but is absent from the bryophyta (true mosses). Gunnison and Alexander (1975) reported that green algae synthesize non-lignin phenolic compounds which may be quantified as lignin by the Klason technique (Effland, 1977). Moreover lignin is found in all higher plants where it acts as a structural component of support and conducting tissues and confers disease resistance (Crawford, 1981).

Lignin contents vary between plants. Cowling & Kirk (1976) found 18 - 25% lignin, on a dry- weight basis, in the stems of woody angiosperms, about 25 - 35% in gymnosperms and a wider range (10 - 30%) in monocotyledons. Plant residues and wastes, such as straws, timber and logging wastes, may have high lignin contents and may constitute disposal problems, instead of yielding valuable raw materials for generation of bulk chemicals and feedstuff (Wayman & Parekh, 1990).

### 1.3.1 Biosynthesis and chemical composition

Lignins are heterogeneous polymers of phenyl propanoid subunits which are predominantly present in the middle lamella and primary wall of plant cells. The thicker secondary cell wall has a lower lignin and correspondingly higher cellulose content (Cowling & Kirk, 1976; Knapp, 1985). The polymer is formed by

enzyme-mediated dehydrogenative polymerisation of the three methoxylated cinnamyl alcohols; p-coumaryl, coniferyl and sinapyl alcohols (Ander & Eriksson, 1978; Fig. 1.1a). The precursor, L-phenylalanine, for cinnamyl alcohol synthesis is generated from carbon dioxide by way of shikimic acid (Higuchi *et al.*, 1977). Cinnamyl alcohols are oxidised by phenol oxidases to form free radicals that polymerise to form a highly-branched, three-dimensional aromatic polymer with a range of intermonomeric bonds (Fig. 1.1b).

Three major types of lignins are recognised:

(A) Guaiacyl (G) lignins, principally of coniferyl units with small amounts of coumaryl and sinapyl units, which are found in most conifers, lycopods, ferns and horsetails.

(B) Guaiacyl - syringyl (GS) lignins, found primarily in dicotyledonous angiosperms, that contain equal amounts of coniferyl and sinapyl with only minor amounts of coumaryl units.

(C) Guaiacyl-syringyl-coumaryl (GSH) lignins, present in grasses and bamboos, which consist of approximately equal amounts of all three cinnamyl alcohols, with part of the coumaryl units being present as esterified acids (Eriksson & Lindholm, 1971; Higuchi *et al.*, 1977). Moreover significant concentrations of etherified p-coumaric and ferulic acids have been identified in extracts of cell walls of tropical grasses and forages (Jung & Shalita-Jones, 1990; Ford & Hartley, 1990).

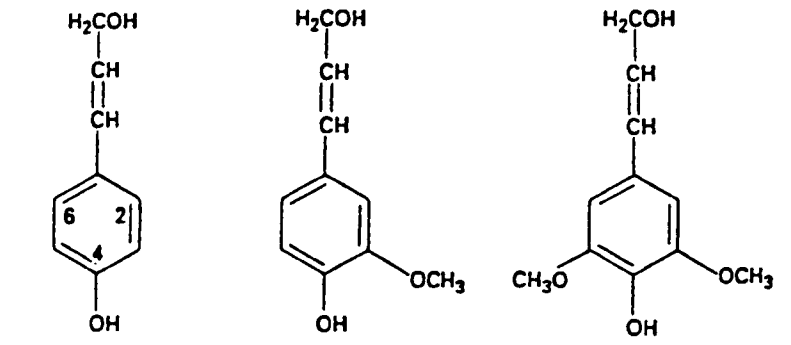
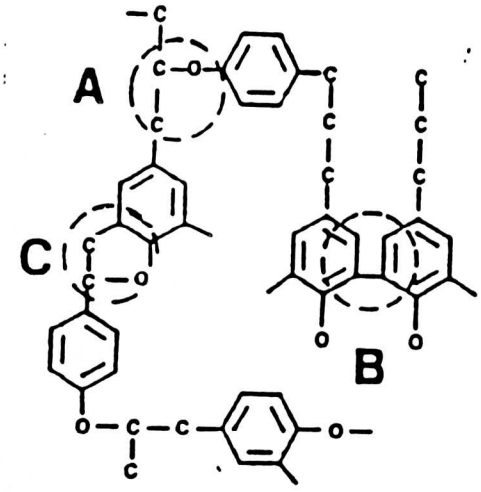



Fig. 1.1a Three lignin monomers.




*Small fragment of lignin to illustrate the three most common types of linkages. Arylglycerol-β-aryl ethers (A) account for almost half the linkages, with diphenyl (B) and phenyl coumarin (C) linkages each accounting for about 10%.*

Prince & Stiefel, (1987).

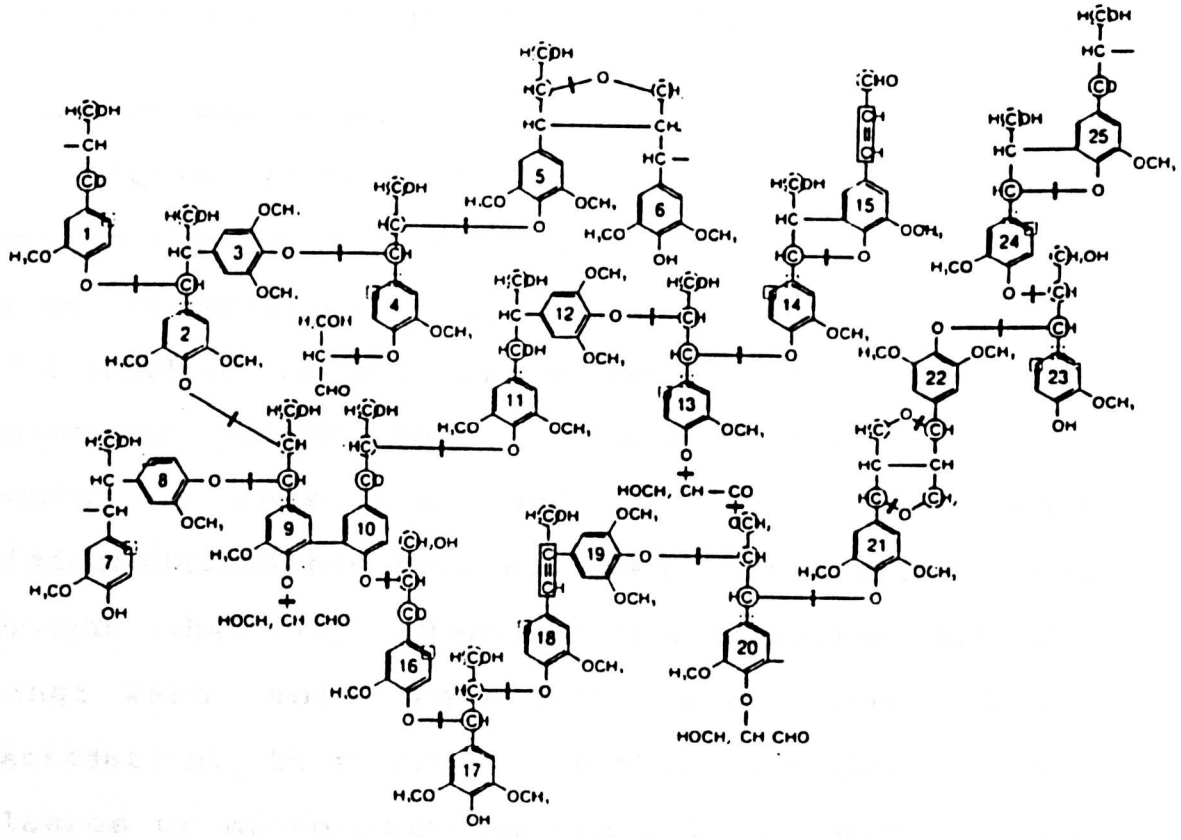


Fig. 1.1b Schematic representation of aspen lignin (Chua and Wayman, 1979). Electrophilic sites: strong O weak ◌; nucleophilic sites: strong □ weak ⬚.

Unlike carbohydrates and proteins, there are few readily hydrolysable bonds in lignin polymers ; monomer units are linked by a number of types of stable chemical bond (Leisola & Fiechter, 1985) amongst which the β-aryl ether(B-O-4) bonds predominate. In contrast, α-aryl ether bonds can be readily hydrolysed (Adler, 1977). Recent studies have revealed that sidechain alpha carbons of phenylpropanoid units are linked to plant non-cellulosic polysaccharides by ester and 4-O-methylglucuronic ether bonds ( Crawford, 1981). However, as yet the effects of such bonds on lignin biodegradation are not fully understood.

### 1.3.2 Biodegradation

Lignin is resistant to biodegradation largely because the majority of its heterogeneous intermonomeric bonds are resistant to hydrolysis. Through the use of $^{14}C$ labelled natural and synthetic lignins and low-molecular weight model compounds containing key bonds, a reasonable understanding of lignin biodegradation has been achieved. Initially it was thought that only phenol-oxidase secreting white rot fungi were able to effect significant lignin degradation. It is now known that a number of other classes of microorganisms are able to degrade and depolymerise lignin to at least a limited extent although it is unclear whether mineralisation is achieved (Table 1.2). These organisms include the brown

Table 1.2 **Representative Organisms that Degrade and/or Modify Natural Lignin**

| Organism | Effect on Lignin | | |
|---|---|---|---|
| | Modify molecular size or weight[a] | Partial chemical modification[b] | Complete chemical decomposition[c] |
| **I Eukaryotic microbes** | | | |
| White rot fungi | | | |
| *Pleurotus ostreatus* | + | + | + |
| *Phanerochaete chrysosporium* | + | + | + |
| *Coriolus versicolor* | + | + | + |
| *Polyporus anceps* | | | |
| Brown rot fungi | | | |
| *Lenzites trabea* | + | + | - |
| *Gloeophyllum trabeum* | + | + | - |
| *Poria cocos* | + | + | - |
| *Lentinus lepideus* | + | ND | - |
| *Poria monticola* | + | ND | - |
| Soft rot fungi | | | |
| *Thielavia terestris* | + | + | ND |
| *Preussia fleishhakii* | + | + | ND |
| *Chaetomium piluliferum* | + | + | ND |
| others | | | |
| *Fusarium solani* | + | + | ND |
| **II Prokaryotic microbes** | | | |
| Actinomycetes | | | |
| *Streptomyces badius* | + | + | ND |
| *Nocardia autotrophica* | + | + | ND |
| *Bacillus megaterium* | + | + | ND |
| *Acetobacter* species | + | + | - |
| *Pseudomonas* species | + | + | ND |
| **III Lignocellulose destroying animals** | | | |
| Steer | + | - | - |
| Millipede | + | - | - |
| Termite | + | + | ND |

[a] from Zeikus (1981)

Abbreviations and code to Table 1.2

\+ occurs

\- not demonstrated

ND not determined.

a As determined by physical or biochemical action on the polymer that decreases size or molecular weight.

b Determined by chemical analysis of decayed wood or lignolytic bioassays with $^{14}C$-natural lignins.

c As determined by detailed chemical-structural analysis of decayed lignocellulose.

and soft rot fungi, and both eubacteria and actinomycetes.

White rot fungi are known to degrade each of the three main polymeric components of wood at differing rates. Strains of *Polyporus versicolor* and *Ganoderma applanatum* have been reported to deplete both lignin and carbohydrate components of wood at similar rates (Kirk, 1973; Buswell & Odier, 1987). However it is known that certain white rot fungi utilise lignin in wood in preference to carbohydrates (Otjen *et al.*, 1987) . Such fungi include *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Polyporus berkeleyi* (Ander & Eriksson, 1976).

The soft rot fungi are either ascomycetes or fungi imperfecti and can decay the surface layers of many hardwoods. Esyln *et al.* (1975) observed that, of six soft rot fungi, four depleted polysaccharides faster than the lignin. However, two strains (*Paecilomyces* sp. and *Allescheria* sp.) preferentially removed lignin. However, Ander and Eriksson (1978) concluded that, in general, soft rot fungi were only able to depolymerise lignin to a limited extent. Hatakka and Uusi-Rauva (1983) obtained 1 - 8% mineralisation of $^{14}C$ -lignin with soft rot fungi as compared to 50 - 60 % with white rot fungi.

The brown rot fungi have been reported to effect modification of lignin structure whilst significantly

depleting carbohydrates resulting in the brownish colour of decaying wood (Ander & Eriksson, 1978). Kirk and Adler (1970) have shown that wood attacked by brown rot fungi had decreases in methoxy and increases in phenolic hydroxyl contents.

The extent to which bacteria depolymerise lignin during degradation is still not fully understood. Bacteria implicated in attack on lignins include strains of the eubacteria *Pseudomonas*, *Flavobacterium*, *Aeromonas*, *Xanthomonas* and *Bacillus* and the actinomycetes *Nocardia*, *Thermomonospora* and *Streptomyces* (Odier & Monties, 1978; Deschamps *et al.*, 1980; Knapp, 1985; Pelmont *et al.*, 1989). Certain of these bacteria have been reported to mineralise lignins. Robinson and Crawford (1978) recovered 11% $^{14}CO_2$ from labelled spruce $^{14}C$-lignin of spruce wood during attack by a *Bacillus* sp.

Invertebrates, or their gut microbes, also appear to effect lignin breakdown. Neuhauser and Hartenstein (1976 a & b) reported that cinnamic acid 2-($^{14}C$), vanillin 5-($^{14}C$), benzoic acid ring -($^{14}C$) and p-methoxy -phenol-$^{14}C$-methoxyl can be mineralised and $^{14}C$ incorporated into body tissues. However subsequently they were unable to show mineralisation of 14-C ring, methoxy or side chain DHP in the alimentary tracts of soil invertebrates such as isopods, snails, millipedes, slugs and earthworms in 10 days of incubation (Neuhauser *et al.*, 1978). In contrast, Butler and Buckerfield (1979) recovered 16.5 to 32.4% $^{14}CO_2$ from labelled $^{14}C$-

DHP lignin fed to a termite (*Nasutitermes exitiosus*) after 27 to 50 days of incubation. Furthermore, Crawford (1981) reported that cellulose is depleted in invertebrate guts. Zeikus (1981) concluded the gut structure of invertebrates, such as termites, is suitable for lignin degradation. Thus a contribution by gut microbes cannot be excluded.

### 1.3.3 Mechanism and enzymology of white-rot fungal degradation of lignins

It has been known for some years that lignins attacked by white-rot fungi have higher carbonyl, carboxyl and hydroxyl group and lower methoxyl and phenolic contents than sound lignins (Ishikawa *et al.*, 1963; Kirk & Chang, 1975). In contrast, radioisotopic labelling experiments have shown that soft rot fungi attack lignin largely by demethylation but also effect limited attack on aromatic rings (Ander & Eriksson, 1975; Haider & Trojanowski, 1975). Brown rot fungi appear to degrade lignin by demethylation with parallel introduction of -carbonyl groups into propanoid side-chains (Ander & Eriksson, 1978).

To date, three classes of enzyme have been proposed as effecting lignin depolymerisation: laccases, peroxidases and ligninases (Ander & Eriksson, 1976 ; Glenn & Gold, 1983; Umezawa & Higuchi, 1989). Secretion of significant concentrations of all of these enzymes is associated with attack on lignins. Ander and Eriksson

(1976) noted that Kraft lignin was not degraded when exposed to mutant strains of *Phanerochaete chrysosporium*, defective in phenol oxidase production whereas the phenotype was suppressed when purified laccase was added to media. These authors also reported that cellulolytic and xylanolytic activities were strongly inhibited by wood phenols in the mutant suggesting that phenol oxidases may act in detoxifying the zone of fungal attack by degrading or polymerising inhibitory phenolic compounds.

Laccases are able oxidatively to demethylate methoxylated aromatic acids contained in lignins yielding methanol and o-quinone (Ishihara & Miyazaki, 1974). Toxic quinones appear to be reduced to phenols by cellobiose; quinone oxidoreductases which simultaneously oxidise cellobiose to cellobiono-d lactone (Westermark & Eriksson, 1974; (Fig 1.2). However it is clear that laccases require free phenolic groups on aromatic rings whereas lignin peroxidases and ligninases are able to attack fully methoxylated rings suggesting that the latter enzymes are the key agents in lignin depolymerisation.

### 1.3.4 Biochemistry of lignolytic enzymes

**Laccases** are blue copper-containing oxidases, widely distributed in higher plants and fungi, that can utilise the full oxidizing capacity of dioxygen, effecting reduction to two molecules of water. Other blue

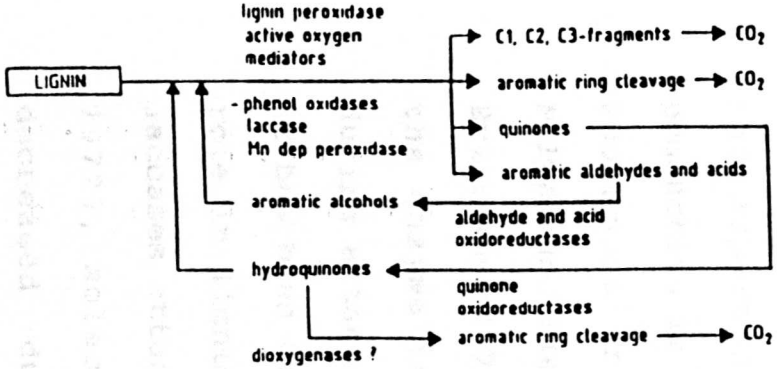



Fig. 1.2a Hypothetical scheme for lignin degradation by *P.chrysosporium*.
From; Wayman & Parekh (1990).

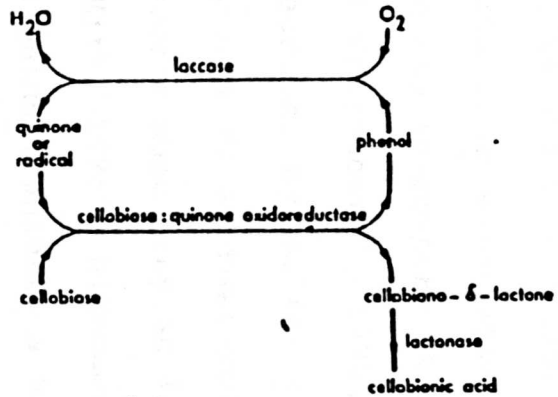



Fig. 1.2b Proposed mechanism for the action of laccase and cellobiose: quinone oxidoreductase.

From: Westermark & Eriksson, (1974) cited after Ander & Eriksson, (1975).

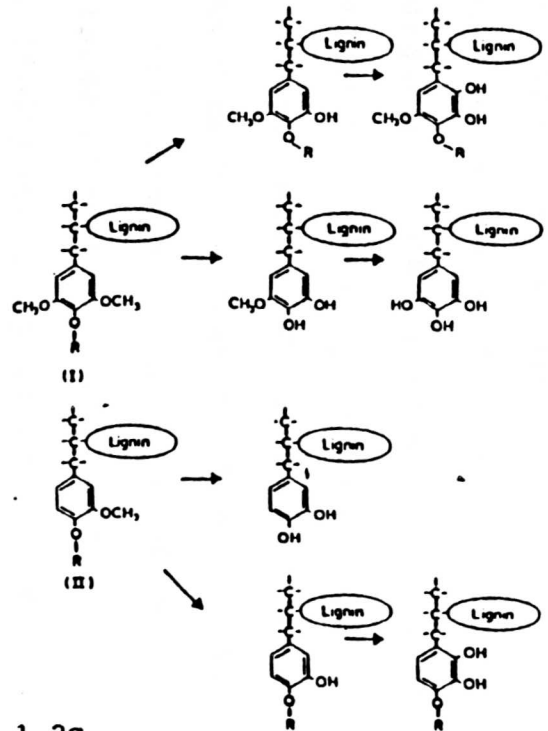



Fig. 1.2c Demethylations and hydroxylations of phenylpropane units within lignin that would produce catechol (o-diphenolic) moieties. Structure (I) represents a C-9 unit of the syringyl type (a 4-hydroxy-3,5-dimethoxyphenyl substitution pattern). Structure (II) represents a C-9 unit of the guaiacyl type (a 4-hydroxy-3-methoxy-phenyl substitution pattern). The R-group may represent H (forming a phenolic hydroxyl) or etherified (alkyl-carbon) substitutions involving an adjacent C-9 unit

oxidases, ceruloplasmin and ascorbate oxidase, have many properties in common with laccases (Reinhammar, 1984) although these are catalytically the most complex with three types of active copper centre (I - III). Laccases can oxidise mono-, di- and polyphenols with a preference for p- over o- or m- diphenols. Certain monophenols inactivate laccases but the presence of gelatine or certain detergents, such as Tween 80, appears to protect the enzyme from reaction inactivation.

Laccases are very important enzymes in fruits and other food plants such as peaches and tea, contributing to both desirable reactions and spoilage (Mayer & Harel, 1968). However these enzymes are also found in certain basidiomycetes (*Polyporus*, *Agaricus*, *Pleurotus* and *Pholiota*) and ascomycetes such as *Neurospora*, *Podospora* and *Aspergillus* (Law & Timberlake, 1980). Laccase production is either constitutive and intracellular as in *Podospora* or as several inducible exoenzymes as in *Polyporus*, *Pleurotus*, *Pholiota* and *Neurospora* (Froehner & Eriksson, 1974a; Leonowitz & Trojanowski, 1978). Since the enzyme is abundantly secreted by white-rot fungi during attack on wood, but absent from brown-rot fungi, it has been concluded that laccase must play an important role in lignin degradation (Kirk, 1971). Although fungal laccases attack milled wood lignins (Ishihara & Miyazaki, 1972), molecular weights are increased rather than decreased due to polymerisation of product quinones.

However, it is possible that laccases are important in regulating expression of genes encoding lignin-degrading enzymes, since in the basidiomycete *Agaricus*, a complex pattern of laccase production is observed (Wood, 1980a ). The molecular weight of laccases range from 60,000 to about 140,000 daltons with carbohydrate on the glycoproteins representing as much as 45% of the *Rhus* laccase. *Neurospora crassa*, *Polyporus versicolor* and *Podospora anserina* laccases have similar amino acid compositions implying strong conservation of fungal protein structure (Reinhammar, 1970; Froehner & Eriksson, 1974b). With the exception of *Podospora* laccase I(a tetrameric enzyme), each laccase consists of a single strand of 500 amino acid polypeptide.

**Ligninases or lignin peroxidases**

Ligninases are extracellular fungal peroxidase enzymes initially isolated as secreted by lignolytic cultures of the white-rot *P.chrysosporium* during idiophasic growth (Leisola *et al.*, 1984; Palmer *et al.*, 1987). Early work suggested the enzymes were only produced in static flasks but subsequently maximum level of production was achieved in agitated cultures (Leisola *et al.*, 1984; Janshaker & Fiechter, 1988). Ligninase activity can be enhanced by gassing of cultures with oxygen and addition of veratryl alcohol. Enzymes have a molecular weight between 39,000 - 43,000 daltons and spectral analyses revealed that they contain single protoporphyrin IX haems as prosthetic group (Tien & Kirk,

1984). In the oxidised states the prosthetic group can accept electrons from a range of donor molecules including potassium iodide, veratryl alcohol and dimeric lignin model compounds (Palmer *et al.*, 1987).

Oxidation reactions catalysed by ligninase can result in cleavage of C-C bonds in the propyl side chains of lignin model dimers and depolymerisation of fully methoxylated lignins (Tien & Kirk, 1983; Umezawa & Higuchi, 1989). Moreover, veratryl alcohol is oxidised to the aldehyde (Tien & Kirk, 1984) and other reactions such as oxidation of benzylic alcohols and methylene groups, hydroxylation of olefinic bonds in styrenes, decarboxylation of phenylacetic acids, cleavage of lignin ether bonds and aromatic ring opening are observed (Palmer *et al.*, 1987).

Tien and Kirk (1984) observed that ligninase activity was dependent upon hydrogen peroxide but that during cleavage of C-C or hydroxylation of benzylic methylene groups, substrates were oxidised by dioxygen rather than hydrogen peroxide, suggesting an oxygenase activity. However, it is known that oxygen uptake often accompanies classical peroxidase activity since oxygen interacts with free radicals produced as reaction intermediates (Yamazaki & Yokota , 1973) to form organic peroxy radicals (Harman *et al.*, 1986). It is also possible that oxygen participates in a shuttle mechanism, reacting with the ferrous form of the peroxidase to yield compound III which oxidises

electron donors to produce an oxidised product and superoxide (Smith *et al.*, 1982). Although oxygen could interact with the enzyme by either or both mechanism, Palmer *et al.* (1987) reported that oxygen utilization by this enzyme was only significant when substrates capable of promoting lignin bond cleavage such as veratryl alcohol or homoveratric acid were present whereas with ferrocyanide or dimethoxybenzene, uptake of oxygen was not observed. Thus degradation of radical cations to a free radical is a prerequisite for oxygen utilisation. Free carbon base radicals, such as produced from oxidation of veratryl alcohol, are highly reactive species undergoing a variety of reactions such as dimerization or polymerisation with other molecules. It is thought that oxidised veratryl alcohol may act as a low molecular weight oxidation species that can penetrate lignin polymers effecting internal depolymerisation reactions.

A further haem containing peroxidase protein was isolated from ligninolytic cultures of *Coriolus versicolor* by Evans *et al.* (1984). This 53,700 dalton polypeptide was presumed to be a glycoprotein with a protoporphyrin IX prosthetic group.

**Manganese-dependent peroxidases**

In 1984 Kuwahara *et al.* isolated a novel extracellular peroxidase enzyme from culture fluid of lignolytic cultures of *P.chrysosporium* . The enzyme, with a

molecular weight of 46 Kd, demonstrated requirements for hydrogen peroxide, manganese and lactate and performed similar catalytic reactions to horse radish peroxidase by oxidising phenol red, o-anisidine and polymeric dyes. A similar enzyme, isolated by Huynh and Crawford (1985) from lignolytic cultures of *P. chrysosporium* had a molecular weight of 45 - 47 Kd and showed a requirement for manganese and hydrogen peroxide but did not require lactate.

Both enzymes have been purified and characterised (Glenn & Gold, 1985) and were found to be identical. According to the authors both contain a single protohaeme IX, with a high spin ferric iron. The enzyme is capable of oxidising Mn(II) to Mn(III), which in turn oxidizes the organic substrates. Manganese peroxidases have been reported to function as phenol oxidizing enzymes and possibly they participate in hydrogen peroxide production.

### 1.3.5 Physiological factors affecting lignin degradation

Supply of alternate carbon and nitrogen sources for growth appears to be central in regulation of lignin degradation in both *C. versicolor* and *P. chrysosporium*. In *P. chrysosporium* rate and extent of lignin mineralisation is related to type and concentration of carbohydrates in growth media (Kirk *et al.*, 1976; Buswell & Odier, 1987). Kirk *et al.* (1976) assessed the influence of carbohydrates such as xylose, glucose,

cellobiose, cellulose and xylan on mineralisation of $^{14}$-C DHP by *P.chrysosporium* and *C.versicolor*. A parallel relationship between concentration of glucose and cellulose and extent of mineralisation was observed. In control cultures when spruce lignin was sole carbon and energy source, mineralisation was very low and no increase of biomass was recorded. However, in a strain of *P. chrysosporium* enhanced ligninolytic activity was observed when glycerol, a relatively poor carbon source, was supplied (Buswell *et al.*, 1984). Delignification was suppressed in solid state fermentation of straw by *Coprinus* species when supplemented with either molasses or whey. The reason why a carbohydrate co-substrate is required is unclear. It is possible that energy may be required either for synthesis of enzymes and biomass (Dagley, 1978; Kuwahara *et al.*, 1984) or alternatively for generation of the hydrogen peroxide required for oxidation of lignin substrates.

The role of veratryl alcohol in lignin degradation by *P. chrysosporium* is also complex since addition of exogenous veratryl alcohol to growth media stimulates lignin degradation, the compound plays a role in the oxidative mechanism of lignin depolymerisation (Faison & Kirk, 1985) and strains secrete the compound as a secondary metabolite (Lundquist & Kirk, 1978).

Ligninolytic activity is also stimulated by nitrogen

limitation of cultures (Kirk *et al.*, 1978; Glenn & Gold, 1983; Hatakka *et al.*, 1983 ). Keyser *et al.* (1978) showed that *P.chrysosporium* cultures, grown in 2.4 mM N media, were nitrogen limited within 48 to 72 h and showed mineralisation of $^{14}C$(ring)-DHP approximately 24h later. In contrast, media containing 24 mM N inhibited release of $^{14}CO_2$ from dimeric lignin model compounds and depolymerisation of aspen lignin by *P. chrysosporium*.

However, regulation of lignin mineralisation and production of lignolytic enzymes appears to be different in other basidiomycetes (Freer & Detroy, 1982; Leatham & Kirk, 1983). Freer and Detroy (1982) reported that nutrient nitrogen had no significant effect on the rate of lignin degradation by a basidiomycete culture isolated from cow dung. Similarly, Leatham and Kirk (1983) observed minimal influence of nutrient nitrogen in *Pholiota mutabilis*, whereas with *Lentinus edodes* and *P. ostreatus*, mineralisation of $^{14}C$-(ring)-DHP was enhanced at higher concentrations of nitrogen. Moreover, alternative nitrogen sources, such as the amino acids glutamate and histidine, also inhibited mycelial binding and DHP depolymerisation (Chua *et al.*, 1983). Reid (1983) reported that supplementation of *P. chrysosporium* culture media with complex nitrogen sources at the onset of lignolytic phase repressed mineralisation and delayed the onset of lignin degradation.

Other media components, such as thiamine, surfactants and mineral ions influence rates of lignin

decomposition (Leatham, 1986; Leisola & Fiechter, 1985; Janshekar & Fiechter, 1988). Leatham (1986) observed that the divalent cations calcium and manganese stimulated lignolytic activity in *P. chrysosporium* and *L. edodes* at 40 ppm and 10 ppm, respectively. In contrast, Janshekar and Fiechter (1988) concluded that absence of minerals does not repress production of lignin peroxidases. It is also known that certain surfactants such as Tween 80, polyethylene glycol and oleic acid enhance lignin peroxidase activity although the mechanism is not clear (Janshaker and Fiechter, 1988).

Lignin depolymerisation is an oxidative process and increased atmospheric oxygen has been reported to enhance attack by wood rotting fungi (Kirk *et al.*, 1978; Bar-lev & Kirk, 1981; Reid & Seifert, 1982). Leisola *et al.* (1984) recorded significant increases in lignolytic activity of *P. chrysosporium* with 30% carbon dioxide and 10% oxygen but inhibition in a 100% oxygen atmosphere. In contrast, Kirk *et al.* (1978) had earlier demonstrated, with this organism, absence of lignin depolymerisation at 5% oxygen but a two to three fold increase in 100% oxygen . Although slow mineralisation of $^{14}$C-(lignin)-lignocellulose and $^{14}$C-DHP has been observed in anaerobic salt marsh sediments and thermophilic (55$^{o}$C) enrichment cultures (Benner *et al.*, 1984; Benner & Hodson, 1985), there are few published reports of the process in the absence of oxygen although conversion to

methane and carbon dioxide by mixed bacterial populations has been recorded (Zeikus *et al.*, 1982; Colberg & Young, 1982; Brunner *et al.*, 1987; Field & Lettinga, 1989 ). Related low molecular weight aromatic compounds are also effectively catabolized anaerobically (Healey *et al* , 1980; Schink, 1988).

Culture conditions are not the sole factors limiting lignin depolymerisation (Zeikus, 1980; Faison & Kirk, 1985). The presence of a warty layer, of unknown composition, which covers the plant cells appears to restricts cell-wall attack by micro-organisms (Engels & Brice, 1985): this stains for protein and can be disrupted by detergent treatments (Morrison, 1986). Moreover compounds, such as halides, or ions that attack covalently bound metals inhibit lignin degradation and ligninases are inactive at neutral and alkaline pH through deprotonation of active site groups in the active sites. Similar effects are observed with laccases (Branden *et al.*, 1973; Andreasson & Reinhammar, 1979).

## 1.4 CELLULOSES

### 1.4.1 Natural cellulosic materials

Cellulose is the most abundant of the biomass polymers and is the principal structural polysaccharide of plant cell walls. It is not often present in a pure form in nature, confers a high tensile strength to plant tissues and is recalcitrant to microbial degradation (Cowling & Kirk, 1976; Eveleigh, 1987). It is normally

found in close association with other cell wall components: hemicelluloses, pectins, lignins, and minerals such as silicates (Table 1.3a). Celluloses are found in lower plants such as algae and fungi and in certain bacteria (Knapp, 1985), and form varying proportions of stems, leaves and all higher plant tissues (Table 1.3b). However celluloses are also major components of sewage solids, domestic wastes and animal excreta, forming the major fraction of solid organic waste in the U.S.A. (Humphrey, 1975)

### 1.4.2 Structure and chemical composition

Celluloses consist of anhydroglucose residues linked by B 1 - 4 glucosidic bonds to form glucan chains. The degree of polymerisation (DP) of such glucans has been calculated as ranging from 15 to 14,000 (Fig. 1.3a). Molecular weight determinations relying upon osmometry and cryoscopy, in which the laws of dilute solutions are used, estimate numbers of solute molecules as number averages of degrees of polymerisation (Pn). More recently, viscometry has been used to obtain weight average of degrees of polymerisation (Pw) (Gascoigne & Gascoigne, 1960). Knapp (1985) estimated glucan chains as in the range $2.5 \times 10^5 - 2.5 \times 10^6$ (7 $\mu$).

Individual glucan chains aggregate into cellulose microfibrils by lateral hydrogen bonding forming a highly oriented crystalline structure (Cowling & Kirk, 1976). Less ordered parts of the microfibrils are referred to as

TABLE 1.3a COMPOSITION OF COTTON AND TYPICAL ANGIOSPERM (BIRCH) AND GYMNOSPERM (SPRUCE) FIBRES.

| CONSTITUENT | COTTON | BIRCH | SPRUCE |
|---|---|---|---|
| | | (% dry weight). | |
| Holocellulose | 94.0 | 77.6 | 70.7 |
| Cellulose | 89.0 | 44.9 | 46.1 |
| Non-cellulosic polysaccharide | 5.0 | 32.7 | 24.6 |
| Lignin | 0.0 | 19.3 | 26.3 |
| Protein(N X 6.25) | 1.3 | 0.5 | 0.2 |
| Extractables | 2.5 | 2.3 | 2.5 |
| Ash | 1.2 | 0.3 | 0.3 |

From: Cowling & Kirk (1976).

TABLE 1.3b MAJOR SOURCES AND RELATIVE ABUNDANCE OF CELLULOSE IN NATURE.

| Source | Cellulose(%) | Lignin(%) | Non cellulosic polysaccharide |
|---|---|---|---|
| Hardwoods(angiosperms) | 40 - 55 | 18 - 25 | 24 - 40 |
| Softwoods(gymnosperms) | 45 - 50 | 25 - 35 | 25 - 35 |
| Monocot stems(Grasses) | 25 - 40 | 10 - 30 | 25 - 50 |
| Parenchyma cells of leaves | 15 - 20 | | 1 - 5 |
| Fibres(cotton/flax) | 80 - 90 | | 5 - 20 |
| Newsprint | 40 - 55 | 18 - 30 | 25 - 40 |
| Waste fibres(from chemical pulping). | 60 - 80 | 2 - 10 | 20 - 30 |

From: Cowling & Kirk, (1976).

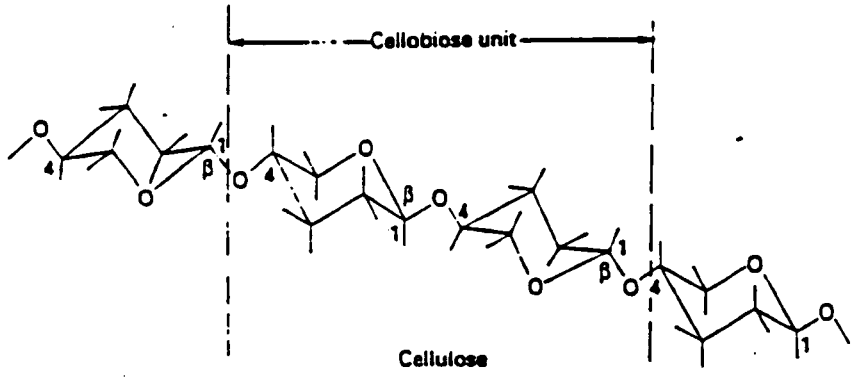



Fig. 1.3a Glucose, cellobiose, cellulose.

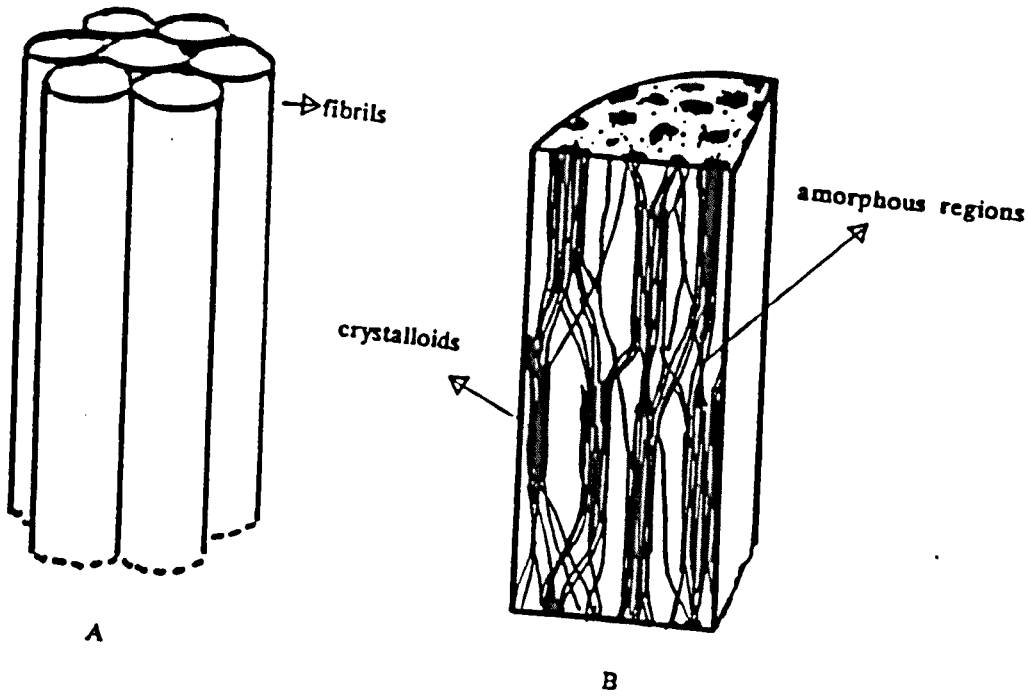



Fig 1.3b Alignment and composition of elementary fibrils of cellulose. A. bundle of parallel fibrils held together crosswise by hydrogen bonds. B. lateral sectional view of one fibril(after Sihtola & Neimo, 1975).

amorphous or para-crystalline cellulose (Fig. 1.3b ). Preston and Cronshaw (1958) estimated microfibrils as 500 nm in cross section suggesting a rectangular crystalline core, surrounded by a para crystalline sheath. X-ray diffraction and density determinations have been used to assess the ratio of crystalline and amorphous cellulose in lignocelluloses (Hermans, 1949). Many methods measure only the rate of penetration of liquids into the fibre, estimating either amorphous material or the accessibility of the sample. A high degree of crystallinity reflects a perfect orientation, conferring great strength and rigidity but a decrease in fibre elasticity.

### 1.4.3 Biodegradation of celluloses

Biological depolymerisation of celluloses is important in ecosystems, ensuring recycling of nutrients. Such breakdown is normally effected by hydrolytic enzymes referred to as cellulases, secreted by many organisms with the exception of mammals. Among microorganisms, fungi such as phycomycetes, ascomycetes, basidiomycetes and fungi imperfecti (Wood, 1980b: Knapp, 1985), appear to be particularly active in aerobic environments, such as soils and water. Strains of *Trichoderma*, *Aspergillus*, *Penicillium*, *Fusarium*, *Chaetomium*, *Rhizoctonia* and *Verticillium* have been noted as cellulolytic (McCrae *et al.*, 1989). Moreover, fungal cellulolytic activity has also been reported in anaerobic habitats such as water-

logged soils, animal guts and aquatic sediments, and anaerobic phycomycetes have a central role in cellulose breakdown in the rumen (Orpin, 1981; Borneman *et al.*, 1989). Three morphologically-distinct phycomycetes, isolated from horse caecum, were similar to *Piromonas communis* and *Sphaeromonas communis* (Orpin, 1981).

Bacteria that effect aerobic breakdown of cellulose include members of *Cellulomonas*, *Myxobacteria*, *Bacillus* and *Actinomycetes* (Edberg & Hofsten, 1975). Anaerobic bacteria are also key cellulose degraders and mammals, notably ruminants, have evolved digestive systems containing specialised fermentations in the upper (bovine rumen) and lower gut (horse caecum). Important cellulolytic bacteria in the rumen are *Bacteroides succinogenes*, *Butyrovibrio fibrosolvens*, *Eubacterium cellulosolvens*, *Ruminococcus albus*, and *R. flavifaciens* (Hobson, 1971) and these are known to depolymerise celluloses to yield a mixture of volatile acids and gases.

The protozoa are the least studied of the cellulolytic organisms. Axenic cultures of protozoa have been shown to depolymerise carboxymethylcelluloses (Yamin & Trager, 1979) and rumen protozoa to metabolise cellulose and xylan (Ushida *et al.*, 1987; Onodera *et al.*, 1988). Cellulolytic flagellate protozoa have been reported in the hind gut of wood-eating termites (Yamin & Trager, 1979).

### 1.4.4 Mechanism and enzymology of cellulolysis.

Cellulolytic fungi appear to depolymerise natural celluloses by a cooperative action of a number of enzymes (Klyosov *et al.*, 1980; Wood, 1980; Eveleigh, 1987). Primary attack on crystalline cellulose is effected by two classes of enzyme; endo -β 1 - 4 glucanases (EC3.2.1.41) and exo-β 1 - 4 glucanases or cellobiohydrolases (EC 3.2.1.91). Cellodextrin products are hydrolysed to monosaccharides by β-glucosidase or cellobiase (EC3.2.1.21). In the first model of cellulase action proposed, Reese *et al.* (1950), postulated the existence of hydrolytic ($C_X$) and non-hydrolytic enzymes($C_1$). It was proposed that $C_1$ initiated hydrolysis of crystalline cellulose by cleavage of hydrogen bonds of glucan chains, enabling chain breakage by $C_X$ (Fig. 1.4a). In this hypothesis $C_1$ enzymes are produced by microorganisms capable of degrading crystalline celluloses but not by those that decompose only soluble derivatives and amorphous celluloses. The exact initial attack on native cellulose was not clear. Recent reports suggest that $C_1$ enzymes are exo β 1 - 4 glucanases while $C_X$ activity is that of endo β 1 - 4 glucanases (Gong & Tsao, 1979; Eveleigh, 1987). In the model of Eveleigh (1987), supported by electron microscopy, endoglucanase($C_X$) acts randomly in hydrolysing bonds within glucan chains, especially in amorphous zones, generating multiple sites for attack by

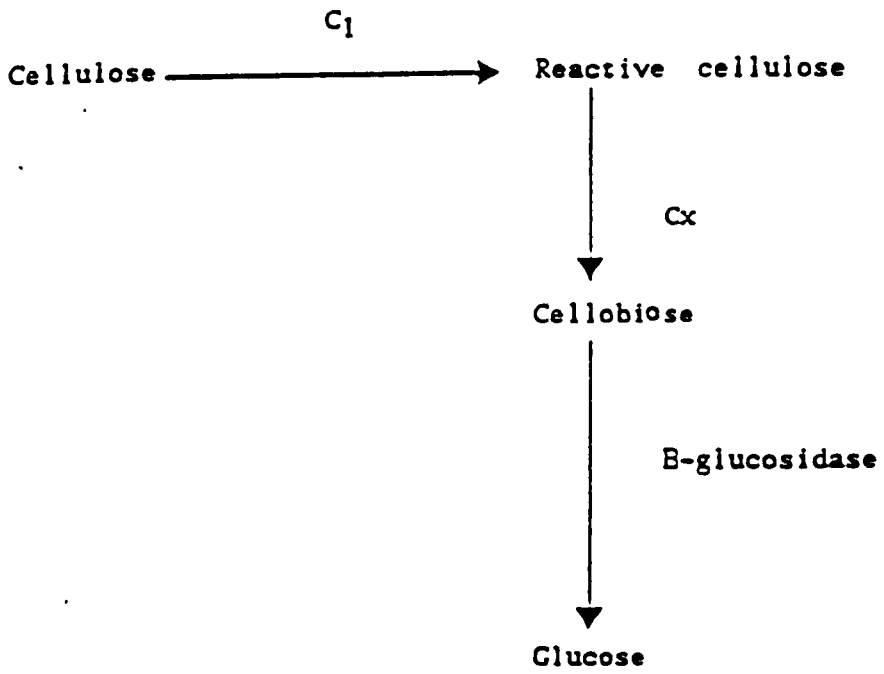



Fig. 1.4a Mechanism of enzymatic hydrolysis of cellulose according to Reese *et al.*, (1950).

cellobiohydrolases($C_1$) (Fig. 1.4b). Exoglucanase($C_1$) attack splits off cellobiose disaccharide units from the non-reducing end of chains. The third enzyme, cellobiase, completes saccharification by degrading cellobiose to glucose (Humphrey *et al.*, 1977; Wood, 1980; Woodward, 1987) alleviating 79% inhibition of depolymerising enzymes at 0.01% concentration; glucose, also a potent inhibitor of depolymerisation, is only 45% inhibitory at 0.01% (Wood, 1980b).

However, oxidative enzymes are also involved in cellulose degradation, minimising catabolite repression of cellulase synthesis and feedback inhibition of cellulase activity (Eriksson, 1981). Two important oxidative enzymes are glucose and cellobiose oxidases which use molecular oxygen to oxidise cellodextrins (cellobiose) to aldonic acids (cellobionic acid), and glucose to gluconolactone (Eriksson, 1978, 1983).

### 1.4.5 Synergy between hydrolytic cellulases

In the seventies, two groups independently proposed schemes to explain the cooperative mechanism of enzymic depolymerisation of cellulose. Wood and McCrae (1979) showed that only two of the three cellulolytic enzymes were required for cellulose saccharification. Enzymes from culture filtrates of *Trichoderma reesei* and *Fusarium solani* were mixed and shown to yield soluble oligomers, cellobiose and glucose whereas individual enzymes were

CELLULASE: A PERSPECTIVE

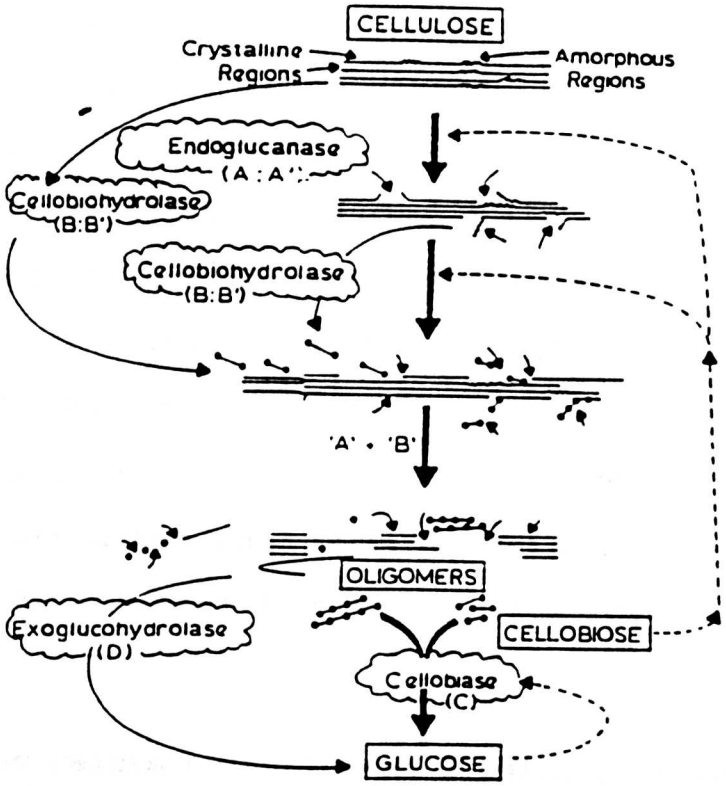



Fig.1.4b . Generalized scheme for cellulolysis. Cellulose is initially attacked in amorphous zones by endoglucanases, (A:A'), thus generating multiple sites for attack by cellobiohydrolases (B:B'). The continued cooperative action between exo- and endo-splitting polysaccharides continues, combined with the terminal action of cellobiase (C) to yield glucose. The cooperative action of cellobiohydrolase and endoglucanase is synergistic. Most individual enzymes do not promote effective hydrolysis, although certain cellobiohydrolases will completely degrade crystalline cellulose. Exo- and endopolysaccharases can each occur in multiple forms and can exhibit different specificities in relation to the degree of polymerization of the substrate. Two forms of each endoglucanase (A:A') and cellobiohydrolase (B:B') are theoretically possible based on the two stereoconfigurations of the cellobiosyl unit in the chain (see text). In contrast, in the attack of soluble oligomers such as by exoglucohydrolase (D) (Klyosov *et al.* 1980), only one enzyme is necessary as the enzyme can initiate attack from either 'side' of the substrate.

From ; Eveleigh (1987)

essentially inactive (Eveleigh, 1987). Wood (1980) also showed that mixtures of endoglucanase and β-glucosidase, or β- glucosidase and cellobiohydrolase had little effect on ordered celluloses (Table 1.4). Wood (1980) and Fagerstam and Pettersson (1980) made important contributions to the understanding of enzyme synergy. Cooperativity may be attributed to the need to attack the two possible orientations of terminal glucan cellobioses (Wood, 1985) since if endoglucanases initiated attack on cellulose crystal surfaces, stereochemistry would dictate that only one of the possible glycosidic linkages would be cleaved by each enzyme (Fig. 1.5). Fagerstam and Pettersson (1980) reported that the two immunologically distinct *Trichoderma reesei* cellobiohydrolases (CBHI and CBH II) also exhibited two-fold synergism in mixtures and this has also been observed in *Penicillium funiculosum* (Wood, 1985).

### 1.4.6 Constraints on cellulose depolymerisation

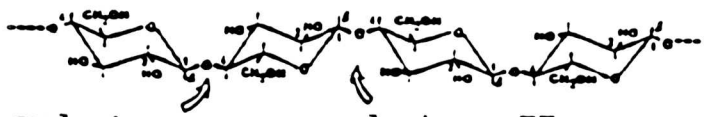
Rate and extent of cellulose degradation is determined by physical and chemical features of natural cellulosic materials. Enzyme attack is limited by low surface area/volume ratio, high degree of polymerisation, and molecular weight, degree of crystallinity, and association with other polymers (Cowling & Kirk, 1976; Wood, 1985). Moreover cotton cellulose glycosidic bonds are less accessible to hydrolytic enzymes at less than

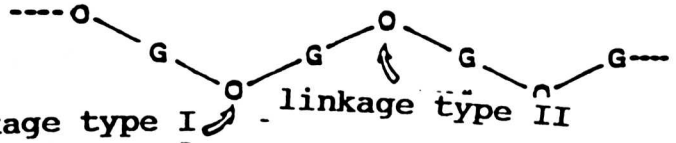
TABLE 1.4 CELLULASE (cotton solubilising) ACTIVITY OF THE COMPONENTS OF *TRICHODERMA KONINGII* AND *FUSARIUM SOLANI* CELLULASE WHEN ACTING ALONE AND IN COMBINATION

| ENZYME | COTTON SOLUBILIZATION(%) | |
|---|---|---|
| | *F. solani* | *T. koningii* |
| Cellobiohydrolase(CBH) | 1 | 2 |
| Endo-(1 -- 4)-B- glucanase | 1 | 1 |
| B-glucosidase | Nil | 1 |
| Cellobiohydrolase + endoglucanase | 53 | 58 |
| Endoglucanase + B-glucosidase | 6 | 4 |
| Cellobiohydrolase + Endoglucanase + B-glucosidase | 72 | 71 |
| Original unfractionated enzyme | 71 | 71 |

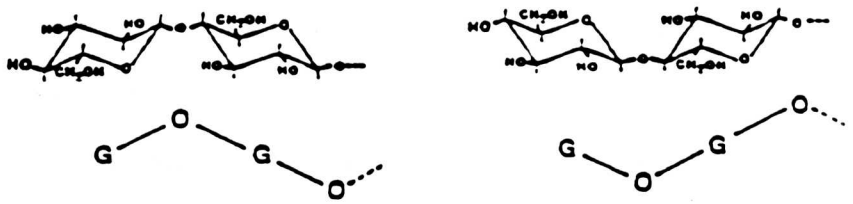
All enzymes were present in the same proportion in which they were in the original cell free culture filtrate. Incubations were for 7 days at 37$^{o}$C.

From: Wood *et al.* (1980).




Cleavage of cellulose chain can be affected by either endoglucanase type I or type II attacking type I or II glucosidic linkages.



non reducing end group (type I)

non reducing end group (type II)

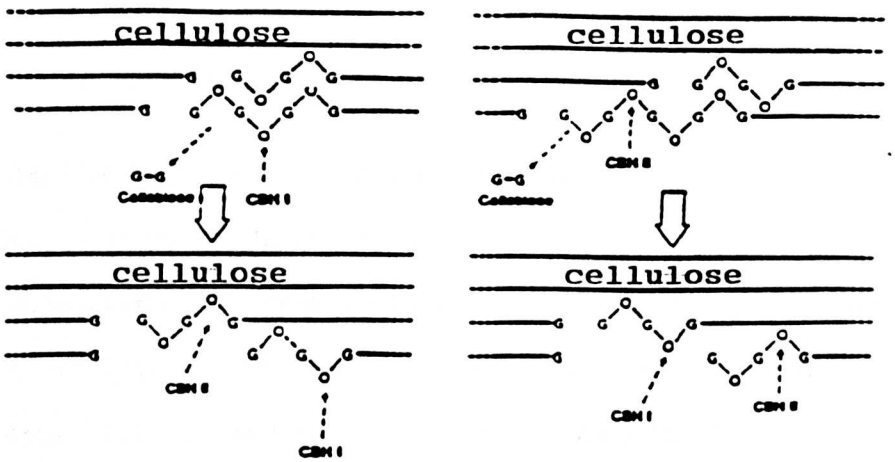



Fig. 1.5 Mechanism of synergistic action of *P.pinophilum* cellulase. From Wood *et al.* (1988).

10% moisture. Cellulases appear to degrade amorphous regions of cellulose molecules rapidly but not crystalline sections.

Gascoigne and Gascoigne (1960) concluded that divalent cations (manganese, cobalt, magnesium and calcium) stimulated microbial attack whereas other metals (mercury, chromium, silver, copper and zinc) were inhibitory and phenolics have been reported to inhibit both microbial growth and enzyme activities (Eriksson, 1978). In contrast, Muller *et al.* (1988) concluded that cellulase production by *Trametes versicolor* was stimulated by phenolic compounds in growth medium. Laccase appeared to oxidise phenols to quinones, reduced by cellobiose quinone oxidoreductases, with conversion of cellobiose to cellobionolactone, reducing product repression of depolymerising enzymes.

## 1.5 NON-CELLULOSIC POLYSACCHARIDES (HEMICELLULOSE)

### 1.5.1 Structure of hemicellulose

Hemicelluloses or non-cellulosic polysaccharides (NCP), are often regarded as being predominantly arabinoxylans and arabinogalactans (Amado & Neukon, 1985). Structurally hemicelluloses are heteropolymers of a range of pentose and hexose monosaccharides linked by glycosidic bonds, and often highly branched (Tsao & Chiang, 1983: Puls & Poutanen, 1990). Monosaccharides found in hemicellulose polymers include xylose, arabinose, mannose, galactose, glucose and a number of

related uronic acids. Xylose is often the major component. Acetic acid is also present in hemicellulose hydrolysates.

Hemicelluloses are often thought of as xylans (B 1 - 4) found in angiosperm lignocellulose (Fig. 1.6), but in softwoods are arabino-4-0 methylglucuronoxylans and in hardwoods 0 -acetyl -4-methylglucuronoxylans. Softwoods differ from hardwood xylans in being less acetylated with arabinose side-chains linked by β -1,3 glycosidic bonds (Puls & Poutanen, 1990). Hardwood xylans appear to have longer chain length (DP = 150 - 200) than those of soft woods (DP = 70 - 130) (Zimbo & Timell, 1967).

### 1.5.2 Enzymology of hemicellulose degradation

Amongst the various types of hemicellulases, xylanases have been most widely studied and found to be endo-acting hydrolases. These enzymes appear to be widely distributed in both bacteria and fungi. Exo-glycosidases, such as β-xylosidase further hydrolyse xylooligomers to component monosaccharides. Endo-xylanases can be differentiated into those yielding primarily xylose and xylobiose and those that produce larger oligomers: both are unable to hydrolyse L-arabinosyl branch points. A further group of enzymes attacks branch points. The hemicellulase α-L-arabinofuranosidases liberate L-arabinose from arabinoxylans, arabinans and arabinogalactans (Kaji, 1984; Biely, 1985). Secretion of these enzymes by strains of certain actinomycetes, such

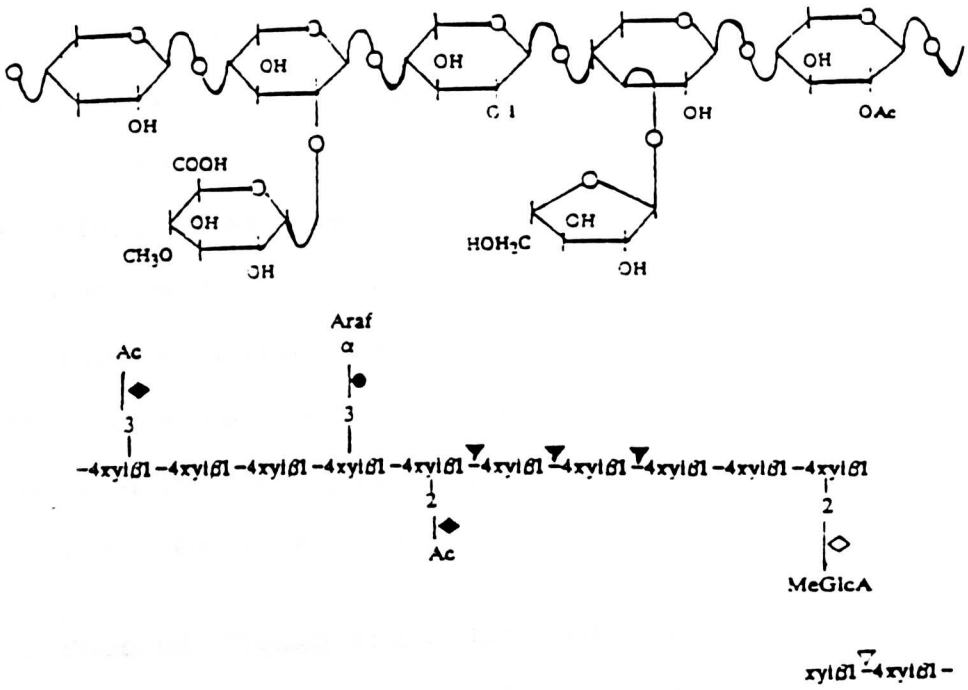



Fig. 1.6 A hypothetical plant xylan and the sites of its attack by microbial xylanolytic enzymes. The fragment comprising five D-xylose units is presented in the upper part of the figure. Ac, acetyl group; Araf, L-arabinofuranose; MeGlcA, 4-O-methyl-D-glucuronic acid; Xyl, D-xylose. (after Biely, 1985)

as *Microspora bispora* and *Streptomyces sp.*, is induced by arabinan, xylan, wheat bran or wheat straw (Poutanen *et al*, 1987; Zimmermann *et al*, 1988). Methylglucuronidases(-4-O-methylglucuronidase) also act synergistically with endo-xylanases, in hydrolysing B-1,2 linkages between glucuronic acid sugar moeities and xylose residues in xylans.

Other important classes of hemicellulase include mannanases, hydrolysing β-1,4 glycoside bonds in mannans (Zimmermann, 1990). and ferulic and coumaric acid esterases. These enzymes, hydrolysing important linkages between phenolic compounds and residues in arabinoxylans in grasses (Muller-Harvey *et al.*, 1986), are secreted by actinomycetes during growth on oat-spelt xylan, wheat bran and maize lignocellulose (Mackenzie *et al.*, 1987; Donelly & Crawford, 1988).

**1.6 Uses of lignin and holocelluloses**

Chemically modified lignins are obtained as byproducts of pulping. Three types are widely available : low molecular weight lignosulphonates from sulphite pulping, kraft lignins such as Indulin AT and thiolignins. The latter products are more condensed than native Bjorkman or milled wood lignins. The value of industrial lignins is primarily in physical rather than chemical properties with lignosulphonic acids, for example being used, in animal feed formulations, mineral

and coal pelletisation and as a glue substitute (Dupin, 1986: Table 1.5). Chemical processing yields low molecular weight compounds such as vanillin, a valuable synthetic flavouring for ice cream, and the polymer is used in production of the pharmaceutical L-dopa, for the treatment of Parkinson's disease (Wayman & Parekh, 1990). Rotstein *et al.* (1981) reported that lignin, when used as a human dietary additive, lowers blood cholestrol and it has been used for treatment of bloat in cattle.

Celluloses and derivatives have wide applications in food, textile and pulping industries. Regenerated celluloses, utilising the viscose process, are formed into artificial silks and other textile fibres, such as rayon, and films like cellophane and cellulose acetates. Moreover viscose solutions of cellulose may be cast into forms such as sponges. Wood pulp products include consumer hygiene products and a range of papers, cards and wood replacements (Wayman & Parekh, 1990) as well as a number of other applications (Fig. 1.7).

Hemicelluloses are also converted into valuable byproducts. For example hydrolysis to xylose can be followed by crystallisation of the sugar and hydrogenation to xylitol or dehydrogenation to furfural. Xylitol is an important sweetener, and furfural is used in oil refining and in furane resins. Hemicellulose solutions can be evaporated to yield wood molasses used as a flavouring and binders in animal feeds.

**Table 1.5** Lignosulphonate sales* in Europe, 1986 (Dupin, 1986)

| | *'000 Tonnes dry matter* |
|---|---|
| Binder for mineral pelletization | 50 |
| Binder for soil stabilization and fertilizers | 15 |
| Binder for organic material (coal) | 25 |
| Binder for animal feed | 105 |
| Binder as glue substitute | 30 |
| Concrete and cement additives | 20 |
| Dispersant for agricultural chemicals | 25 |
| Dispersant and use in ceramics | 25 |
| Drilling and additive | 25 |
| Dye dispersant, pigment | 3 |
| Miscellaneous | 20 |
| Total | 343 000 tonnes |

* Average lignosulphonate prices have value from $/t 45–50 from the mill for the cheapest liquid to $/t 300–400 for the most efficient powder and $/t 600–700 for specific products used in dye industry

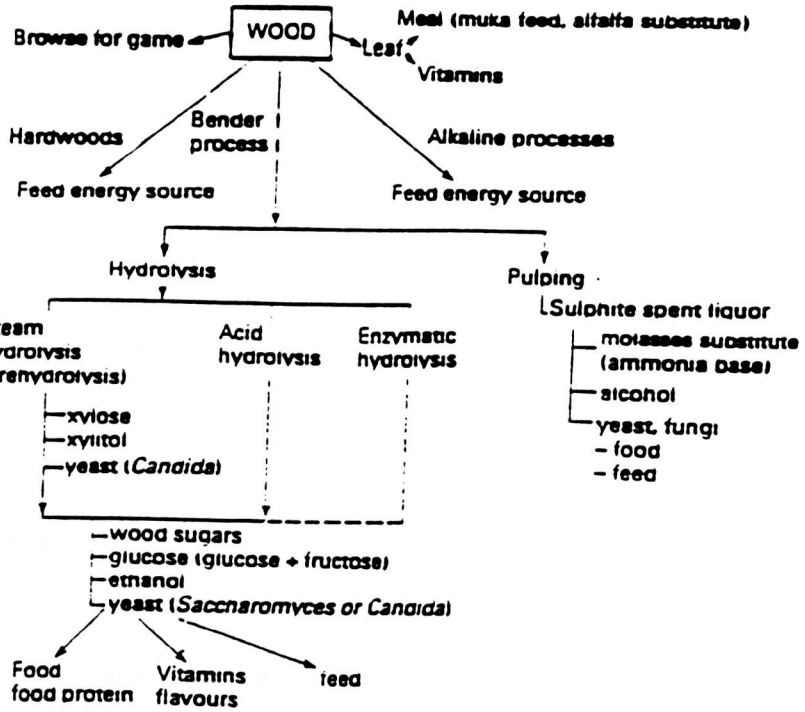



Fig. 1.7a Food and feed products from wood
From; Wayman & Parekh (1990).

PRODUCTS FROM WOOD CELLULOSE

**Cellulose**

- papers
  - cultural
  - industrial
- hygienic products
- fillers for plastics

**Regenerated cellulose**

- viscose rayon
  - cuprammonium rayon
  - film ('cellophane')
  - tyre cord
  - sponges

**Esters**

- acetate
  - fibre
  - photofilm
  - cigarette filters
  - plastics
- butyrate plastics
- nitrate
  - explosives
  - varnishes

**Ethers**

- carboxymethyl cellulose
- methyl cellulose
- ethyl cellulose
- hydroxyethyl cellulose

**Sugars**

- feed
- ethanol

**Grafts**

- acrylates

Fig. 1.7b Products from cellulose
From; Wayman & Parekh (1990).

## 1.7 ENHANCING DIGESTIBILITY OF LIGNOCELLULOSES

### 1.7.1 Physical treatments

Physical treatments reduce the effects of crystallinity and lignin in limiting enzymatic hydrolysis (Norkrans, 1950; Millet *et al.*, 1975; Fan *et al.*, 1981a). A major limitation in cellulose depolymerisation is steric exclusion of cellulases from plant cell wall capillaries (<10 nm diameter), limiting enzymic attack to the exterior of polymers (Whitaker *et al.*, 1954). Physical pretreatments can reduce crystallinity and increase exposed surface area. Such pretreatments can be divided into mechanical, including the various forms of milling, and non-mechanical such as irradiation, pyrolysis and steaming.

#### 1.7.1.1 Milling

Shearing and impacting forces yield a powdery substrate of low crystallinity index, with enhanced access to enzymes (Fan *et al.*, 1981b). Such mechanical pretreatment methods include ball, " two roll", hammer, colloid and vibroenergy milling and extrusion (Mandels *et al.*, 1974; Han & Callihan, 1974; Tassinari & Macy, 1977).

Fan *et al.* (1981b) observed a four-fold increase on the rate of enzymatic saccharification of ball-milled wheat straw and Humphrey *et al.* (1977) reported 72, 80 and 89% digestion of different size fractions (>149μ, >74μ, >45μ) of Avicel exposed to 24h attack by a strain

of *Thermoactinomyces*. Puri (1984) noted that although ballmilling of cotton linters for 15 min decreased crystallinity from 80 to 68% with bagasse increases in digestibility could be ascribed primarily to reductions in particle size and increased surface area. In contrast, Fan *et al.* (1981b) recorded appreciable increases in rates of hydrolysis following moderate reductions in crystallinity indices of ball-, Fitz- and roller-milled wheat straw (Table 1.6) although they were unable to explain the enhanced rate of hydrolysis of roller milled straw.

Using municipal solid waste exposed to culture filtrates of *Trichoderma reesei*(QM 9414), Gracheck *et al.* (1981) recorded enhancement of saccharification from 6.7% to 22.4% with hammer milling, considered to give size reduction in newsprint without increasing exposure of cellulose to hydrolysis (Mandels *et al.*, 1974).

#### 1.7.1.2 Steaming and freeze explosion technique

Steaming and freeze explosion have long been used as pretreatments for lignocellulosics to enhance susceptibility to enzyme attack (Jurasek, 1979; Ulmer *et al.*, 1981; Rai & Mudgal, 1987). Ulmer *et al.* (1981) used a 19 l pressure steam generator, connected to a 3.8 l stainless steel treatment vessel by 6.35 mm steel line, and reported complete solubilization of hemicelluloses, a slight increase in lignin and no appreciable change in cellulose and crude protein contents of steam treated

TABLE 1.6 EFFECT OF PHYSICAL PRETREATMENT ON HYDROLYSIS RATE AND CRYSTALLINITY INDEX.

| TYPE OF PRETREATMENT | EXTENT OF HYDROLYSIS AFTER 8h | RELATIVE EXTENT OF HYDROLYIS AFTER 8h | MAXIMUM CONVERSION (%) | CRYSTALLINITY INDEX |
|---|---|---|---|---|
| BALLMILLING | | | | |
| 4h | 7.2 | 3.4 | 23.6 | - |
| 8h | 9.1 | 4.3 | 23.3 | 66.9 |
| 16h | 7.2 | 3.4 | 19.1 | - |
| 24h | 9.8 | 4.6 | 19.1 | - |
| FITZ MILLING | | | | |
| Coarse | 2.7 | 1.3 | 8.2 | - |
| Fine | 3.0 | 1.4 | 9.1 | 73.0 |
| ROLLER MILLING | | | | |
| 0.25h | 5.3 | 2.5 | 12.4 | - |
| 0.50h | 6.5 | 3.1 | 14.5 | 70.8 |
| Extrusion | | | | |
| Under pressure | 2.7 | 1.3 | 5.8 | - |
| without pressure | 2.5 | 1.2 | 5.8 | - |

From: Fan *et al.* (1981b).

feedlot waste fibres after 10 min at 200$^{o}$C.

Puri and Mamers (1983) enhanced cellulase digestibilities of wheat straw, bagasse and *Eucalyptus regnans* wood chips to 81, 78, and 75% of wheat straw, bagasse and *Eucalyptus regnans* wood chips, respectively, utilising a Siropulper pilot plant equipped with a 115mm diameter pretreatment vessel or digester with a working volume of 6 l connected to an 8.3 l gas storage tank by an 18 mm diameter line. Digester contents were discharged into a 60 l collecting tank at the base of the digester after cooking or autolysis at 200 $^{o}$C for times between 0 and 60 min with pressurisation from carbon dioxide and nitrogen. This treatment, which rendered celluloses completely susceptible to saccharification, was, at 200$^{o}$C, intermediate between the softening of lignin (140 - 160 $^{o}$C) and transition of cellulose (220 - 250 $^{o}$C).

Dale and Moreira (1982) employed ammonia freeze explosion (AFEX) pretreatments, which do not have the disadvantage of degrading plant saccharides, with a 4.5 l autoclave vessel packed with 400 g of lignocellulose. Anhydrous ammonia (99.5%) was injected into the autoclave through a valve, pressure was monitored and released after periods of contact. The lid of the autoclave was then removed and residual gaseous ammonia allowed to evaporate. This process operated at a lower temperature than steam explosion and residual ammonia serves as a nitrogen source for microbial growth. Dale *et al.* (1985) obtained 4 - 5 times enhancement of sugar

yields with AFEX but concluded that lignocellulosics varied in susceptibility with a typical optimum treatment of 1:1 ratio of ammonia at 250 psi for 30 min.

Rai and Mudgal (1987) steamed wheat straw with 50% water in a laboratory autoclave at 2 Kg $cm^{-2}$ for 4 h and concluded that maximum steam penetration into fibres was achieved if the lignocellulose was moistened with water prior to steaming. These authors also recorded increases in acid detergent fibre (ADF) and lignin contents with treatments both after both 4 and 2 hr treatments (Rai & Mudgal, 1986). Similarly, Kling *et al.* (1987) injected saturated steam at 15 Kg $cm^{-2}$ into a reactor containing sugar cane bagasse preheated to 190$^{o}$C and observed marked increases in solubilization of hemicelluloses and susceptibility to enzymic saccharification.

However, Hart *et al.* (1980) recorded losses of 6 - 16% of dry matter as volatiles when an autoclave, operating at 28.1 Kg $cm^{-2}$, containing steamed substrate was vented to the atmosphere. However, Milstein *et al.* (1987) criticised the use of high pressure steam explosion pretreatments, concluding such processes were expensive, give rise to toxic phenolics and losses of nutritious hemicelluloses so that they were not suitable for farmers. They reported that with an alternative technique, solar pasteurization, they obtained increases in available cellulose of 20 - 40% and 6 - 8% in protein from wheat straw.

### 1.7.1.3 Irradiation

Although gamma and electron beam irradiation techniques have no compelling economic advantage over other methods, pilot studies by Lawton *et al.* (1951), showing that both crystalline and amorphous cellulose structures were affected, stimulated further work. Glegg and Kertesz (1957) showed that cellulose depolymerisation continues up to 30 days after radiation treatment at low moisture content and Blouin and Arthur (1960) reported the effect of irradiation was greater in oxygen than in inert atmospheres. Recently, the effect of irradiation treatments on *in vivo* digestibility of lignocellulosics has been studied ( Beardmore *et al.*, 1980; Lillehoj & Han, 1983; Khan *et al.*, 1987). Beardmore *et al.* (1980) noted surface areas of irradiated cellulose were increased at high dosages (106.2 $m^2g^{-1}$ at 500 Mrad) although crystallinity was not affected. Increases in *in vitro* digestibilities (Table 1.7) of gamma and electron radiated straw (wheat, oat, barley, and rye) of up to 80% (Leonhardt *et al.*, 1983) were attributed to decreases in crude fibre, cellulose and neutral detergent fibre contents of treated materials. Duran *et al.* (1986) reported structural modification of commercial and rice hull dioxane lignins after photochemical treatment resulted in 2-fold enhancement in yields of single cell protein. Decreases in lignin fluorescence at 400nm after irradiation treatment,

TABLE 1.7 IN VITRO DIGESTIBILITY OF STRAW IRRADIATED BY MEANS OF 1 mev ELECTRONS AT DOSES OF 1 MGy AND 2 MGy .

| STRAW | DIGESTIBILITY(%) | | |
|---|---|---|---|
| | I Mev electrons irradiated materials | | |
| | D=0 | D=1 MGy | D=2 MGy |
| Rye | 9.3 | 53.6 | 74.3 |
| Wheat | 16.0 | 71.7 | 83.0 |
| Oat | 11.9 | 64.8 | 78.7 |
| Barley | 15.3 | 77.9 | 85.0 |

From: Leonhardt *et al.* (1983).

Mev = millielectron volts.

MGy = mega gray

indicate such treatments induce modification of the lignin structure (Duran *et al.*, 1988).

However, carbohydrates are also modified : decreases in tensile strength and pore radius of gamma irradiated cellulose membranes are often observed. Khan *et al.* (1987) showed that electron beam irradiation of newsprint and pulp at doses of 1.0 MGy and 1.5 - 2.0 MGy, respectively, dissociated cellulose from lignin, enhancing enzymic saccharification.

### 1.7.2 Chemical pretreatment techniques

Chemical pretreatment has received considerable attention and although sodium hydroxide treatment is commercially viable and currently in the U.K., the effects of acids, organic solvents and oxidizing agents have also been investigated. Although chemicals are in widespread use in the delignification and structural modification of lignocelluloses in pulping (Fan *et al.*, 1981b) the disadvantages of cost and effluent treatment problems have limited its application to enhancement of digestibility of feeds (Table 1.8a).

#### 1.7.2.1 Oxidizing agents

Oxidizing agents are particularly effective in chemical oxidation of lignins (Smith *et al.*, 1970; Toyama, 1976). Goering *et al.* (1973) reported decreases in cellwall content and increases in digestibility of straw exposed to sodium chlorite. Han and Callihan (1974)

# MISSING PAGES ARE UNAVAILABLE

observed decreases in crystallinity of cellulose without a corresponding increase in digestibility of sugar cane after treatment with oxidizing agents. Decreases in lignin content, recorded with wheat straw treated with sodium hypochlorite in an autoclave, were attributed to conversion of the polymer to low molecular weight aromatic compounds (Fan *et al.*, 1981b). *Cochliobolus specifer* fermentation of chlorite-delignified wheat straw gave enhanced yields of protein (Chahal *et al.*, 1979; Chahal, 1982). Ibrahim and Pearce (1983) observed enhancements of *in vitro* digestibility of barley straw from 37.2 to 43.2%, for pea straw from 40.9 to 45.8%, and for bagasse from 27.0 to 38.5% with 9 g $kg^{-1}$ chlorite. However, Goering *et al.* (1973) reported poor palatability and acceptance of sodium chlorite- treated straw due to production of sodium chloride.

Treatment of wheat straw with peracetic acid gave a 10- fold enhancement of enzymic saccharification (Fan *et al.*, 1981). Similarly gaseous sulphur dioxide has been used for disrupting lignin cellulose complexes in plant residues yielding significant improvements in digestibility of treated aspen, birch and oak woods with decreases in lignin but no change in polysaccharide contents (Millet *et al.*, 1975).

### 1.7.2.2 Organic solvents

Since mineral acids oxidise a significant proportion of hemicellulose saccharides to inhibitory furfurals

(Sarkanen, 1980), delignification by organic solvents has been investigated and shown to generate cellulosic fibres for either paper making or enzymatic conversion to glucose. Solid low molecular weight lignins for fuel or feedstock for chemical conversion and unmodified hemicellulose sugars are obtained as valuable by-products. Commonly used organic solvents include ethanol, butanol, ethylene glycol, tri-ethylene glycol and dimethyl sulfoxide. Sarkanen (1980) showed that hydrolysis of glycosidic bonds is accelerated in ethanol-water mixtures, which was attributed to the swelling of lignin that subjects the polysaccharide component to stress as the acqueous ethanol penetrates the cell walls. Lee (1979) concluded that volatile solvents such as acetone and isopropanol have no major advantages over aqueous ethanol but tri-ethylene glycol and dimethylsulfoxide have been reported to be more effective (Orth & Orth, 1977). Although the high boiling point of tri-ethylene glycol (190 $^{o}$C), and other reactions of dimethyl sulfoxide, limit recovery, these reagents delignify at high efficiencies at low digester pressures. Furthermore, Fan *et al.* (1981b) concluded that pretreatment of wheat straw with ethylene glycol prior to autoclaving was particularly effective (Table 1.8a) and rates of enzymic saccharifications of bagasse and rice straw were significantly increased after ethylene glycol treatment.

Although Sarkanen (1980) concluded that butanol was

particularly suitable for organosolv treatments, no significant decrease in lignin content of wheat straw was observed with butanol extraction at elevated temperature and pressures (Fan *et al.*, 1981b). Despite many shortcomings, organic solvents have the apparent advantage of recovering lignins with less carbohydrate than with other methods of delignification (Fig 1.8).

**1.7.2.3 Mineral acids**

Hydrolytic removal of hemicelluloses is often effected with strong acids (Han & Callihan, 1974) and Chahal (1982) reported that high yields of glucose and xylose, and high quality lignin, were obtained when wood chips were treated with small amounts of strong acids such as hydrochloric and sulphuric acids. The rate of enzymic saccharification of wheat straw was enhanced four-fold by treatment with dilute sulphuric acid (Fan *et al.*, 1981b) although there was an apparent increase in lignin content, attributed to preferential removal of hemicelluloses. Moo-Young *et al.* (1978) reported increased protein production in the first 16h of fermentation of wood after mild acid hydrolysis although cellulose content remained constant during the fermentation, suggesting preferential utilisation of hemicellulose sugars. When the experiment was repeated with strong acid, appreciable decreases in cellulose content were recorded suggesting greater disruption of lignocellulose and cellulose structure. Although dilute

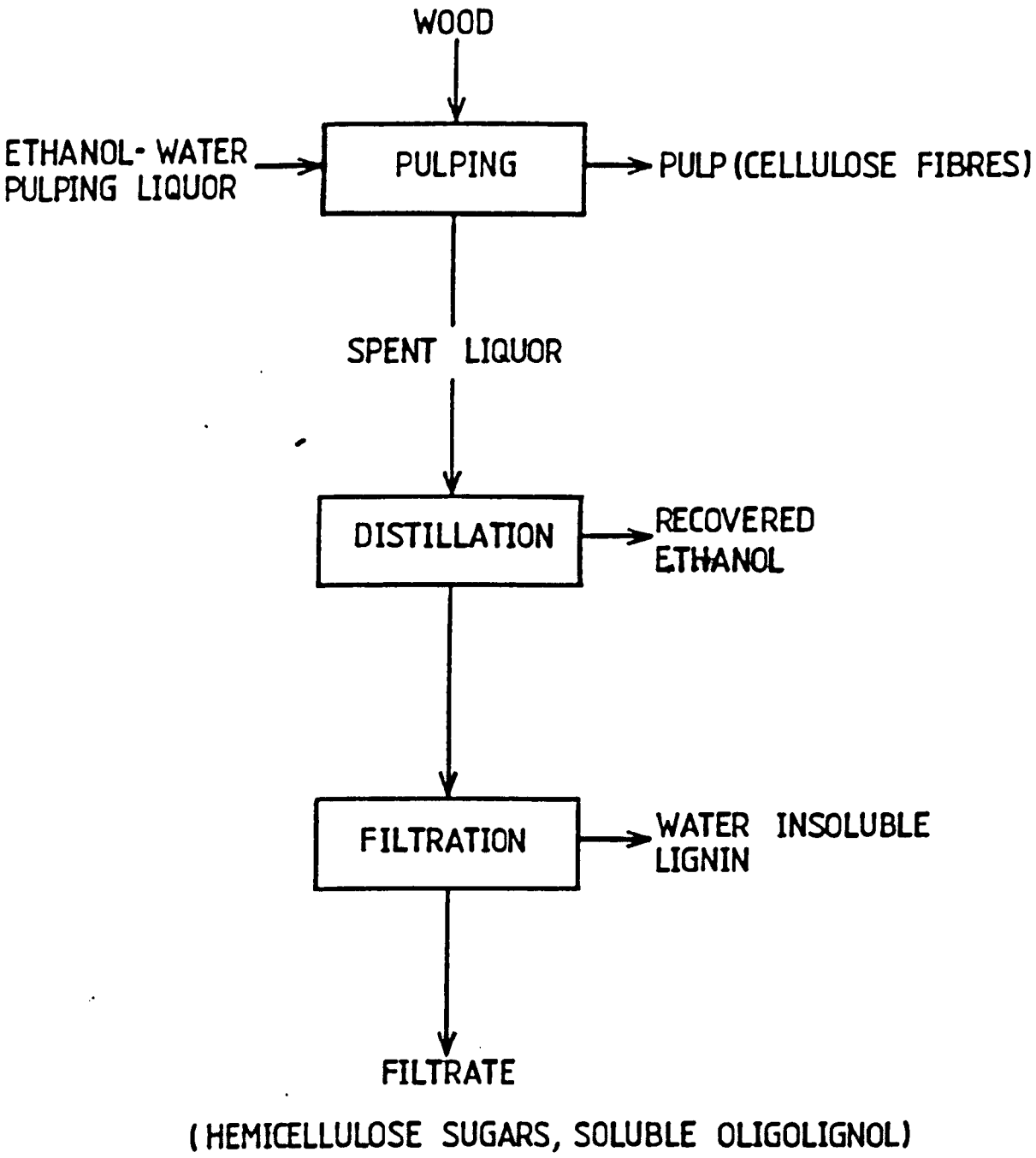



Fig. 1.8 PRINCIPLE OF RECOVERY IN ORGANOSOLV. PULPING. (FROM SARKANEN, 1980).

sulphuric acid produced glucose and xylose with treatments of poplar wood at between 162 and 222 $^{o}$C, cellobiose was found to accumulate during hydrolysis with *Trichoderma reesei* C30 cellulase suggesting reaction products inhibit B -glucosidases (Knappert *et al.*, 1981). Detroy *et al.* (1980) reported 69 to 75% decreases in lignin contents of acid treated wheat straw with 98% conversion of carbohydrates in enzymic saccharifications. Thus to summarise, acid delignifications have significant limitations when subsequent bioconversion of carbohydrates to simple sugars is required (Table 1.8 b & c).

#### 1.7.2.4 Sodium hydroxide

Beckman (1921) was the first to report that digestibility or feed value of straw was increased from 40 to 70% by soaking chopped lignocellulose in 1.5% sodium hydroxide at atmospheric temperature and pressure, followed by washing with water to remove residual alkali. However this discovery was not exploited until the 1960s as the cost of treated straw was more than that of conventional feeds (Homb, 1956). However the spraying technique, introduced by Wilson and Pidgen (1964), revived interest in this approach and Jackson (1977) reported that such treatment results in solubilization of the cellwall natably hemicelluloses, lignin and silica, swelling of cellulose, and saponification of uronic and acetic acid esters attached to xylans (Chahal, 1982),

TABLE 1.8b ADVANTAGES AND DISADVANTAGES OF USING ACIDS FOR THE SACCHARIFICATION OF LIGNOCELLULOSIC MATERIALS.

**ADVANTAGES**

1. Pre - treatment of feedstock not necessary
2. Short reaction times
3. Cheap and readily available catalysts
4. Low temperatures with concentrated acid

**DISADVANTAGES**

1. Concentrated acid recovery essential but expensive
2. High temperatures($180^{o}C$) required with dilute acid
3. Sugar product decomposition to furfural and organic acids
4. Neutralization required before fermentation of hydrolysates
5. By products of hydrolysis toxic to yeast
6. Lower potential yield of ethanol
7. Expensive construction materials

From: Woodward (1987).

TABLE 1.8c ADVANTAGES AND DISADVANTAGES OF USING ENZYMES FOR THE SACCHARIFICATION OF LIGNOCELLULOSIC MATERIALS

**ADVANTAGES**

1. Mild conditions of temperature and pH
2. No degradation products
3. Simultaneous saccharification/ fermentation possible
4. Expensive corrosion resistant reactors not required.

**DISADVANTAGES**

1. Pre treatment of lignocellulosic substrate material
2. Products of saccharification inhibit hydrolysis
3. High levels of enzyme required
4. Adsorption and loss of enzyme on undigested residue limits recycling
5. Satisfactory recovery and recycle of enzyme technically difficult.

From: Woodward (1987).

thus increasing the accessibility of polysaccharides to microbial or enzymic attack. Han (1978) reported that a *Cellulomonas* sp. was able to utilise 73% of rice straw and sugar cane bagasse carbohydrates after treatments with 4% NaOH for 15 min. at 100°C. Similarly, rates of saccharification of wheat straw with *Trichoderma viride* (Qm 9414) cellulase was significantly increased after treatment with 1% sodium hydroxide (Fan *et al.*, 1981b); the lignocellulose appeared light brown and fluffy after autoclaving but light yellow after treatment at room temperature. Moo-Young *et al.* (1978) showed that such alkaline treatments resulted in significant losses of hemicellulose sugars (xylose 4 - 25%, mannose 1.5 - 12%, glucose 2 - 5.4%, galactose, 0.8 - 2.5% and arabinose 0.4 - 1.6%) and lignin-related compounds from the substrate. These authors also reported that growth of *Chaetomium cellulolyticum* (ATCC 32319) was depressed on alkali-treated wood which was attributed to the inhibitory effects of soluble lignin and carbohydrate products. Rai and Mudgal (1987) found that 5-day treatments of wheat straw with differing concentrations of sodium hydroxide at room temperature reduced contents of non-cellulosic cellwall components and a stepwise enhancement in *in vitro* dry matter digestibility was observed. However, Ibrahim and Pearce (1983) concluded that digestibility of bagasse, barley straw, sugar cane and sunflower hulls was decreased at high NaOH concentrations, which

was attributed to sodium toxicity and the alkalinity of the treated material. Similar observations were made by a number of authors (Gharib *et al.*, 1975a & b; Sharma and Jackson, 1975; Jackson, 1977). Gharib *et al.* (1975a) found that residual unreacted alkali increased the pH of poplar bark from 4.7 to 12.2 after treatment with 16g NaOH/100g dry matter and Sharma and Jackson (1975) reported that the sodium content of straw increased by 0.6 percentage units for each 1% NaOH. A major constraint in alkaline treatments of straw is that reagents are toxic, corrosive, difficult to handle and create severe effluent disposal problems.

**1.7.2.5 Other chemicals**

Chemical reagents such as sodium carbonate, calcium hydroxide, ammonia, calcium oxide, hydrogen peroxide and ozone have been reported to delignify lignocelluloses (Chandra & Jackson, 1971; Neely, 1984; Dale *et al.*, 1985; Takagi, 1987). A 1:1 mixture of $Na_2CO_3$ and NaOH was found to give similar increases in roughage digestibility to sodium hydroxide although sodium bicarbonate was not as effective (Chandra & Jackson, 1971). Similarly, mixed $Ca(OH)_2$ (1%) and NaOH (3%) were found to be more effective than sodium hydroxide at 4% in enhancing feed efficiency and daily weight gain in lambs (Waller & Klopfenstein, 1975). However, lime or calcium oxide treatments of rice straw reduced contents of crude protein, ether extract and gross energy with increases in

ash and crude fibre contents (Dumlao & Perez, 1976).

Decomposition of wheat straw by *Lentinus edodes* and *Pleurotus sp.florida* was enhanced with addition of low concentrations of ammonium nitrate but decreased at higher concentrations although *in vitro* digestibility of rice straw was reduced when $NH_4NO_3$ was added (Zadrazil & Brunnert, 1980). On the other hand, with addition of urea, Jayasuriya and Perera (1982) showed *in vitro* dry matter digestibility of rice straw could be significantly increased. Moreover, Bakshi *et al.* (1987) observed that the nutritive value of urea treated straw was substantially enhanced after a 9-day fermentation following addition of 3.5 kg urea to 96.5 kg of wheat straw at moisture contents between 40 and 70% and the resulting lignocellulose was both palatable and able to maintain adult buffaloes without adversely affecting body weight or health. Bakshi *et al.* (1986) suggested that the large part of the urea was converted to microbial protein although ammonia released by catabolism effected partial disruption of lignocellulose structure.

Gould (1984) studied delignification of wheat straw by hydrogen peroxide and showed that the extent of subsequent enzymic saccharification of cellulose was dependent upon the pH of the pretreatment solution and that delignification by alkaline $H_2O_2$ proceeded more rapidly at room temperature than at 5 $^oC$ or 60 $^oC$. Similarly, a pH >10 was optimal for cellulose digestibility of straw treated with $H_2O_2$ although treated

substrates varied in susceptibility to enzymic saccharification. Takagi (1987) observed that a solution of hydrogen peroxide, containing manganese sulphate, enhanced the susceptibility of municipal solid wastes to enzymatic saccharification to a much greater extent than either compound alone.

### 1.7.3 Biological pretreatment

#### 1.7.3.1 Fungal Fermentation

Fungal fermentation is one of the oldest arts (Aidoo *et al.*, 1982) although microbes have largely been utilised in production of foods and alcoholic beverages (Smith, 1985). Growth of micro-organisms on solid matrices widely referred to as **"SOLID SUBSTRATE(STATE) FERMENTATION"** (Hesseltine, 1977, 1987) has been defined as microbial growth in the "absence or near absence of free water" (Smith, 1985). Products of such fermentations are listed in Table 1.9.

Based upon growth conditions and process control, there are four distinct forms of solid state fermentations (Smith, 1985; Hesseltine, 1987):

(A) Static fermentations in growth of inoculated "mushrooms", such as *Lentinus edodes* on oak logs.

(B) Fermentations with occasional agitation without forced aeration as has been utilised in Koji production of *Aspergillus oryzae* on mixtures of rice and soyabeans.

(C) Fermentation with slow continuous agitation,

TABLE 1.9 SOME EXAMPLES OF SOLID SUBSTRATE FERMENTATIONS

| EXAMPLE(S) | SUBSTRATE | MICRO-ORGANISMS INVOLVED |
|---|---|---|
| Mushroom production | straw, manure | *Agaricus bisporus*<br>*Lentinus edodes*<br>*Volvariella volvaceae* |
| Oriental fermentation | wheat & soyabean | *Aspergillus oryzae* |
| Soy - sauce | Soya - bean | *Rhizopus* species |
| Tempeh | peanut press cake | *Neurospora sitophili* |
| Ontjom cheese | Milk cord | *Penicillium roquefortii* |
| Leaching of metals | Low grade ores | *Thiobacillus* sp |
| Organic acids | Cane sugar, molasses | *Aspergillus niger* |
| Enzymes | Wheat bran etc | *Aspergillus niger* |
| Composting | Mixed organic material | Fungi, bacteria, actinomycetes |
| Sewage treatment | Components of sewage | Bacteria, fungi protozoa. |

From: Smith (1985).

utilised in production of mycotoxins on grain by fungi. (D) Fluidised beds in which solids are suspended in a bioreactor by an air current.

### 1.7.3.2 Solid substrate bioreactors

Despite adaptation of a wide range of equipment (Table 1.10) a shortage of data on the physiological basis of the processes limits the application of the process and has resulted in scale-up problems. However at the laboratory level, it is often possible to monitor the biochemical changes in the chosen reactor vessels using a range of sensors. Overall, it can be concluded that, despite technical constraints, the processes have certain advantages over conventional liquid fermentations (Table 1.11).

Bioreactors can be regarded as containment systems in which materials are transformed by enzymes and or living cells (Moo-Young & Blanch, 1981; Smith, 1985). Bioreactors, used for many decades in food processing, beverage fermentations and more recently in effluent treatment and biomedical applications, can be divided into 3 categories: batch, semi-continuous (fed-batch) and continuous reactors (continuous stirred tank or plug flow). Transformations can be effected by enzymes (soluble or immobilised) or whole cells (growing or immobilised or static) in either aerobic or anaerobic environments (Atkinson, 1974; Cooney, 1983).

Individual bioreactors are normally tailored to the

TABLE 1.10 TYPES OF SOLID STATE FERMENTATION BIOREACTORS

| Bioreactor | Capacity | Application | Reference |
|---|---|---|---|
| Fernbach flask | 2.8 L | production of aflatoxin with A.parasiticus | Hesseltine *et al* (1968) |
| | | production of fungal spores | Sehgal *et al*(1966) |
| Rectangular reactor (box kiln) | 1 ton | Single cell protein | Durand and Chereau (1988) |
| Two stage SF - SSF bioreactor | - | bioconversion of lignocellulose | Viesturs *et al* (1987) |
| Microporous film bag | - | mycotoxin production | Cuero *et al* (1985) |
| Cement mixer | (a) 70 L | feedlot waste/corn fermentation | Hesseltine (1977) |
| | (b) 114 L | straw fermentation with *Trichoderma viride* | Han & Anderson (1975) |
| Wooden trays | - | Koji production with *Aspergillus oryzae* | Church (1923) |
| Rotating drum | (a) 55 gallons | composting | Schulze (1962) |
| | (b) - | koji production | Arima (1964) |
| | (c) 5 gallons | enzyme production with *A.oryzae* | Underkoffler (1939) |
| Horizontal drum | 2.3 Kg | Feedlot wastes/corn fermentation | Hrubant *et al* (1976) |
| Biological support particles | - | fluidized bed reactor | Atkinson *et al* (1979) |
| Tubular fermentors | - | SCP | Moo-Young *et al* (1979) |
| Waterloo process | 200 litre | SCP | Moo-Young *et al* (1979) |

TABLE 1.11 ADVANTAGES AND DISADVANTAGES OF SOLID STATE FERMENTATION PROCESSES

**ADVANTAGES**

1. Bacterial contamination rarely occurs because of low moisture content of substrates
2. Use of simple and cheap raw materials as media components.
3. Pollution problems are not common, since effluent production does not occur.
4. Space requirement for the reactor vessels is small compared to submerged fermentation.
5. Products may be recovered directly from the fermenters by addition of small volume of extraction solvent.
6. The conditions for cultivating the organisms are similar to that of their natural habitat.
7. The particulate nature of the substrate makes aeration easier due to air spaces between each particle.
8. The product yields are quite high and reproducible too.
9. The energy requirement is very minimal. And the risk of inhaling spores are low in vessels with constant agitation due to reduction in sporulation rate.

**DISADVANTAGES**

1. Generation of so much heat. This may inhibit microbial activity and also affect reactor performance.
2. Difficulties in process control as regards to monitoring changes in pH oxygen and carbon dioxide levels, moisture content and product concentration
3. The energy cost could be quite high in large scale "SSF" processes requiring agitation
4. The cost of chemical and physical pretreatments of substrates to enhance their susceptibility to microbial attack may be very expensive.
5. The "SSF" conditions permit mainly the growth of moulds because only moulds can thrive at very low moisture content of the substrates.

From : Woodward, (1987)

requirements of the process. However major constraints of most biological reactors are:

(a) Flocculation, resulting from microbial aggregation and attachment to polymeric substrates that results in viscous reaction mixtures imposing engineering constraints such as in mass and heat transfer.

(b) Aggregation, notably with filamentous microbes forming clumps, pellets or mycelia, which create intra-particle diffusional resistances resulting in anaerobiosis (Moo-Young & Blanch, 1981). Calleja (1984) noted that aggregates form at cell or cell-substrate interfaces by covalent bonds although strain variation is known to be important (Bruce & Hunt, 1988).

(c) Instrumentation and process control of environmental factors such as temperature, pH and dissolved oxygen and nutritional parameters are often required.

(d) Most bioreactors require spargers or mechanical mixers to ensure adequate supply of oxygen for aerobic systems and removal of toxic gaseous by-products in anaerobic fermentations.

### 1.7.3.3 Design considerations of bioreactors for solid state fermentation

**(A) Rotating drum fermenters** are mounted on rollers that act both as supports and for rotation. Lonsane *et al.* (1985) reported that such fermenters included 5 gallon Pyrex glass containers, iron pans, ceramic jars or cement

mixers of up to 70 litres rotating at between 1 and several revolutions per minute. Such bioreactors are normally equipped with inlets and outlets for gas so that inlets reach the bottom of the vessel or alternatively branch and end in a spray nozzle. Aeration can be accomplished by use of a suction fan at the inlet port or by a compressor. Air can be sterilised by bubbling through sulphuric acid and subsequently humidified by passing through water. Originally, drum fermenters were produced so that initial steps like steaming, inoculation, incubation and final drying were performed *in situ*. However, provision of baffles or sectioning of the fermenter into parts has significant advantages and a typical modern four-part drum fermenter would consist of four simulated baffled stainless steel drums placed rotating on a common shaft powered by a variable speed drive motor, provided with a 5-cm diameter port for charging, water addition, inoculation and sampling (Lindenfesler & Ciegler, 1975). Such units can be readily dismantled for cleaning and sterilisation. Aidoo (1979) evaluated the efficiency of a 20 litre horizontal fermenter equipped with paddles and other workers have reported on the value of such bioreactors (Schulze, 1962). However, there have been reports of difficulties in scale up as mycelia can be destroyed during early growth stages due to abrasion (Underkofler *et al.*, 1947). Moreover other problems in temperature

control, microbial contamination, aggregation of mycelia into balls and growth retardation through particle attrition also create problems.

**(B) Wooden cell fermenters**, such as boxes of dimension 30 X 30 X 30 cm, filled to 25 cm in depth with 4.5 kg brans were reported by Underkofler *et al.* (1947). This container was provided with air inlet and outlet, thermometer pockets, an open tube manometer and an infrastructure for saturating air with moisture at the desired temperature, and the facility for reversing air flow. The bioreactor supported good microbial growth and reproducibility but difficulties were encountered in emptying the fermented solids.

**(C) Covered pan fermenters**, equipped with perforated bottoms, have been used extensively especially at laboratory scale (Hao *et al.*, 1943). Such bioreactors can have provision for aeration under pressure and the reversion of direction of air flow, and temperature control. Procedures such as bran-moistening, sterilisation, inoculation and incubation can be performed *in situ* and such bioreactors require little space, and generate uniform growth, and consistent and higher yields of biomass and enzymes. The absence of mechanical devices for rotation eliminates disturbances of mould mycelia during growth.

**(D) Conveyor fermenters** can be designed so that a sequence of conveyors are used to give a production process. Jeffries (1948) described a plant in which, on

the first conveyor, bran was heated to 85°C and held at this temperature for 15 min before transfer to a second conveyor using sterile cool air. The substrate was then inoculated by spraying spores or continuous feeding of dry inoculum and inoculated solids were then transferred into sterile perforated metal trays by a mechanical spreader. These trays entered a growth tunnel attached to an overhead trolley; on completion they were transferred to a drying tunnel consisting of a 2-stage centre exhaust drier, utilising a counter current and concurrent air drying. Finally, empty trays were sterilised, prior to reuse, by passage through a 155°C oven for 3 min. A major disadvantage was that sterilisation at 85°C for 15 min was not sufficient to eliminate contamination and temperature control was difficult.

**(E) Column fermenters** formed from glass or plastic columns with provisions for aeration at both top and bottom were described by Raimbault and Alzard (1980). Temperature control was effected using a thermostatic jacket or incubation in a temperature controlled room. Humidity was maintained by use of moist air.

**(F) Butler-type corn storage bin fermenters** were described by Silman *et al.* (1979). A bin of 5.5 m diameter, with a capacity of 1266 bushels of corn, was coated internally with epoxy paint and equipped with a slotted floor and a single auger stirrer, rotating from

the centre in a helical path. Aeration was effected utilising a squirrel cage blower, PVC pipe, followed by sheet metal transition into a plenum under the slotted floor and an air compressor so as to effect air circulation. The substrate (corn) was moistened by water from spray nozzles, installed in the auger, while humidification was achieved by spraying water into the air intake or recirculation leg. Temperature probes could be inserted at various depths and control achieved by evaporative cooling of the surface by covering the roof with burlap soaked with water. However fungal growth was found to be non-uniform and evaporative cooling insufficient to cope with rapidly growing organism. In these bioreactors, used for aflatoxin production, temperatures as high as 47.2 $^{o}$C have been observed.

**(G) Tray fermenters** consisting of sets of trays designed to hold 2.5 - 5 cm layers of the substrate were incubated in suitable racks in cabinets or chambers where optimum growth parameters could be maintained. Temperature and humidity control systems could be employed and trays humidified by saturated air. Trays could be either wooden frames, provided with a false bottom of wire mesh, or metallic with perforated bottoms or screens, to ensure adequate aeration of substrates. Tray fermenters are simple in operation since forced aeration is not required and have been used successfully in commercial production of mouldy bran (Underkofler *et al.*, 1946). A major constraint is the requirement for space to house

the relatively bulky processing plant.

**(H) Other types of fermenters** have been described by Raimbault and Alzard (1980) for bioconversion of cassava starch granules using *Aspergillus niger*. The Waterloo process for single cell protein production from cellulosic substrates, described by Moo-Young *et al* (1979a), and the process of Durand and Chereau(1988) for protein enrichment of sugar beet pulp have each required the development of novel bioreactors.

### 1.7.3.4 Fungi used for lignocellulose bioconversion

A successful microbial delignification process will depend not only on optimal bioreactor conditions but on the organism employed. Selectivity for lignin degradation over polysaccharide would be desirable and to date most studies have been performed with white rot fungi although it is known that these vary in ability to delignify or enhance the digestibility of lignocelluloses (Reid, 1989a).

### 1.7.3.5 Use of strain construction in lignocellulose bioconversion

Use of conventional strain construction in white-rot fungi to enhance selective delignification of wood and other lignocellulosics has met with limited success. Eriksson *et al*. (1983) concluded that only white-rot fungi effectively degraded all wood polymers, and strain construction should permit selective

enhancement or elimination of activities of interest (Eriksson & Goodell, 1974; Eriksson *et al.*, 1983). Selective delignification of wood using suitable, efficient mutant strains would reduce the energy requirement in thermomechanical pulping and enhance nutritional value of high fibre plant residues.

Eriksson and Goodell (1974) produced the first white-rot mutant (*Polyporus adustus*) defective in cellulolytic activity, but showed that this strain was also defective in mannanase and xylanase activities. Subsequently, Ander and Eriksson (1975) developed a cellulase-deficient strain 44 of *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum* strain 44). This *P. chrysosporium* strain retained the ability to degrade xylan but was unable to saccharify cellulose and had reduced phenol oxidase activity. Similar observations were reported with other fungi, with a cellulase-deficient strain 26 of *Phlebia radiata* being able to depolymerise xylan whereas a similar cellulase deficient strain of *Phlebia gigantea* did not metabolise non-cellulosic polysaccharide (Eriksson & Goodell, 1974).

Improvements in the properties of cellulase-defective mutants were reported by Eriksson *et al.* (1983) with two derivatives of the original mutant being high in phenol-oxidase activity (44-18) and able to depolymerise xylan (44-9). In subsequent studies, a new mutant (63-2) of *P. chrysosporium* that was unable to degrade cellulose in 15 days of growth, and had high phenol oxidase and

xylanase activities, and a cellulose-depolymerising non-sporulating (44-2) revertant strain of 44 were isolated. The ability of such strains to degrade mono and dimeric lignin model compounds and native lignins and induce weight loss in lignocellulosics has been studied by a number of workers (Eriksson *et al.* 1983; Al-Ani, 1985; Al-Ani & Smith, 1986). Eriksson *et al.* (1983) reported that mineralisation from a dimeric lignin model compound was higher with wild type than with any of the mutants, although 63-2 was more efficient than derivatives of mutant 44. The new mutants produced similar amounts of acid-soluble lignin in degraded wood to the wild type and were much superior to strain 44. However, fermentation of wood with 63-2 also resulted in greater weight loss than either wild type or mutant 44. Al-Ani (1985) studied delignification of sugar cane bagasse using both wild type and three mutant strains(44, 44-2, 63-2) and concluded that digestibility of bagasse was more enhanced with wild type than any of the mutants.

### 1.7.3.6 Physicochemical aspects of solid state fermentation (SSF)

To optimise delignification in solid substrate fermentations lignocelluloses should be in a physical form facilitating air circulation, minimising bioflocculation and aggregation of microbial cells and substrate particles (Hesseltine, 1977 & 1987; Smith, 1985). Temperature control, and exchange and control of

gases, are always a problem in solid-substrate fermentations. Microbial contamination is reduced at the low moisture contents used since fungi can sporulate, germinate and grow at Aw values of 0.6 - 0.7 whereas bacteria only thrive actively in substrates with higher water activities (Smith, 1985).

Furthermore, inoculum is also important in solid state fermentations and Hesseltine (1972) observed that at high concentration of spores there was rapid germ tube production, particularly with *Aspergillus oryzae*. Unfortunately, most fungi do not sporulate well in semisolid culture media, and inocula must be prepared as spawn as for production of *Agaricus*, *Lentinus*, *Pleurotus* and *Volvariella* mycelia. Fungi are initially inoculated into a fermentable solid matrix such as rye kernel, and colonised kernels are dispersed evenly on the test substrate.

Use of microbial monocultures in solid substrate fermentation processes has received significant attention since this strategy can be used to enhance production of specific products whilst minimising formation of toxic metabolites (Miall, 1975; Aidoo, 1979; Raimbault & Alzard, 1980; Moo-Young *et al*, 1983). Aidoo (1979) described a novel scale-up process for the preparation of koji, fermenting steamed rice or other cereals with pure culture of *Aspergillus oryzae* to provide a starter for such oriental foods as miso, sake and soya sauce.

However, there is still interest in mixed culture solid substrate fermentations since these often show synergistic action and resist contamination. For example, a mixed culture of *Chaetomium cellulolyticum* or *Trichoderma lignorum* and *Candida lipolytica* was found to be more effective in bioconversion of wheat straw than monocultures (Viesturs *et al.*, 1981). Subsequently, Viesturs *et al.* (1987) reported development of a two-stage bioreactor for combined submerged and solid substrate fermentation. Milled wheat straw in the top section of a bioreactor was inoculated with a mycelial culture of *Trichoderma reesei* whilst the bottom, containing considerable quantities of sugars and other metabolites in recirculated liquor, was inoculated with *Endomycopsis fibuliger*. It was observed that the combined system produced an enhanced yield of biomass and cellulase over either fermentation alone.

## 1.8 BIOTECHNOLOGY OF LIGNOCELLULOSES

### 1.8.1 Silage

Ensiling is a preservation method for forage crops used for centuries but still common practise in modern agriculture (Linden *et al.*, 1987). In the natural fermentation lactobacilli convert sugars in plant residues to lactate and acetate. The acidic pH of the fermented biomass (pH 3.8) coupled with the anaerobic nature of the ensiling process protects forage from other deleterious bacteria and fungi(McDonald, 1976).

In forage fructose and glucose are the major fermentable carbohydrates although sucrose and fructans are also present (McDonald, 1976). Lactic acid bacteria, present in fresh herbage in very low numbers (Table 1.12) (Gibson *et al.*, 1958; McDonald *et al*, 1964), begin to dominate the ecosystem as pH and available oxygen decrease, also actively inhibiting growth of other bacteria (Langston & Bouma, 1960; Gibson *et al.*, 1961). Lactic acid bacteria are divided on a metabolic basis into homofermenters and heterofermenters. Homofermentative lactic acid bacteria produce lactate from hexoses whereas heterofermenters convert glucose to a mixture of lactate, ethanol and carbon dioxide (Table 1.13). Organic acids such as citrate and lactate are changed during ensilage: citrate and malate for example are fermented by various pathways to yield such products as lactate, acetate, formate, ethanol, 2,3 butanediol and acetoin (McDonald & Whittenbury, 1973). Such organic acids, together with other anions, and plant proteins buffer herbage to pH 4 - 6. Proteins in herbage are rapidly hydrolysed to amino acids which are deaminated and decarboxylated by lactic acid bacteria. Clostridia, also present on herbage, can ferment to form a silage but the product is of relatively high pH and contains significant amounts of butyrate, branch chain fatty acids and ammonia (Table 1.13).

Ensiling with lactic acid bacteria is very efficient in terms of recovery of dry matter with a 5 - 6% higher

TABLE 1.12 SOME SPECIES OF LACTIC ACID BACTERIA COMMONLY FOUND ON FRESH HERBAGE AND SILAGE.

| Homofermentative | Heterofermentative |
|---|---|
| *Lactobacillus plantarum* | *Lactobacillus brevis* |
| *Pediococcus acidilactici* | *Lactobacillus buchneri* |
| *Streptococcus durans* | *Lactobacillus fermentum* |
| *Streptococcus faecalis* | *Lactobacillus viridescens* |
| *Streptococcus faecium* | *Leuconostoc mesenteroides* |
| *Streptococcus lactis* | |

From : McDonald (1976).

TABLE 1.13 **MAIN PRODUCTS OF SOME NUTRIENTS BY SILAGE BACTERIA**

**Homofermentative lactic acid bacteria**

| | | |
|---|---|---|
| Glucose | ---- | 2 Lactate |
| Fructose | ---- | 2 Lactate |
| Pentose | ---- | Lactate and Ace---- |
| 2 Citrate* | ---- | Lactate and 3 Acetate + 3 $CO_2$ |
| Malate* | ---- | Lactate + $CO_2$ |

**Heterofermentative lactic acid bacteria**

| | | |
|---|---|---|
| Glucose | ----- | Lactate + Ethanol + $CO_2$ |
| 3 Fructose | ----- | Lactate + 2 Mannitol + Acetate + $CO_2$ |
| 2 Fructose + Glucose | --- | Lactate + 2 Mannitol + $CO_2$ |

***Clostridia***

| | | |
|---|---|---|
| 2 Lactate | ---- | Butyrate + 2 $CO_2$ + 2 $H_2$ |
| Alanine + 2 Glycine | ---- | 3 Acetate + 3 $NH_3$ + $CO_2$ |
| 3 Alanine | --- | 2 Propionate + Acetate + 3 $NH_3$ + $CO_2$ |
| Valine | --- | Isobutyrate + $NH_3$ + $CO_2$ |
| Leucine | ---- | Isovalerate + $NH_3$ + $CO_2$ |
| Histidine | ---- | Histamine + $CO_2$ |
| Lysine | ---- | Cadaverine + $CO_2$ |
| Arginine | ---- | Putrescine + 2 $CO_2$ + 2 $NH_3$ |

* Pathways are similar for heterofermentative lactic acid bacteria.

From: McDonald (1976).

metabolizable and net energy content than hay. Methane production is also slightly higher with silage than hay although this may be through methanogen catabolism of formic acid used as a silage additive. Consumption of silage by ruminants was found to increase in parallel with dry matter content (Thomas *et al.*, 1961) although lower palatability of wet silages may reflect higher contents of aldehydes, histamines and organic acids which are appetite depressants (Harris *et al.*, 1966). Palatability of silage may also be reduced by products of clostridial breakdown of proteins and by high concentration of free acids in low pH silages (Mcleod *et al.*, 1970; Wilkins *et al.*, 1971).

Ensiling is also affected by the presence of sufficient oxygen to enable plant cells to continue to respire by oxidation of sugars (McDonald, 1976). Takaishi (1970) reported that high levels of oxygen support growth of yeasts reducing lactate production, and other factors altering the metabolic pattern can reduce the nutritional value of silages.

### 1.8.2 Composting and mushroom fermentation

Currently, the major U.K. commercial processes utilising lignocellulosic residues are composting for organic manure and soil conditioners (Zadrazil, 1990) and cultivation of the edible white-rot basidiomycete *Agaricus bisporus* (Tautorus, 1985; Hayes and Nair, 1975). A variety of lignocelluloses are used for production of

fungal fruiting bodies. Shitake mushroom (*Lentinus edodes*), the most important cultivated mushroom world-wide, is grown on felled, aged deciduous hardwoods such as oak, beech, chestnut and alder although successful production of shitake under controlled indoor condition with sawdust or wood-chip based synthetic mixtures has been reported (Farr, 1983). Oyster mushrooms (*Pleurotus* sp.) have also been produced commercially on wastes from pulp and paper mills (Mueller & Gawley, 1983). Rice straw, residues from tapioca manufacture and straw from *Bombax melaboricum* have been used in production of the paddy straw mushroom (*Volvariella volvaceae*).

In production of the mushroom *Agaricus bisporus* three distinct production stages are recognised: composting, spawning and sporophore formation (Fig. 1.9 ). Composting is an aerobic process carried out by a sequence of microorganisms to yield a friable plant derivative (Table 1.14) which is pasteurised to eliminate insects, nematodes, competitive fungi and seeds of higher plants (Tautorus, 1985). The partially degraded lignocellulose is inoculated with mushroom spawn, pre-grown on cereal grains. After colonisation by mycelia the compost is covered with a 2 - 4 cm casing layer of soil of high moisture retaining properties which induces fruiting as well as acting as a mechanical support for the developing sporophores.

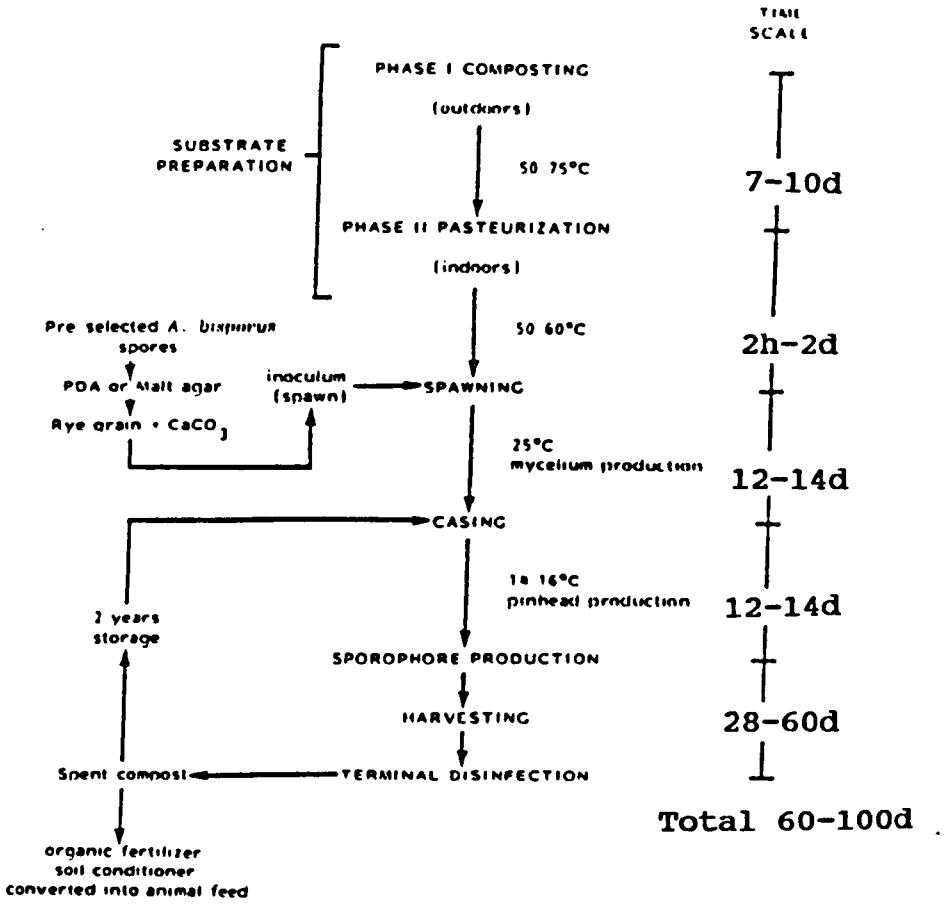



Fig. 1.9 Current techniques in ***Agaricus bisporus*** solid state fermentation.
From; Tautorus (1990).

Table 1.14 Typical Components of Natural (horse manure-based) and Synthetic Mushroom Composts

| Type | Fresh weight (kg) | Purposes |
|---|---|---|
| A. Natural | | |
| Horse manure | 900.0 | Activation of fermentation, bulk density; extremely variable in nonprotein N and water; slow release of carbohydrates |
| $(NH_4)_2SO_4$ | 9.0 | Nitrogen enrichment |
| Superphosphate | 9.0 | Mineral deficiency |
| $CaCO_3$ | 22.0 | pH balance |
| Gypsum | 22.0 | Improvement of physical characteristics; disperses colloids; decreases greasiness may also decrease pH and $NH_3$ |
| B. Synthetic | | |
| Hay | 68.0 | Physical structure for aerobic conditions; resevoir of cellulose, hemicellulose, lignin |
| Corncobs | 68.0 | Concentrate meal; both N and C available relatively slowly (approximately 1% N) |
| Brewer's grain | 13.6 | Readily available carbohydrate-rich nutrients |
| Poultry manure | 11.33 | Nitrogen enrichment |
| Urea | 1.18 | Inorganic nitrogen enrichment; quick release of N |
| Potash | 1.63 | Mineral deficiency |
| Gypsum | 4.53 | |

From; Tautorus (1985).

### 1.8.3 Single cell protein(SCP).

Numerous reports have suggested the technical feasibility of producing high quality single cell proteins from biomass substrates (Kharatyan, 1978; Solomons, 1986; Senez, 1987). The term "single cell protein" was coined at Massachausett Institute of Technology in 1966 to describe proteins from micro-organisms, including yeast, bacteria, algae and multicellular moulds (mycoproteins). Large scale bioconversion of non conventional food substrates into quality protein is almost commercially viable in the U.K. and an additional benefit is that the microbes used in such bioconversion processes are rich in B-group vitamins. Single cell proteins such as Quorn, a mycoprotein, can compete in nutritional value with conventional protein (Table 1.15 a, b & c).

Virtually any plant residue from the food and brewery industries can be used for SCP production. Such by-products often create waste disposal problems but could be enhanced in nutritional value by fermentations to generate by-product human or animal feed ingredients (Table 1.16).

In particular, plant wastes such as cocoa hulls, coffee hulls, cotton residues, sawdust, straw, bark, bagasse, molasses and bran have been explored as substrates for microbial cultivation (Table 1.16 ).

A considerable range of microbes have been evaluated for single cell protein production. Bacteria have the

TABLE 1.15a NUTRITIVE VALUE OF SCP AS COMPARED WITH THOSE OF ANIMAL AND PLANT SOURCES (%)

| Protein source | Biological value | Digestibility | Net protein utilization |
|---|---|---|---|
| **ANIMAL** | | | |
| egg | 100 | 97 | 97 |
| milk | 93 | 97 | 90 |
| PLANT | | | |
| oat meal | 79 | 60 | 47 |
| corn meal | 72 | 76 | 55 |
| potatoes | 69 | 74 | 51 |
| **YEAST/SUBSTRATE** | | | |
| *Candida lipolytica*(Alkane) | 61 | 96 | 59 |
| *C. lipolytica*(gas oil) | 54 | 94 | 51 |
| *C. utilis*(sulphite waste) | 32 - 48 | 85 - 88 | 27 - 42 |
| *S.cerevisiae*(brewers) | 58 - 69 | 80 - 90 | 47 - 62 |
| *S.cerevisiae*(dry) | 52 - 87 | 70 - 90 | 36 - 78 |
| *S.cerevisiae* | 70 - 89 | 91 - 92 | 64 - 82 |
| **BACTERIA** | | | |
| *Micrococcus cerificans* | 76 | - | - |
| *Hydrogenomonas eutropha* | 78 | 84 | 64 |
| *Bacillus megaterium*(whole) | 62 | 56 | 35 |
| *Bacillus megaterium*(broken) | 70 | 67 | 47 |
| FUNGI | | | |
| *Fusarium* sp. | 70 - 75 | - | - |
| ALGAE | | | |
| *Chlorella scenedesmus* | 54 | 65 | 35 |
| *Spirullina maxima* | 72 | 84 | 60 |

From: Chen & Peppler (1978)

TABLE 1.15b ESSENTIAL AMINO ACIDS IN PROTEINS COMPARED TO FAO GUIDELINES.

| AMINO ACID | FAO GUIDELINE | g amino acid per 100g protein | | | | |
|---|---|---|---|---|---|---|
| | | YEAST | FUNGI | BACTERIA | SOYABEAN | COWS MILK |
| Cystein | 2.0 | 0.3 | 2.0 | 0.4 | 1.2 | 0.9 |
| Isoleucine | 4.2 | 7.3 | 3.4 | 3.0 | 5.8 | 6.4 |
| Leucine | 4.8 | 8.1 | 5.0 | 4.7 | 7.6 | 9.9 |
| Lysine | 4.2 | 10.7 | 4.4 | 4.1 | 6.6 | 7.8 |
| Methionine | 2.2 | 1.4 | 1.5 | 1.7 | 1.1 | 2.4 |
| Phenylalanine | 2.8 | 4.1 | 5.2 | 2.4 | 4.8 | 4.9 |
| Threonine | 2.8 | 4.8 | 10.0 | 3.2 | 3.9 | 4.6 |
| Tryptophan | 1.4 | 0.5 | - | 0.6 | 1.2 | 1.4 |
| Tyrosine | 2.8 | 1.4 | 3.5 | 2.1 | 3.8 | 5.1 |
| Valine | 4.2 | 5.7 | 5.4 | 3.6 | 5.2 | 6.9 |

From: Forage and Righelato (1978).

TABLE 1.15c **VITAMIN CONTENTS IN PROTEIN SOURCES**

| VITAMIN | VITAMIN CONTENT(ug/g) dry weight | | | | |
|---|---|---|---|---|---|
| | *Aspergillus niger* | *Fusarium moniliforme* | *Torula* sp. | Basidiomycetes | Soya bean |
| Biotin | 0.24 | 0.31 | 2.3 | 0.37 - 2.32 | - |
| Riboflavin | 6.7 | 9.5 | 45.0 | 7 - 96 | 3.5 |
| Pyridoxine | 25.4 | 36.8 | 33.4 | 0.6 - 98 | - |
| Pantothenic acid | 25.0 | 25.5 | 37.0 | 3 - 52 | 15 |
| Cyanobalamin | 35.0 | 33.0 | - | - | - |

From: Forage and Righelato (1978)

advantage of rapid biomass production, with the highest protein contents, utilising such diverse energy sources as oil hydrocarbons and sewage without pathological effects (Bhattacharjee, 1970). Reddy *et al.* (1976) produced fermented ammoniated condensed whey (FACW) after whey treatment with *Lactobacillus bulgaricus* 2217, which was rich in protein (56% cp) and could form a nitrogen supplement for cattle feeds. Reddy and Erdman (1977) reported that anaerobic fermentation of feedlot waste filtrate by rumen bacteria reduced residual carbohydrates forming a protein-rich product.

Fungi have been used for production of mycoproteins because they can utilise complex substrates or fibrous wastes, such as lignocellulosics. The Finnish paper industry developed the "pekilo" process for production of ascomycete biomass on sulphite liquor effluent. Fungal mycelia are rich in protein (30 - 50% on dry weight), contain all the essential amino acids in addition to vitamins B1, B2, B12 and pantothenic acids, and contain more unsaturated fatty acids (74 - 84%) than meats (Huang, 1982).

However, yeasts, the oldest source of single cell protein, are widely used in foods and feeds as a dietary supplement. A typical example is the commercial production of "Bel yeast", a strain of *S. cerevisiae*, on whey in France (Moebus & Teuber, 1983).

TABLE 1.16a PRODUCTS OBTAINED AS A RESULT OF MICROBIAL ACTION ON NATURAL AND INDUSTRIAL WASTES AT DOMESTIC LEVEL.

| COUNTRY | PRODUCT | RAW MATERIAL USED | REACTIVE ORGANISM |
|---|---|---|---|
| Chile | microbial protein | fruit peels, papaya waste | yeast |
| Egypt | microbial protein | bagasse, rice mills distiller slops. | yeasts and bacteria |
| Quatamala | animal feeds | bagasse, cotton cakes | yeasts and bacteria |
| India | organic accidulants | domestic and industrial wastes | bacteria |
| Israel | fodder yeast | citrus peels | *Candida tropicalis* |
| Malaysia | poultry feed | fish wastes, tapioca | bacteria, chlorella |
| Nigeria | SCP, protein compost, poultry feed. | cassava wastes, rice hulls & straws. | *Candida* sp. |
| Senegal | compost, animal feed | millet, sorghum & groundnut shells | bacteria |
| Thailand | SCP | municipal waste, coconut water, cassava & vegetable wastes. | *Torula* sp *Chlorella* sp |

From : Sanni (1981).

TABLE 1.16b **INDUSTRIAL PRODUCTION OF SCP FROM REWABLE RAW MATERIALS**

| SUBSTRATE | ORGANISM | PROCESS | TONS/YEAR |
|---|---|---|---|
| Molasses | *Candida utilis* | Cuba Speichim(France) | 80,000 |
| Molasses & starch | *Corynebacterium melasicola.* *Brevibacterium lactofermentum* | France orsari[a] | 10,000 |
| | | France eurolysine[a] | 8,000 |
| Starch hydrolysate | *Fusarium graminarium* | UK Rank Hovis McDougal | 50 - 100 |
| Fecula plant wastes | *Endomycopsis fibuliger* + *Candida utilis* | Sweden -symba | 10,000 |
| Corn wastes | *Trichoderma viride* | USA, Denver | Pilot |
| Confectionery wastes | *Candida utilis* | UK, Tate & Lyle | 500 |
| Sulphite liquors | *Paecillomyces variottii* | Finland -Pekilo | 10,000 |
| | | USA - Rhinelander | 5,000 |
| | | USA - Boise cascadeco | 5,000 |
| | | Finland - Metsaluton co. | 10,000 |
| Whey | *Kluyveromyces fragilis* | USA - Amber laboratories | 5,000 |
| Cellulosic wastes | *Chaetomium cellulolyticum* | Canada - Waterloo Univ. | Pilot |

[a] By-products of amino acid production.

From : Senez (1987).

### 1.8.4 Enriching protein value of lignocellulosic feeds

Nutritional value, including proteins and vitamins, of animal feeds may be enhanced by microbial fermentations (Table 1.16 a & b). Han (1975) obtained a net protein yield of 20% from fermented rice straw and observed that microbial protein amino acid profiles were similar to that of soyabean meal. Similarly, barley straw fermented with *Trichoderma viride* yielded a product containing 18 - 24 % protein and 30% lignin (Peitersen, 1975 a & b). Han *et al.* (1976) obtained 42.6% crude protein, 0.4% fat and 6.4% nucleic acids in dried biomass of *Aureobasidium pullullans*, cultivated on acid hydrolysates of rye grass straw, which could be fed to rats without harmful effects.

Moo-Young *et al.* (1977) reported that amino acid profiles of *Chaetomium cellulolyticum* treated biomass were similar to those of alfalfa and soya bean products and that albino rats fed a diet containing 40% of the fermented substrate showed no adverse pathological effects. Pamment *et al.* (1979) investigated protein enrichment of pulp and paper mill wastes by fermentation with a cellulolytic fungus (*C. cellulolyticum*) and obtained a product, with 28% protein, at a specific growth rate of 0.12 $hr^{-1}$ on these substrates. Moo-Young *et al*, (1979 a & b) used the same organism in the "Waterloo" process for bioconversion of lignocellulosics into feeds that were highly nutritious and non -toxic.

Feeds including enriched wastes have been reported to give high weight gains in animals (Fig 1.10; Bhattacharjee, 1970).

Alkaline pretreatments produced optimal substrates for solid state fermentation with *C. cellulolyticum*. After 10 - 12 days fungal fermented caustic treated straws contained 19.7% crude protein whereas controls of fermented untreated straw contained only 9.8% protein. Karapinar and Worgon (1983) examined the feed value of bioconverted olive oil press cake and obtained 75.9% and 31.5% increases in protein yield of alkali treated and milled press cake, respectively, after 3 days of fermentation with *Phanerochaete chrysosporium* at 30$^{o}$C. This basidiomycete was more effective than *Aspergillus oryzae*, *A. niger*, or *T. viride* in the bioconversion .

Viesturs *et al.* (1981) converted wheat straw into a protein enriched ruminant feed with 50% *in vitro* digestibility, using mixed cultures of *C. cellulolyticum* and *Candida lipolytica* with a 3-litre horizontal stirred fermenter, whereas Laukevics *et al.* (1984) concluded that use of a stirrer reduced protein enrichment of wheat straw fermented with a mixed culture of *T. reesei* and *Endomycopsis fibuliger* R-574. Protein yields of 13% were achieved in a stationary layer fermenter as opposed to 5.3% in horizontal paddle and 7.2% in horizontal hollow shaft-stirred fermenters in 120 h incubations. Similarly, a five fold increase in protein content of

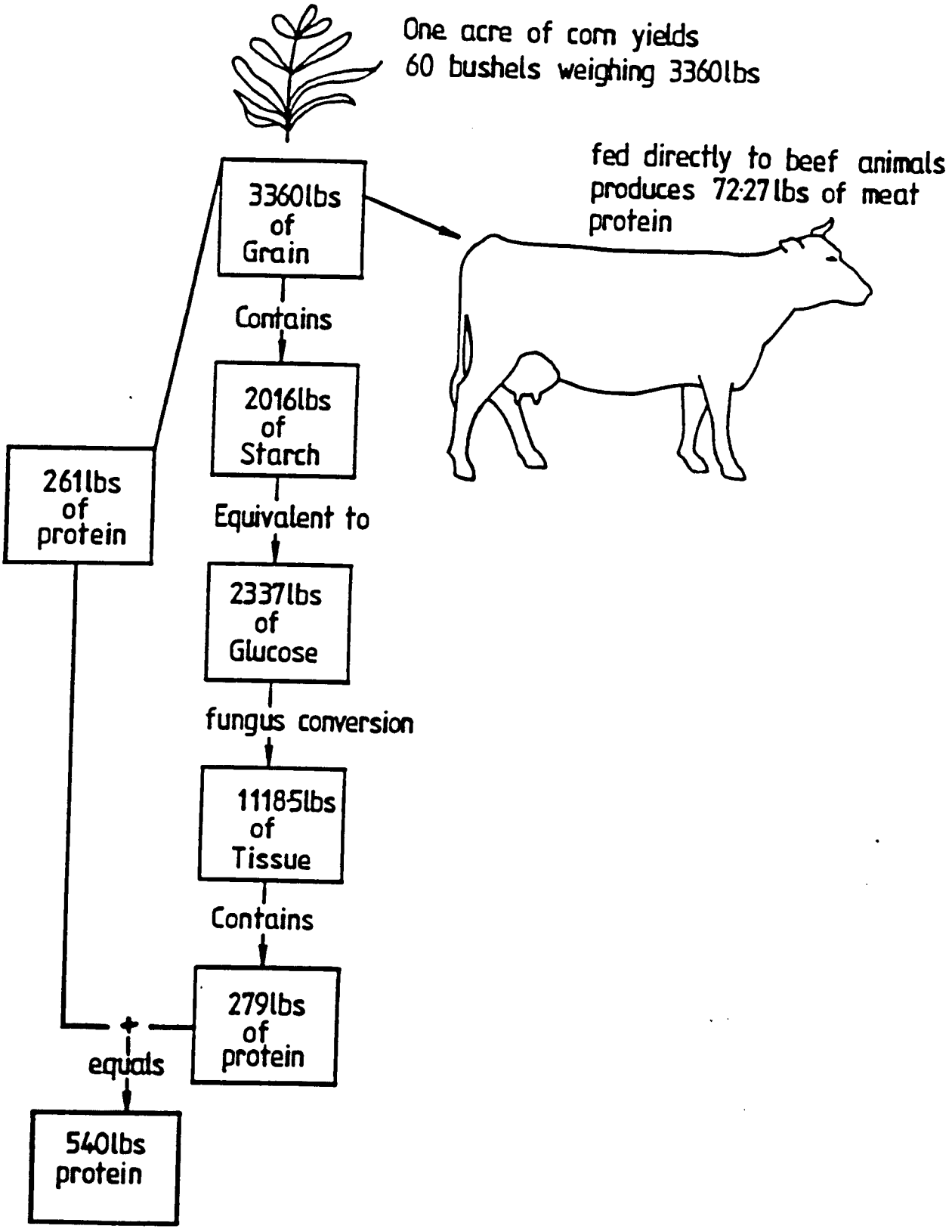



Fig. 1.10 Efficiency of protein production from an acre of corn when fed directly to animals.

From; Bhattacharjee (1970).

citrus wastes was recorded after 7-day fermentations at 30°C with mixed cultures of *P. chrysosporium* (CBS 67171) and *A. niger* (Suhasukan & Yasin, 1986).

Yadav (1988) examined bioconversion efficiencies in wheat straw fermented with an alkalophilic basidiomycete (*Coprinus* sp. 366). In a 7-day fermentation at 37 °C, in polypropylene bags, 40 g straw aliquots gained 3.7 % protein. In whey fermentations, supplementation with poultry, swine and cattle feedlot waste filtrate prior to fermentation gave enhancements of 62 %, 47 % and 72 % protein, respectively (Erdman & Reddy, 1987).

Kassim and Ghazi (1987) concluded that nutrient pH affected both fungal growth and protein yield with *Myrothecium verrucaria*, grown on cellulosics, with an optimum at pH 5.0. For this organism corn steep liquor was better than inorganic and alternate organic nitrogen sources, such as peptone and gluten.

In a lengthy set of experiments, Chavez *et al.* (1988a) examined composition and nutritive value of *C. cellulolyticum* biomass protein produced on either molasses (MBP-2) or glucose (MBP-1). When compared with other single cell protein products such as Torula yeast, brewers' yeast and dried mushrooms, in a 4-week feeding trial on weanling male rats, glucose-grown biomass gave greater weight increases than rats fed Torula yeast but lower increase than either brewers' yeast and or the control casein diet. Rats fed either mushrooms or molasses-grown *C. cellulolyticum* had the lowest body

weights suggesting low palatability. Although, of the test feeds, brewers' yeast gave the highest protein efficiency ratio this was lower than that of the control casein diet. The amino acid composition of both *C. cellulolyticum* biomass products compared favourably with that of dried skimmed milk. However, oven-drying at 60$^{o}$C had adverse effects on both biomass protein yield and amino acid composition when compared to freeze drying at -25$^{o}$C. and rats fed oven-dried protein had lower growth rates than those fed lyophilised material (Chavez *et al.*, 1988b). Blending of *C. cellulolyticum* protein with soya bean protein, in equal proportions, improved growth performance of chicks and piglets in terms of weight gain and increased PER values (Chavez *et al.*, 1988c).

### 1.8.5 Feed value of biodelignified lignocelluloses

It is widely believed that the low rumen digestibility of lignocelluloses arises from lignin in the cell wall impeding cellulolytic attack (Han, 1978; Paterson, 1989). Both chemical pretreatments (Al-Ani, 1985) and biological transformations have been evaluated for reduction of lignin and enhancement of digestibility of lignocellulosics but, to date, results have been inconclusive (Kirk & Moore, 1972; Han and Anderson, 1975; Zadrazil, 1985).

Kirk and Moore (1972) examined reductions in lignin content and increases in digestibility of wood by nine white rot fungi. Although it was confirmed that

digestibility was inversely correlated with lignin content, preferential removal of any polysaccharide was not observed. Han and Anderson, (1975) treated rye grass straw with culture filtrates of several white rot fungi and obtained reduction in lignin content but no increase in *in vitro* digestibility. These authors concluded that lignin depolymerisation products, such as quinones, may inhibit rumen microflora by either binding to substrate proteins or inactivating microbial enzymes.

Zadrazil and Brunnert (1980) evaluated a range of white rot fungi (*Pleurotus* sp., *Lentinus* sp., *Flammulina velutipes*, *Ganoderma applanatum*, *Agrocybe aegerita*) for mineralisation of lignin and enhancement of digestibility of wheat straw with differing levels of ammonium nitrate supplementation. It was concluded that, whereas rate of lignin breakdown varied greatly, no relationship could be established between lignin degradation and *in vitro* digestibility of fermented straw. In particular, *Ganoderma applanatum*, in a 90-day fermentation, reduced lignin content by 44.9% but also reduced *in vitro* digestibility. In contrast a *Flammulina* strain did not reduce lignin content but reduced digestibility to 30.4% less than control straw. These authors also studied the effect of moisture content on both lignin degradation and *in vitro* digestibilities (Zadrazil & Brunnert, 1981). A number of strains, (*P. sajo-caju*, *P. serotinum*,

*Abortiporus biennis*) reduced lignin contents more efficiently at high moisture contents of 75 - 125ml/25g but decreased *in vitro* digestibility of wheat straw at this $a_W$. *Lentinus edodes* reduced lignin content of wheat straw by 61% when supplemented with 0.25% $NH_4NO_3$ and increased *in vitro* digestibility. In another organism (*P. florida*) greater delignification through ammonium nitrate supplementation was accompanied by decreases in digestibility (Zadrazil & Brunnert, 1980). Zadrazil (1980) also evaluated the feed value of five natural lignocellulosics (beech sawdust, reed, rape, sunflower and rice husk) after fungal pretreatments, and concluded that all fungi tested decreased digestibility of rice husks but that lignin reduction did not correlate with increases in digestibility.

Reid and Seifert (1982) assessed the effect of atmospheric oxygen on fungal delignification of aspen wood. It was concluded that lignin degradation by a number of white-rot fungi (*P. chrysosporium, C. versicolor, Pycnoporus cinnabarim, Lentinus edodes, Grifola frondosa, Polyporus brumalis* and *Merulius tremellosus*) was greater in an atmosphere of 100% oxygen than in air. Moreover,wood rotted in 100% oxygen was found to be more digestible except for that treated with *P. ostreatus* and *G. frondosa*. A correlation was observed between increase in digestibility and reductions in lignin content of rotted wood: *C. versicolor* reduced lignin content of aspen wood by 46% and increased

digestibility to 32.8%, in 100% oxygen. However *Pcynoporous cinnabarium* reduced lignin content by 37% and increased substrate digestibility to 38.6%.

Hatakka (1983) studied the effects of white rot fungal pre-treatment of wheat straw on susceptibility to enzymic saccharification and reported that *P. ostreatus* (535), *Pycnoporus cinnabarim* (115), *Ischnoderma benzoinum* (108) fermentations gave significant enhancements. It was concluded that organisms had degraded or modified the lignin component. It was also observed that straw treated with *Pleurotus* species yielded sugar concentrations three times higher than controls. Unfortunately lignin contents of treated substrates were not quantified. Levonen-munoz *et al.* (1983) also evaluated pre-treatment of lignocellulosics with lignin degrading fungi to enhance enzymic saccharification. It was reported that sugar yields were increased from 24.8% to 34 - 38% after pretreatment with *P. chrysosporium* and a *Polyporus* species.

Bakshi *et al.* (1986) investigated the use of fermented straws as a basal ration for ruminants. A 9-day fermentation of wheat straw enhanced *in vitro* rumen digestibility of residual carbohydrates and buffaloes fed fermented straw had a live weight gain of 431 kg compared to 360 kg for controls. However, the lignin content of fermented straw was reported as 80 - 95% greater than controls.

Similar observations were reported by Al-Ani and Smith (1986) using bagasse supplemented with a range of nitrogen sources (malt extract, peptone, yeast extract, $NH_4NO_3$, $NH_4Cl$) followed by fermentation with *P. chrysosporium*. Apparent increases in lignin content were recorded although both malt extract and peptone enhanced the *in vitro* digestibility of the substrates whereas decreases were recorded with $NH_4NO_3$, yeast extract and asparagine supplementation. Fermentations with mutant *P. chrysosporium* neither reduced lignin content nor enhanced digestibility (Al-Ani & Smith, 1986). Pretreatment of sugarcane bagasse with alkali followed by fungal fermentation enhanced digestibility of substrates although no reduction in lignin content was observed (Al-Ani & Smith, 1988).

Buswell and Odier (1987), in a review, concluded that the nature of lignocellulose substrate determines susceptibility to hydrolysis by rumen microbes. For example, straw and hardwoods are found to be more digestible than soft woods. Thus although *in vitro* digestibility of fermented lignocellulosic materials may be enhanced there is no reason why there should be a direct relationship existing between weight loss and *in vitro* digestibility of fermented substrates .

Agosin *et al.* (1989) reported that lignin content of radiata pine was not reduced in fermentations with the brown rot fungus, *Gloephyllum trabeum*, and that increases in *in vitro* digestibility during the first two

weeks of fermentation were followed by substantial decreases following further incubation. In contrast, Zadrazil and Kamra (1989) reported that reductions in organic matter and lignin contents in the initial stages of wheat straw fermentation by *P. sajo-caju*, *P. eryngii* and *Stropharia rugosoannulata* depressed digestibility but this was followed by an increases in digestibility correlated with further degradation of both organic matter and lignin.

However, it should be pointed out that these studies were laboratory based and it is often difficult to simulate a complex biological processes in the laboratory. Natural biotransformation of lignocelluloses into cattle feed has been carried out in the valvidian rain forests in southern Chile for centuries (Gonzalez *et al.*, 1989). Such natural fermentations of wood yield a partially delignified material, initially called "huempe", which is consumed by grazing cattle (Phillipi, 1893). This bioconversion of wood into "Palo-Podrido" (Knoche *et al.*, 1929) is effected through action of successive populations of filamentous fungi and yeasts (Gonzalez *et al.*, 1986, 1989). The importance of such microbial associations, including yeasts, in wood decay has also been highlighted by Blanchette and Shaw (1978).

### 1.8.6 Constraints on the acceptance of microbial biomass enriched substrates as food & feed.

Use of microbial biomass in foods or feeds may have such problems as ensuring palatability, and consumer acceptance despite religious beliefs and cultural attitudes. Many of these problems can be overcome by modern food technology and use of colouring and flavourings. It is also important that pathological effects and protein functionality are characterised. A further problem is the high nucleic acid content of microbial cells which, with large-scale ingestion, has been reported to interrupt mammalian physiology (Li & Chang, 1982; Scrimshaw, 1985). According to Scrimshaw (1985), about 1.75 g $d^{-1}$ of uric acid is excreted through the human kidneys for each 16 - 18 g of RNA in single cell protein although the effect of DNA was only 50 % of that figure. The permissible limit of SCP nucleic acid is considered to be 2.0 g per day (Anon, 1975) for most adults and less than 0.5 g per day for children. Daly and Ruiz (1974) reported that the nucleic acid content of *Cellulomonas* sp biomass, grown on alkali-treated bagasse, could be reduced by passing a 10% cell suspension through a Manton - Gaulin homogeniser for 4 - 6h. Spinning of the homogenate yielded a product containing less than 4% nucleic acid. Moreover, RNA content of microbial biomass can be reduced to safe levels by both heat treatments and alkaline hydrolysis (Scrimshaw & Dillon, 1979).

### 1.8.7 Safety testing

To improve acceptability of microbial biomass products, the International Union of Pure and Applied Chemists (IUPAC) and the protein advisory group of the United Nations have published guidelines for assessment of nutritional value and safety of novel microbial proteins. These were initially concentrated on human food applications (Protein Advisory Group (PAG), 1970 & 1972; IUPAC, 1974) but later focused on animal feeds (PAG, 1974; Stringer, 1985). Essential requirements to be met before novel protein sources could be accepted into human diets were summarised by Udall and Scrimshaw (1986) as: lack of allergenicity, mutagenicity, carcinogenicity, or teratogenicity; favourable organoleptic and functional properties; and cultural acceptability. For animal use, safety of species, substrates, and derived animal products and nutritional value must be demonstrated.

## 1.9 ASSESSMENT OF RUMEN DIGESTIBILITY OF ANIMAL FEEDS

### 1.9.1 The rumen

The rumen may be described as a "continuous fermentation vessel" in the gut of many herbivorous animals (Hungate, 1966; Hobson, 1971) and plays a central role in the digestion of plant cell-wall polysaccharides, which are not attacked by mammalian intestinal enzymes. Hobson (1971) concluded there were two major types of digestive system where microbes contribute significantly

to digestion (Figs. 1.11 a & b). In the horse, microbial breakdown of cellulosics occurs in the caecum and large intestine (Fig. 1.11a), after passage through the stomach and small intestine. Consequently, microbial proteins are not hydrolysed and taken up by the animal; absorption of microbial derived nutrients through the walls of the small intestine appears limited. In ruminants such as cattle, sheep, camels and certain marsupials, microbial attack is in a specialised compartment of a complex stomach (Fig. 1.11b), and nutrients are absorbed prior to passage of fibre into the small intestine.

### 1.9.2 Rumen micro-organisms

Micro-organisms playing the central role of attack on cell wall materials include eubacteria, fungi and protozoa.

**Rumen bacteria** have a growth optimum of 39 + 1$^{o}$C and pH optima of 5.5 - 7.0. The rumen can provide a relatively stable habitat due to continuous supply of similar foods for days or weeks and constant removal of fermentation products. The result is a stable mixed cultures with diversity maintained by the complexity of animal feed nutrients, including carbohydrates, proteins and fats (Hungate, 1966).

Despite the predominantly anaerobic nature of the rumen environment, traces of oxygen are still observed above rumen digesta (Hungate, 1966; Czerkawski and

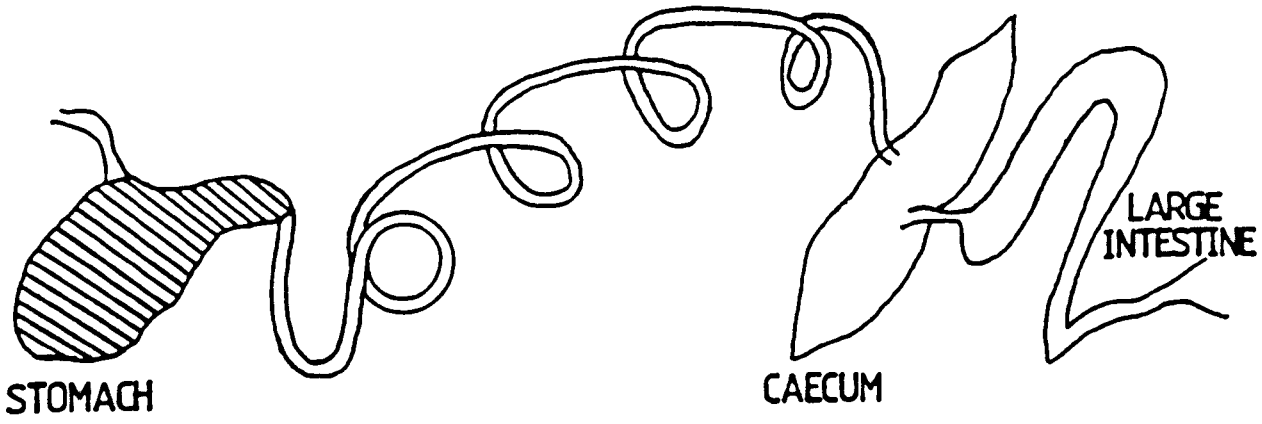



Fig. 1.11a Digestive system of horse.

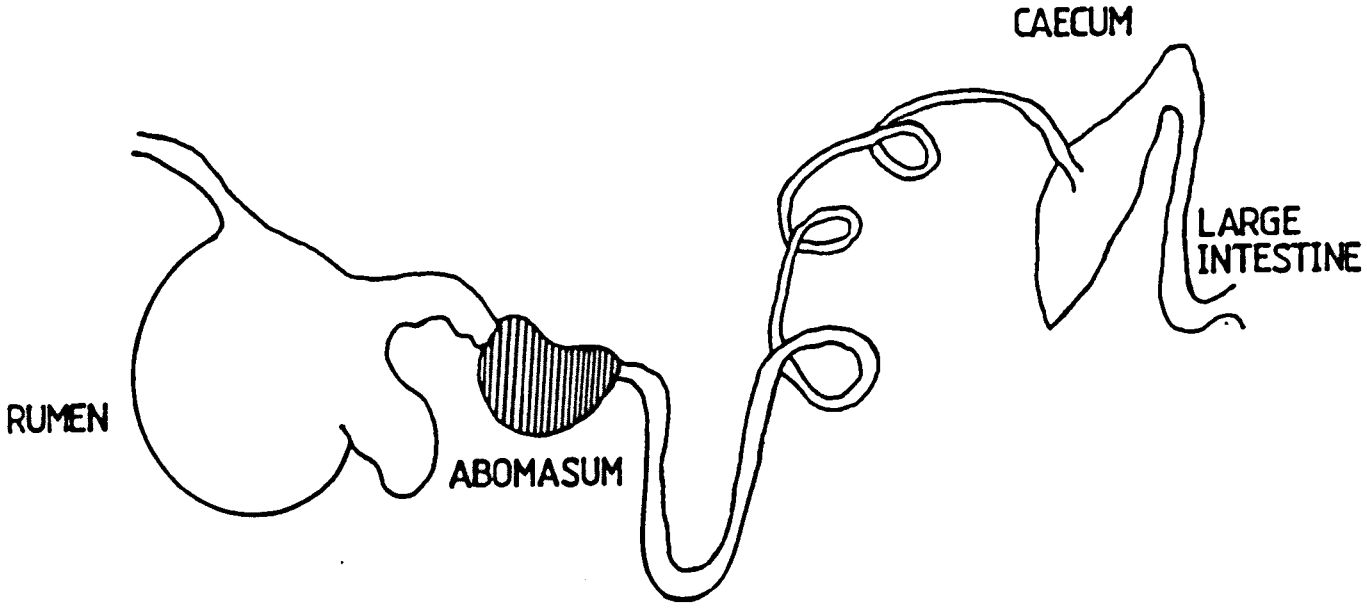



Fig. 1.11b Digestive system of a ruminant.

From ; Hobson (1971).

Clapperton, 1968; Czerkawski and Breckenridge , 1979 a & b) which have been attributed to small populations of aerobic or microaerophilic micro-organisms (Hungate, 1966). Most rumen bacteria are restricted to that habitat, though Bryant and Small (1960) have reported the presence of rumen-like bacteria in other habitats such as the faeces of mammals, and caecum contents of rabbits and porcupine. Within the rumen, micro-organisms may be unevenly distributed between solid particles and the liquid phase (Akin, 1979; Orpin & Letcher, 1978; Orpin, 1981).

Most rumen bacteria are short rods and cocci and range in size from 0.4 - 1.0 μm in diameter and 1 - 3 μm in length. Certain species are distinctive under microscopic examination (Moir & Masson, 1952 ; Hobson, 1971). The typical rumen bacteria are obligate anaerobes; some are gram positive cocci and many are gram negative rods. .

Rumen bacteria have been categorised, on the basis of substrates attacked into cellulose, starch and hemicellulose digesters. Among the characteristic species are cellulose decomposers ; *Bacteroides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus albus,* and *Lachnospira muliparus* (Pelczar *et al.*, 1977). The cellulolytic bacteria play an important role in the

ruminal breakdown of plant structural polysaccharides (Hazlewood *et al.*, 1990).

In ruminants such as the high artic Svalbard reindeer *B. fibrisolvens* is the major cultureable cellulolytic bacterium in both summer and winter representing 66% and 55% of the cellulolytic population respectively (Orpin *et al.*, 1985). Recently Kopecny (1986) reported that the cellulase of *B. fibrisolvens* comprised extracellular and cell associated enzymes, including an endo - 1,4-β glucanase and three B-glucosidases. Ong *et al.* (1989) revealed that cellulases produced by *Cellulomonas fimi* contain distinct structural regions responsible for cellulase binding and catalytic activity. Although pectin and starch are utilised by certain cellulolytic strains, xylans are not fermented by these organisms (Dehority, 1965)

Amylolytic bacteria in the rumen include strains of *Streptococcus bovis*, *Bacteroides amylophilus*, *B. ruminicola*, *Succinomonas amylolytica* and *Selenomonas ruminantium*. *Megasphaera elsdenii* strains are the dominant starch-degraders in the rumen although these bacteria have not been isolated from ruminant feeds. Strains of *B. amylophilus* attack starch and other polysaccharides but do not appear to ferment monosaccharides such as glucose whereas *B. ruminicola* and *S. amylolytica* ferment both sugars and starch.

Hemicelluloses such as xylan are fermented by many strains of *Bacteroides ruminicola*, *B. amylogenes*, *Butyrivibrio fibrisolvens*, *Ruminococcus flavefaciens* and *R. albus*. However certain strains of *Ruminococcus* are known to be unable to utilise xylose (Hungate, 1966).

Oligosaccharides appear to be broken down by bacteria such as *Lactobacillus bifidus* which secrete dextranases. Other bacteria, such as strains of *Eubacterium ruminantium* play a key role in the conversion of sugars into carbon dioxide and the volatile fatty acids: acetate, formate, lactate and butyrate (Fig. 1.12). Acids such as succinate and formate appear to be catabolised by undescribed organisms since these acids are produced by many strains of rumen bacteria yet are not observed to accumulate unlike other volatile acids. *Streptococcus bovis* has been reported to ferment starch to lactate which *Megasphaera elsdenii* catabolises to acetate, propionate, butyrate, valerate, caproate, carbon dioxide and traces of hydrogen.

Methanogenic bacteria, normally very sensitive to oxygen, grow well at pH 7.0 in media with a very low oxidation - reduction potential (-0.35 Ev) which has delayed isolation and characterisation of methanogenic bacteria in pure culture. However, a Gram-positive, coccoid to rod- shaped, non-motile methane producing bacterium, *Methanobacterium ruminantium*, which was able

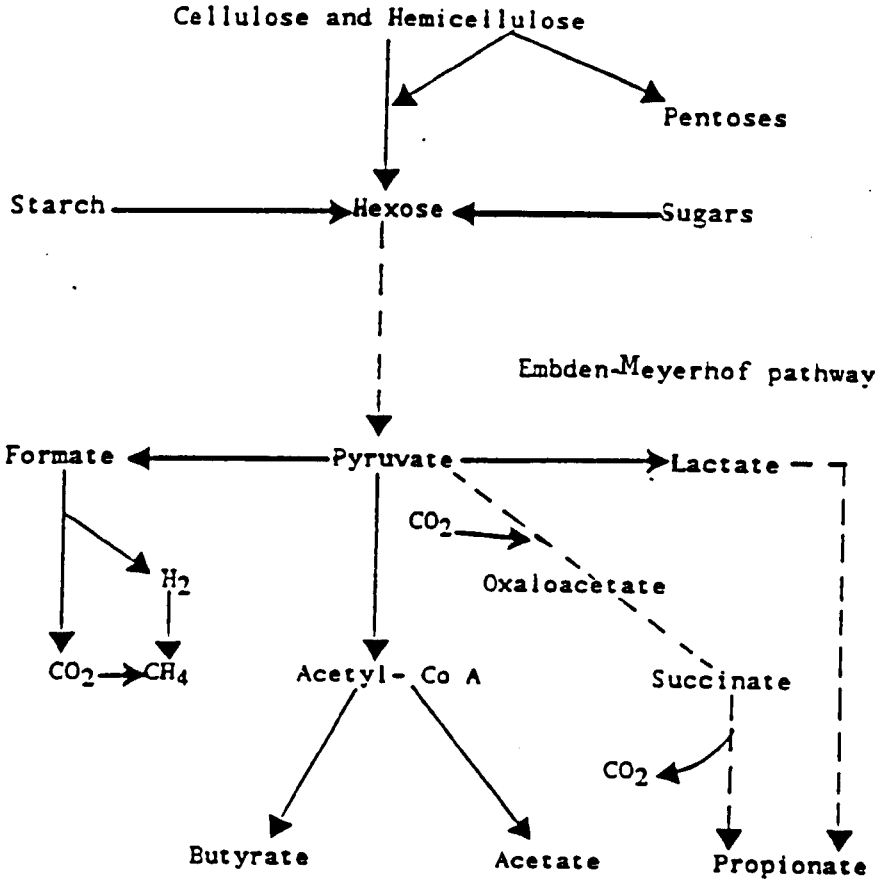



Fig. 1.12 Carbohydrate breakdown in the rumen
From ; Davis & Clark (1981).

to utilise both ether-soluble and insoluble rumen substrates and metabolised hydrogen and carbon dioxide to form methane and water has been isolated (Smith & Hungate, 1958; Pelczar *et al.*, 1977 ). Rumen methanogenic bacteria do not appear to be able to utilise acetate, propionate and butyrate which can thus be absorbed through the mammalian the gut wall.

Proteolytic bacteria do not appear to be important in the rumen, although a number of proteolytic spore forming bacteria, of unknown origin have been reported. Such bacteria include strains of *Proteus*, *Corynebacterium* and *Micrococcus* (Hungate, 1966). Czerkawski and Breckenridge (1985) attributed increases in concentrations of ammonia, free amino acids and soluble peptidic compounds to proteolytic activity of uncharacterised rumen bacteria.

Similarly, dietary lipids are hydrolysed by rumen bacteria into glycerol and volatile acids (including acetate, propionate, butyrate, and succinate) although formate and lactate are not produced (Hobson & Mann, 1961). Lipolytic bacteria can metabolise only ribose, fructose and glycerol (Hungate, 1966) although glycerol can also be metabolised by *Veillonella pervula* (Johns, 1953). Rumen fermentation of lipids does not produce large volumes of gases, although hydrogen sulphide has been reported as one of the few gases formed.

**1.9.2b Rumen protozoa**

The origin of rumen protozoa is not clear, although organisms can survive as cysts in hay diets. Active and motile unicellular eukaryotes are found in the rumen, whereas the omasum and abomasum harbour only immobile and disintegrating protozoal cells and examination of duodenal contents reveals no traces of protozoa (Hungate, 1966). Rumen ciliates have adapted to the anaerobic rumen environment, contributing to breakdown of polysaccharides (Gijzen *et al.*, 1986), but are unable to tolerate acid pH.

The majority of rumen protozoa are ciliates, generally asymmetrical, with only a few species of the smaller flagellates, typically strains of *Chilomastix*, *Tetratrichomonas* sp, *Pentatrichomonas homini*, *Monocercomonas bovis*, *Monocercomonas ruminantium*, *Callimastic frontalis*. Ciliates fall into two groups: Holotrichs and Entodiniomorphs. Holotrichs (*Isotrichia* sp) range in size from 80 - 160 μm by 53 - 100 μm to 97 - 131 by 68 - 87 μm and are very conspicuous, highly ciliated and swim more rapidly than Entodiniomorphs under the microscope. The Entodiniomorphs possess specialised bands of syncilia, used both for locomotion and food ingestion, but have less abundant cilia. Important organisms include *Entodinium* sp, *Diplodinium* sp, *Epidinium* sp and *Ophryoscolex* sp.

It has been concluded that many protozoa exhibit host specificity so that the fauna of the farmed red deer gut differs markedly from that of wild deer of the same species. However some cattle rumen protozoa have been shown to be widely distributed (Hungate, 1943).

The holotrichs utilise disaccharides, storing carbohydrate as starch. Howard (1959) showed that strains of *Dasytrichia* possess enzymes for hydrolysis of cellobiose, maltose and sucrose (Howard, 1959) and *Isotrichia* secrete amylases. Walker and Hope (1964) concluded that protozoa secrete more amylases than bacteria, preferentially utilising soluble starch. Coleman (1964) observed that *Entodinium caudatum* assimilates maltose and soluble starch but was less effective in utilising cellobiose, sucrose and glucose.

*In vitro* studies of rumen contents containing powdered dried grass and cellulose revealed that *Eudiplodium neglectum*, *Eudiplodium magii* and *Diplodinium dentum* ingested substantial quantities of the cellulose (Jouany & Senaud, 1982). However, no cellulolytic activity was observed with cultures of *Epidinium* sp and *Ophryoscolex* sp. although Bailey *et al*, (1962) demonstrated cellobiase activity and the ability to ingest hemicelluloses in *Epidinium*. Literature reports have suggested that protozoa contribute significantly to fermentation of soluble rumen carbohydrates (Howard, 1963; Ushida *et al*., 1987). Similarly protozoa have been reported to obtain energy from proteinaceous materials,

and have been observed to increase when feeds are supplemented with protein. It is also thought that under starvation conditions protozoa may utilise bacteria as a source of nitrogen (Hungate, 1966). Ultimately, much of the nutrients accumulated in protozoa, especially starch stored in holotrichs, is digested in the abomasum and intestine contributing to the nutrition of the host ruminant.

**1.9.2c Anaerobic fungi**

Until the report of Orpin (1981) on isolation of three morphologically distinct strains of anaerobic phycomycetes from the caecum contents of horse, little was known of gut fungi. The horse isolates were found to be similar to *Piromonas communis* and *Sphaeromonas communis*, isolated from the ovine rumen. In the past decade, the role of anaerobic fungi in rumen breakdown of dietary fibre has received considerable attention (Borneman *et al.*, 1989; Gordon & Philips, 1989; Joblin *et al.*, 1990).

Borneman *et al.* (1989) investigated fermentation products and cell wall degrading enzymes in five anaerobic fungi and concluded that major fermentation products in all isolates were formate, acetate, lactate, ethanol, carbon dioxide and hydrogen. The fungi were also able to depolymerise cell-wall carbohydrates, exhibiting such enzymic activities as endoglucanase, exoglucanase, B-glucosidase, xylanase and B-xylosidase. The ability of

two anaerobic *Neocallimastix* isolates, from sheep, to degrade untreated poplar was assessed by Joblin and Nay (1989) and it was concluded that *N. frontalis* could solubilize all three lignocellulosic polymers- cellulose, hemicelluloses and lignin.

### 1.9.3 Physiology of ruminal fermentations

In the rumen, micro-organisms ferment susceptible feed carbohydrates into volatile fatty acids and gases (Fig. 1.12). These acids act as precursors for synthesis of carbohydrates, such as glucose from propionate, and lipids (Hungate, 1966). Less digestible carbohydrates, notably crystalline cellulose, are carried through the gut, forming part of the faeces (Hobson, 1971). Production of volatile acids results in a lowering of rumen pH, usually controlled by neutralisation with saliva secreted in the ruminant mouth. It has been estimated that the daily saliva output of sheep is 6 - 16 litres and about 98 - 190 litres in cattle (Hobson, 1971).

Feed proteins are degraded into peptides, amino acids, and ammmonia which are converted into microbial biomass and recovered by the host in subsequent digestion of microbial cells. However a large part of feed amino acids are incorporated into microbial cell wall peptides, not metabolised in the ruminant intestine. Another loss is conversion of feed nitrogen into microbial hexosamines and nucleic acids also not utilised

by the animal (Hobson, 1971). All classes of feed lipid have been shown to be metabolised by rumen microbiota (Garton *et al.*, 1961) although rumen bacteria also synthesise lipids from fatty acid precursors (Fig.1.13a). Hobson (1971) reported that a significant proportion of milk lipids are of microbial origin. However, most long chain fatty acids are not fermented by rumen bacteria but are absorbed directly by the intestine.

Rumen bacteria appear to play key roles in detoxification of pesticides in feeds and reduce sulphate and nitrate from fertilisers to sulphide and ammonia. Sulphides can be re-used in synthesis of amino acids by rumen bacteria. Vitamins are also synthesised in the rumen, notably Vitamin B12 and Vitamin K which are utilised for growth by both rumen microbes and the animal.

Although lignin in forage feeds is not thought to be substantially degraded by rumen microbiota (Morrison, 1986), it has been shown that lignin or phenolic constituents of orchard grass are converted to either carbon dioxide or methane by rumen bacteria (Akin & Benner, 1988). However, the biochemical pathways for the degradation of these organic biopolymers in the rumen are likely to be similar to that occuring in landfill site (Fig. 1.13 a & b).

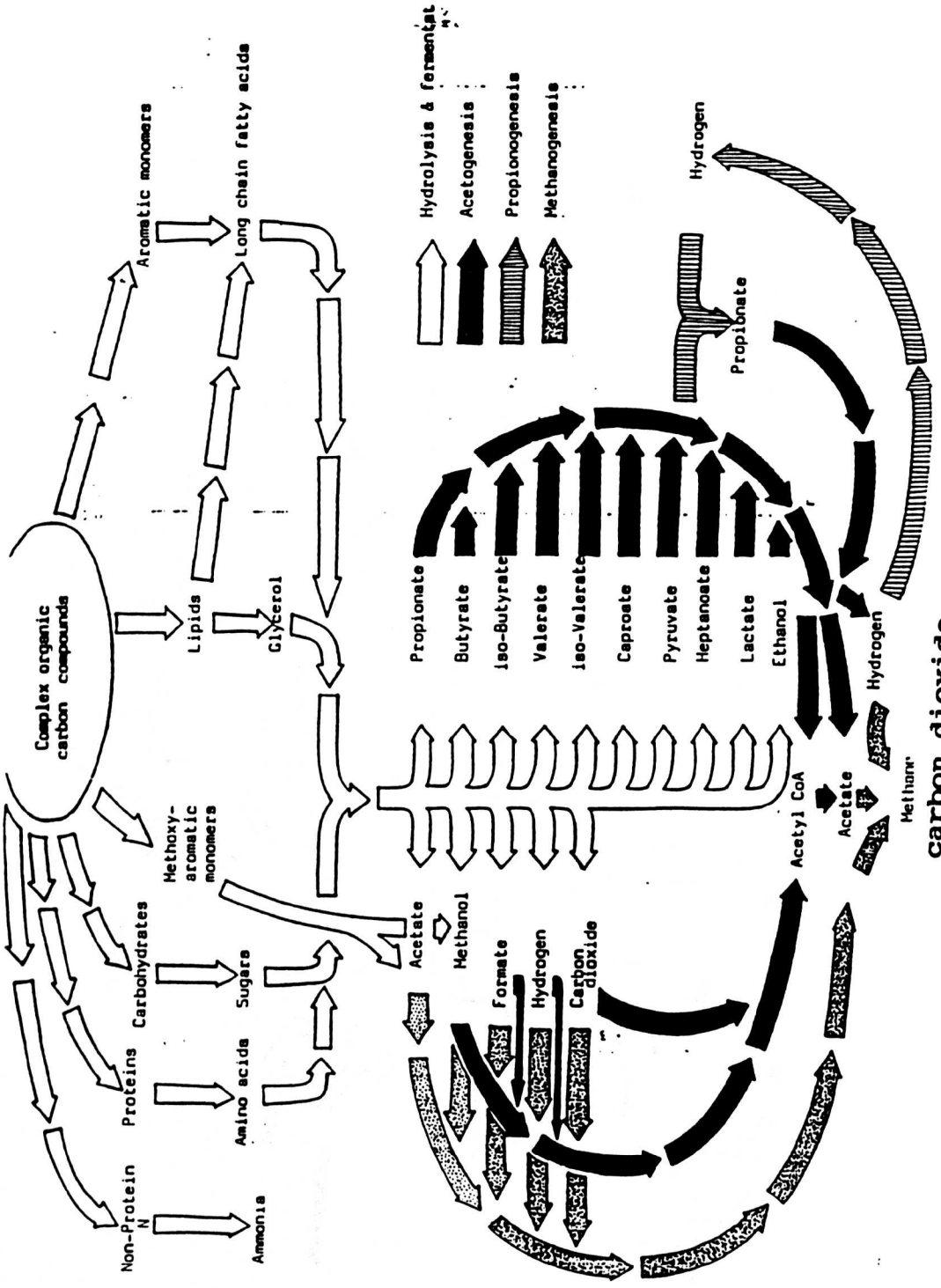
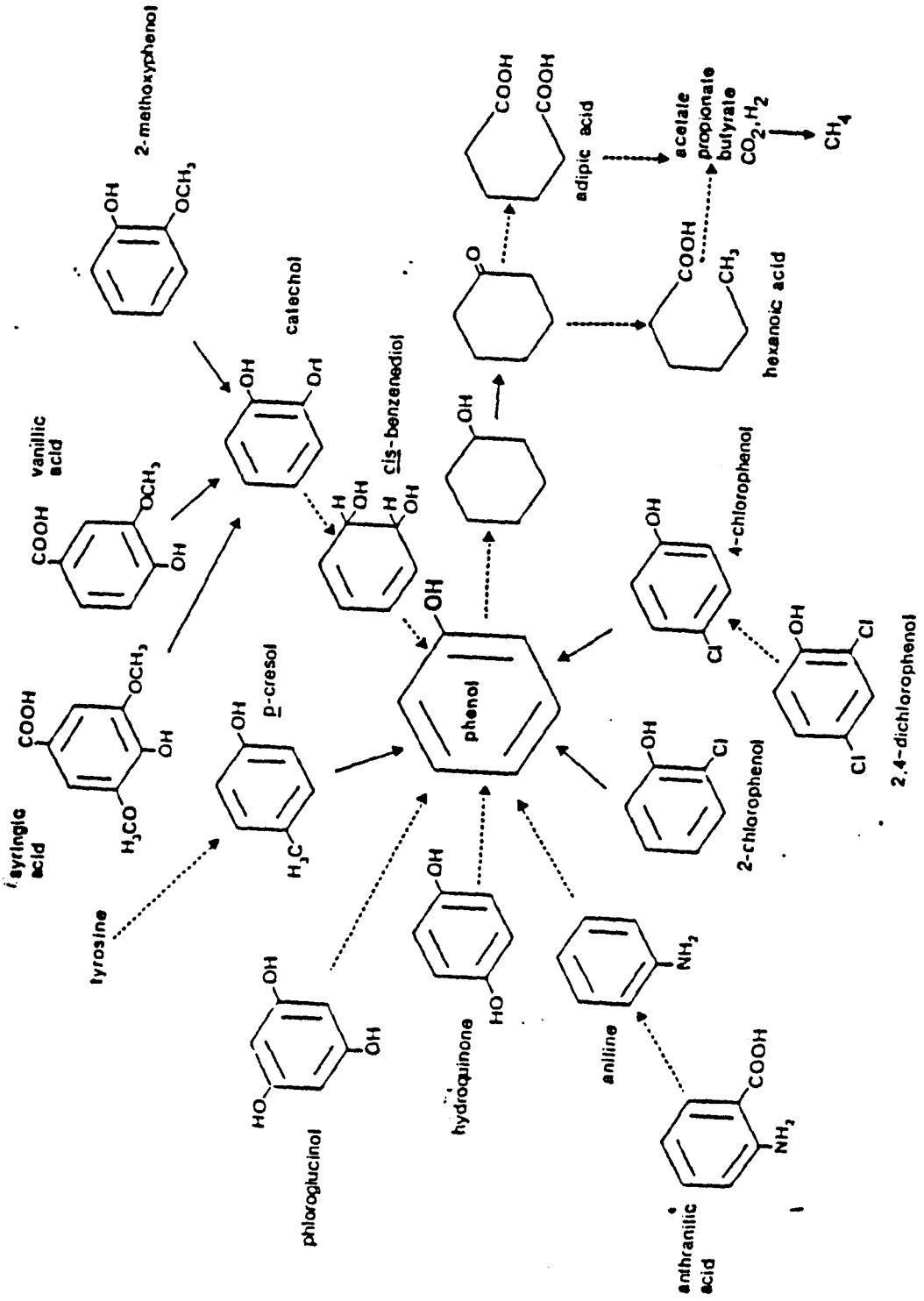



Fig. 1.13a Pathways of anaerobic catabolism of organic polymers. From; Senior, (1989).

Fig. 1.13b Pathways for anaerobic cleavage of aromatic compounds. From; Watson-Craik (1987).



### 1.9.4 Rusitec : the artificial rumen.

Feeding trials on dietary fibres are often impractical for economic reasons (McBee, 1953; Hobson, 1971; Czerkawski and Breckenridge, 1979). Thus simulations of the animal rumen have been developed for *in vitro* assays of digestibility. McBee (1953) employed a Warburg respirometer, inoculating flasks with 1 ml aliquots of rumen fluid from fistulated sheep, mixed with an equal volume of 2% sodium bicarbonate buffer, in the upper part of vessels, and 0.5ml of buffered substrate in side arms, flushing with carbon dioxide prior to and after addition of rumen fluid. The reaction vessel was incubated in a water bath at 37$^{o}$C and fermentation rate expressed as gas evolution at 5 to 10 minute intervals. Bently *et al.* (1955) inoculated a heavy cell suspension, derived from rumen contents, into a culture tube containing urea, a buffered salt solution, bacterial nutrients (vitamins and fatty acids) and a test substrate for *in vitro* digestibility assay; this was incubated anaerobically for an 24 hr and periodic adjustment of pH. This culture system did not support the growth of protozoa but allowed multiplication of cellulolytic bacteria. The widely used *in vitro* digestibility assay of Tilley and Terry (1963) is based upon incubation, at 30$^{o}$C, of substrates in 100ml centrifuge tubes containing phosphate buffer, urea solution and rumen fluid supplemented with pepsin. The major disadvantage of such methods is that microbial activity may be suppressed due

to accumulation of metabolic products.

To overcome such problems the approach developed by Fina *et al.* (1962) was to utilise a semi permeable membrane (VIVAR) which permitted dialysis of soluble metabolic products from digestion sample into the rumen fluid with exchange for other growth nutrients. The package containing test substrates was placed in a fistulated rumen with the aid of a canula. This incubation ensured fluctuations in temperature and pH exactly replicating those taking place in the animal.

Morris and Bacon (1977) assessed the fate of acetyl groups and sugar components during ruminant digestion by suspending nylon bags containing food in the rumen. Dinsdale *et al.* (1978) used a similar technique to establish modes of microbial attack on a range of cellulosics in the ovine rumen. However, a fundamental problem in such techniques was the limited incubation period which was not long enough to ensure adaptation of the microflora to the novel substrate. To achieve the balanced biological and physiological interaction of the rumen *in vitro*, a reproducible continuous culture system was required. Such a system was described by Czerkawski and Breckenridge (1977) and Balasubramanya *et al.* (1987).

Balasubramanya *et al.* (1987) employed an anaerobic digester vessel to determine rumen digestibility of cellulosics. A 10 l borosilicate vessel was equipped with an inlet on the upper and an outlet at the lower side. Nylon bags (10 X 5 cm; 5 u pore) containing 1 g aliquots

of powdered feeds were suspended in the digester. The vessel was inoculated with cattle waste (Balasubramanya *et al.*, 1981) and gas production was found to be most active during the second and third weeks of incubation. However, the RUSITEC technique developed by Czerkawski and Breckenridge (1977) facilitated construction, reliability, and durability and standard accessories could be used for monitoring pH, effluent and gas production. Consequently, "RUSITEC" has remained one of the most effective means of assessing *in vitro* digestibility of feed fibres and monitoring a number of metabolic activities in the rumen although the exact rumen balance of microbial population and activities may not be maintained. In practice this equipment has provided important data on the biological activities important in the rumen.

### 1.9.5 Metabolism of nutrients in the simulated rumen.

The nature of ruminant feeds has a marked effect on their estimated digestibility, especially when conditions are removed from those to which rumen microbes are accustomed (Czerkaswki & Breckenridge, 1977). A typical experiment is infusion of soluble food (composed of carbohydrate 120 g ; organic acids 0.73 g; protein 2.0 g; lipid 0.75 g; salts 1.22 g and trace compounds 0.025 g per litre of artificial saliva) into four "RUSITEC" vessels containing an inert solid matrix, strained rumen solids and rumen fluid followed by incubation for several days at 39 $^{o}$C (Czerkawski & Breckenridge, 1979b). It was

observed that the soluble substrate enhanced fermentation rate but protozoa disappeared from the effluent. Jouany and Senaud (1982) studied the effects of rumen ciliates on digestion of feeds rich in cell wall carbohydrates or starch, using fistulated sheep and found that ciliate biomass was greater in animals fed a high starch diet than in those receiving diets rich in cell-wall carbohydrates. It was also observed that bacterial cellulolytic activity was improved by the presence of rumen ciliates but was decreased with the starch diet. Ushida *et al.* (1987) demonstrated that the presence of protozoa in the rumen stimulated multiplication of cellulolytic bacteria and was thus indirectly responsible for enhanced cellulolysis and xylanolysis. Similarly, Karasawa *et al.* (1983) investigated the effects of carbohydrates on cellulolytic activity of rumen micro-organisms *in vitro* by incubating mixed rumen microflora in anaerobic culture media containing 0.5% cellulose and a range of supplementary carbohydrates. Glucose, cellobiose, maltose and soluble starch stimulated respectively, 36.5 %, 24.5 %, 22.0 % and 9.7 % increases in cellulolytic activity in 12 h incubations at 39 $^{o}$C. Moreover, Gijzen *et al.* (1986) reported similar ciliate populations, fibre degradation and volatile acid production to those found *in vivo* after 65 days of continuous fermentation in an artificial rumen.

Boran and Czerkawski (1983) investigated the effect of substrate concentration on fermentations using a small

artificial rumen consisting of a 50 ml glass syringe fitted with a 3-way plastic stopcock. It was observed that, in general, more propionate, butyrate and less acetate were produced with increasing concentrations of glucose but at high levels, glucose accumulated and enzymic activities were suppressed. Krishna *et al.* (1986) used RUSITEC to evaluate rumen diets containing spent hops and observed that whereas significant increases in total volatile fatty acids and methane production were recorded, mixing of hops with hay resulted in lower methane output, a slight increase in propionate and a marked increase in acetate.

Wedig *et al.* (1987) estimated breakdown of hemicellulose components of alfalfa and orchard grass hay in rumen fluid for 72h and established that xylose concentration increased more rapidly with the alfalfa than with the hay. However, xylose to arabinose ratios increased with digestion for both substrates, indicating arabinose components were more digestible than xylose. Parallel increase in xylose and glucose were attributed to preferential digestion of cellulose over hemicellulose components.

The influence of nitrogen source on the fermentation was investigated by Czerkawski and Breckenridge (1979) who infused various concentrations of ammonium chloride into RUSITEC vessels containing a basal ration (hay) and rumen contents. In duplicate experiments the basal ration was supplemented with either whey powder or soyabean

meal. It was established that shortage of nitrogen decreased digestibility of the basal ration, but substantial increases in protein were observed if both glucose and a nitrogen source were added. Changes in volatile acid and gases were found to be consistent with variation in digestibility of hay dry matter. Fish and soybean meal contributed substantially to the output of both gases and volatile fatty acids (Czerkawski & Breckenridge, 1985) which was attributed to microbial attack of the proteinaceous feeds. However, casein enhanced only the output of C5 acids with a significant decrease in acetate production, suggesting this was not readily hydrolysed in the rumen. In simulated rumen fermentations of starch and cellulose, Al-Akbar *et al.* (1987) observed that supplementation of starch with casein resulted in limited gas and volatile acid production and addition of urea depressed the rate of cellulose fermentation. However incubation of starch with poultry waste enhanced yields of gas, total volatile acids and ammonia compared to controls.

Komisarczuk *et al.* (1986), in a study of the effects of phosphorous deficiency on microbial degradation of dietary components *in vitro* concluded that cellulose digestion was markedly affected and hemicellulose degradation, and volatile acid and gas production were slightly reduced, with a pH increase from 6.5 to 7.3. However phosphorus concentration had no significant effect on protozoan population production. In addition,

it was established that low concentrations of phosphorus(<1mgL$^{-1}$) reduced metabolism of xylose, cellulose and glucose (Komisarczuk *et al.*, 1987).

### 1.9.6 Constraints on the digestibility of fibre in the rumen

Certain constituents of feeds, notably lignin-related compounds, repress breakdown of lignocellulosics in the rumen. Varel and Jung (1986) investigated the effect of cinnamic acid and vanillin on *in vitro* digestibility of cellulose and xylan and concluded that depolymerisation was suppressed by 14 and 49%, respectively. However, a threefold increase in a cellulolytic bacterial population (*Bacteroides succinogenes*) was observed with vanillin although this compound reduced the growth rate of *Ruminococcus albus* and *R. flavefaciens*, p-coumaric acid had a more significant effect. Both vanillin and cinnamic acid appeared to reduce attachment of *B. succinogenes* cells to cellulose particles as observed by Borneman *et al.*, (1986). In a study of a range of phenolic compounds (sinapic, syringic, ferulic, vanillic, p-coumaric and p-hydroxybenzoic acids; syringaldehyde, vanillin and p-hydroxybenzaldehyde) it was observed that microbial growth was not supported in the absence of carbohydrates and none of these compounds stimulated cellulose breakdown. Furthermore, methylcellulose, reported to inhibit growth of *Ruminococcus flavefaciens*, reduced

breakdown of native celluloses by a number of cellulolytic bacteria (Rasmussen *et al.*, 1988).

Other compounds are also known to influence rumen fermentations (Wallace *et al.*, 1980; Sinha & Arora, 1982). Wallace *et al.* (1980) investigated the effect of the antibiotic monensin, incorporated into animal feeds to enhance conversion, on fermentation characteristics in a RUSITEC simulation. The antibiotic suppressed production of acetate and butyrate but enhanced production of propionate and decreased methanogenesis and dry matter digestibility. These authors postulated that the beneficial effect of monensin on feed conversion could be due to the energetic advantages of propionic acid fermentation, increased feed protein by-passing microbial breakdown and reduced loss of carbon through methanogenesis (Wallace *et al*, 1981).

Sinha and Arora (1982) studied the effect of aflatoxins on rumen fermentation. Aflatoxins at 0.1, 0.25, 0.5, 1.0 and 1.25ppm decreased cellulose breakdown and resulted in a stepwise reduction in protein synthesis.

## 1.10 THE OBJECTIVE(S) OF THIS STUDY

This project had a number of distinct objectives. The first was to evaluate the effect of lignocellulose components and fungal breakdown products, both phenolics and sugars, on growth of lignocellulose degrading fungi and the enzymes important in the breakdown of the plant material (Fig. 1.14a).

A second objective was to establish physiological factors determining the rate of breakdown of lignocellulose components in fungal solid state fermentations and to assess the effect of the depolymerisation reactions on the potential of the product as a ruminant feed.

Since fungal attack on lignin would release not only lignin-related aromatic compounds but also secondary metabolites, such as mycotoxins, into substrates, a third objective was to establish the effect of such substances on rumen performance of fermented lignocellulosics (Fig 1.14a).

The overall aim of this project was thus to evaluate fungal fermentations as treatments to enhance the digestibility of lignocellulosic plant residues (Fig 1.14b), regarded as low value fibre feeds but available in significant quantities in both developed and developing agroindustrial environments.

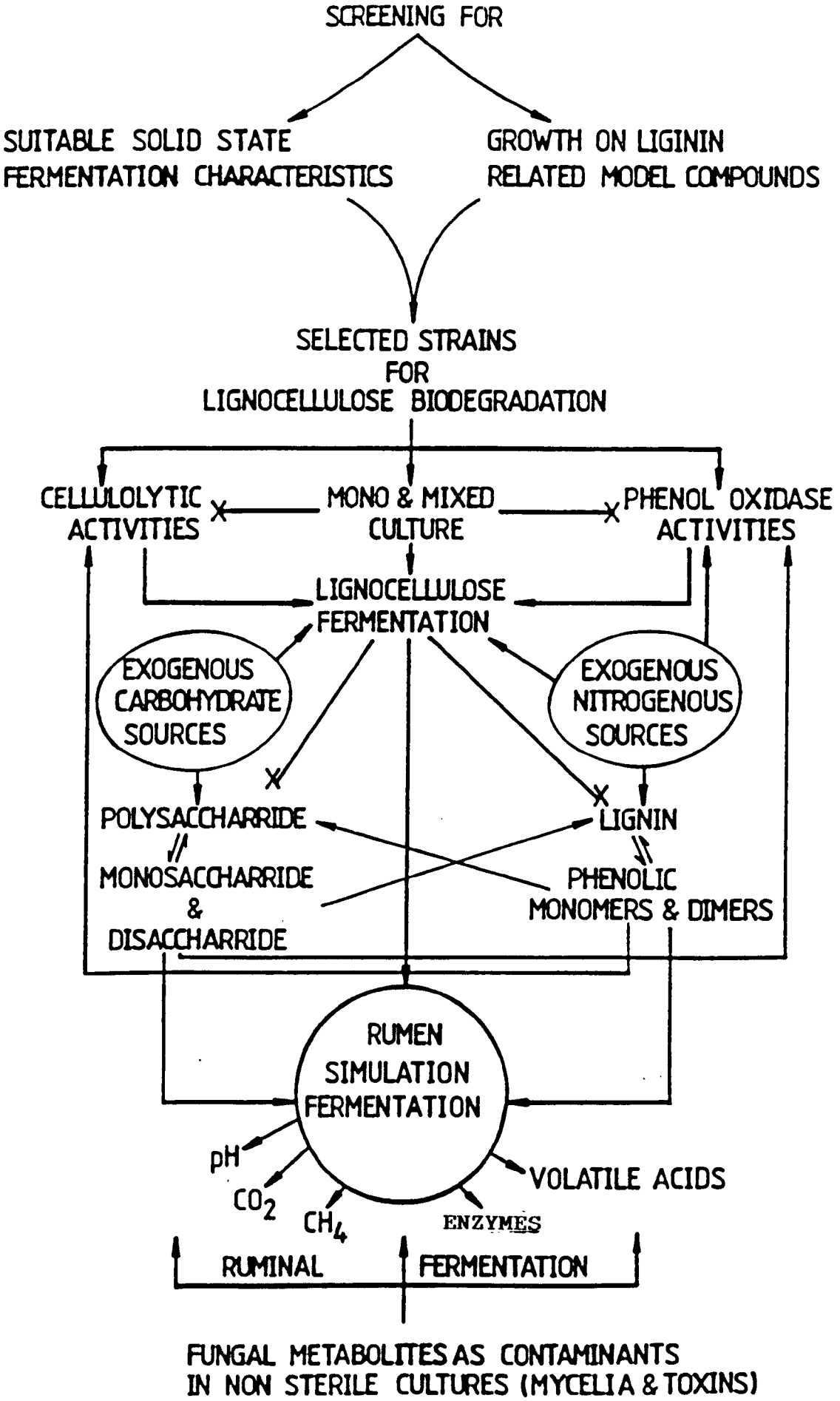



Fig. 1.14a Flow diagram for experimental design.

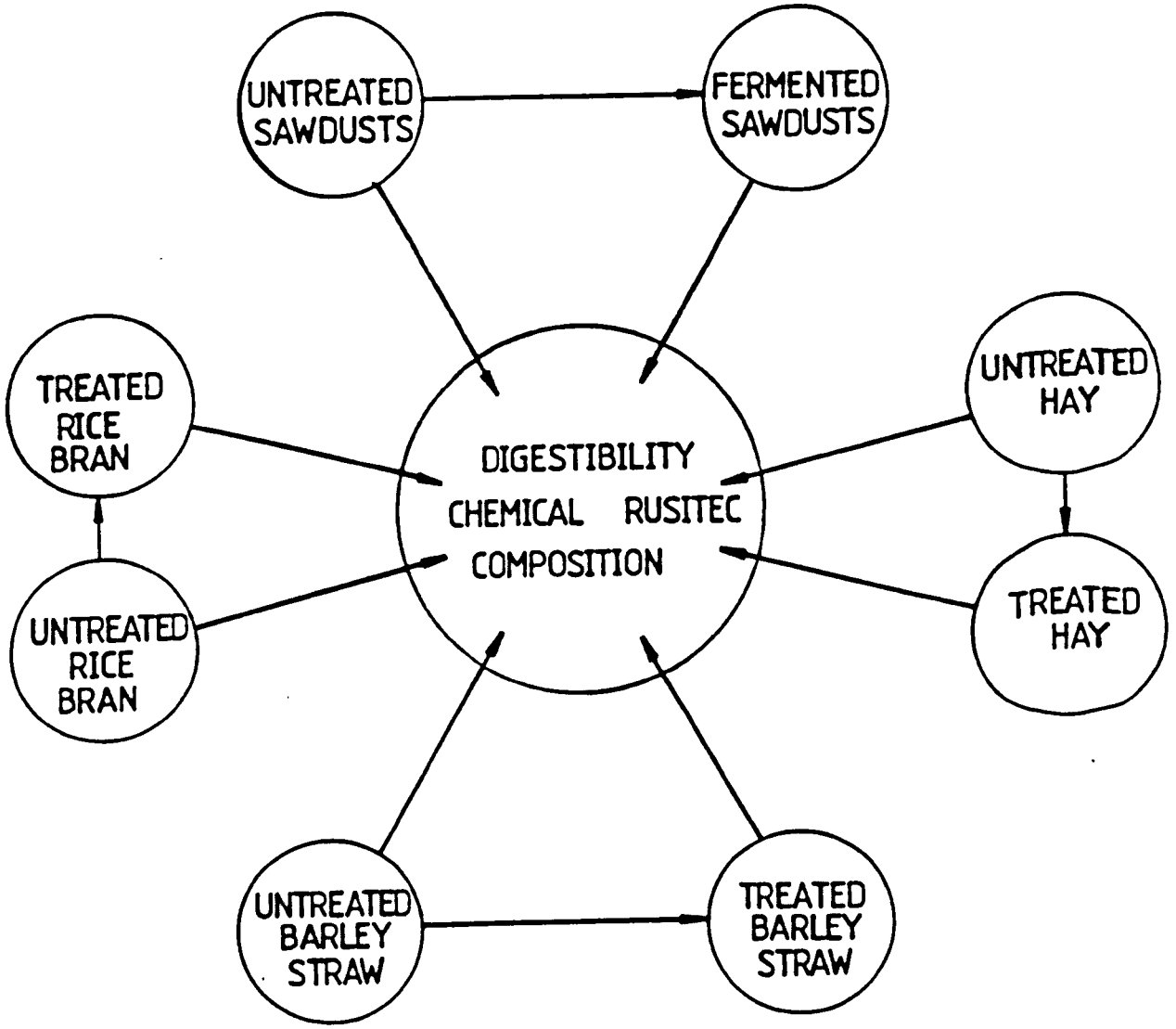



Fig. 1.14b Flow diagram for experimental strategy design.

# SECTION 2

## 2.1 : MATERIALS AND METHODS

### 2.1.1 Substrates

Five substrates were evaluated in this study : (a) spruce sawdust (*Picea sitchensis*), variety - Queen Charlotte Ireland, aged 60 - 65 years (b) birch sawdust (*Betula pendula*) a hardwood, variety - Pendula, aged 25 - 30 years (c) hay (variety-ATEM), (d) barley straw (variety - Golf) and (e) rice bran. Detailed fermentation studies were performed mainly on spruce sawdust. The substrates (spruce shavings & birch wood shavings) were obtained from Economic Forestry Commission, Dunoon, Scotland whereas hay and barley varietal straws were gifts from Hannah Research Institute, Ayr, Scotland. Rice bran was supplied by the courtesy of Tilda Rice Ltd, Leicester. The wood shavings were dried, milled in a Pin mill (KEK Ltd, UK.) then in a Cyclotec 1093 mill (Tecator, Sweden) and sieved through a 500 $\mu$ mesh (Endecotts Ltd , UK). Hay and barley straw were milled in a Pin mill only and used directly without sieving.

### 2.1.2 Micro-organisms

The following fungal isolates were used during this experiment : *Scytallidium lignicola*, *Trichoderma harzianum*, *Aspergillus terreus* and *Aspergillus fumigatus* isolated from decayed Nigerian palm wood;

*Absydia corymbifera* isolated from Tilda rice bran ; *Chaetomium cellulolyticum* (CMI 188965), *Pleurotus sajo-caju* (IMI 199761), *Coriolus versicolor* (IMI 210864), *Phanaerochaete chrysosporium* (CMI 747691) and *Trichoderma reesei* (CMI 192654) were obtained from International Mycolological Institute, Kew London. The organisms were maintained on malt extract agar slants supplemented with 0.1% glucose except for *C. cellulolyticum* which was maintained on modified Czapek Dox agar slants supplemented with either filter paper strips or crystalline cellulose as sole carbon source (Moo-Young *et al.*,1978). All the organisms were incubated at 30°C except *A. corymbifera*, *A. fumigatus*, *A. terreus*, *P. chrysosporium* and *C. cellulolyticum* which were incubated at 37 °C.

### 2.1.2.1 Chemicals

Chemical compounds used during this study were obtained from the following company ;

(1) **BDH Ltd** - guaiacol (28464); xylose( 10372); 3,5 Dinitrosalicylic acid (28235); carboxymethylcellulose (27649); Cetyltrimethylammonium bromide (27665).

(2) **Sigma Ltd** - Indulin lignin (AT)(I-6384); p-hydroxycinnamic acid ; D(+) glucosamine (G- 4875 ); Oat spelt xylan (X- 0627); Remazol Brilliant Blue dye (R - 8001); Congo Red (C-6767); protocatechuic acid (P-5630); p-hydroxybenzoic acid (H-5376); chitin from crab shells (C-3132); polygalacturonic acid (P- 3889); pectin (P-

9135); syringaldehyde (S- 7602).

(3) **Aldrich Ltd** - ferulic acid (12,8708); cellobiose (C1,770-5), catechol (13,501-1); vanillin (V110-4); benzoic acid (10,947-9); trans cinnamic acid (13,376-0); syringic acid (S-800-5); syringaldazine (17,753-9); Catechol (13,501-1); Coumarin (C8,555-7).

### 2.1.3 Media preparation

#### 2.1.3:1 Basal mineral medium

A basal minimal medium modified from that of Ander and Eriksson (1976) was used for both solid and submerged cultures. The composition of the nutrient medium is listed in Table 2.1. The nitrogen content of the medium was adjusted to 0.5 $gl^{-1}(NH_4H_2PO_4)$ for enzyme studies. The pH of media was adjusted to 5.5 using 1 M $H_2SO_4$. Phosphates, sulphates and chlorides were dissolved separately, then mixed and made up to volume.

2.1.3.2 **Water agar** ; About 0.5 ml of steamed water agar was dispensed into small screw cap vials prior to sterilisation (Table 2.2).

#### 2.1.3.3 Buffer

**Citrate buffer - pH 4.8**

Citrate buffer (pH 4.8) was prepared by mixing 23 ml of 0.1 M citric acid and 27 ml of 0.1 M sodium citrate in a volumetric flask. The mixture was made up to 100 ml with distilled water.

TABLE 2.1 COMPOSITION OF MINIMAL MEDIUM

| NUTRIENTS | $gl^{-1}$ |
|---|---|
| $NH_4H_2PO_4$ | 2.0 |
| $KH_2PO_4$ | 0.6 |
| $K_2HPO_4$ | 0.4 |
| $MgSO_4.7H_2O$ | 0.5 |
| $CaCl_2.2H_2O$ | 0.1 |
| Ferrous sulphate | 0.01 |
| Thiamine | 0.001 |

| Micro-nutrient | $gl^{-1}$ | |
|---|---|---|
| $ZnSO_4.7H_2O$ | 6.6 | 1ml |
| $MnSO_4.4H_2O$ | 5.0 | |
| $CoCl_2.6H_2O$ | 1.0 | |
| $CuSO_4.5H_2O$ | 1.0 | |
| distilled water | | 1 Litre |

pH was adjusted to 5.5 using 1 M $H_2SO_4$ or 2 M NaOH.

TABLE 2.2 COMPOSITION OF WATER, MALT EXTRACT AND CZAPEK DOX AGAR MEDIA

| NUTRIENTS | WATER AGAR $gl^{-1}$ | MALT EXTRACT $gl^{-1}$ | CZAPEK DOX $gl^{-1}$ |
|---|---|---|---|
| Tween 80 (0.1%) | 0.5ml | - | - |
| $NaNO_3$ | - | - | 2.0 |
| KCl | - | - | 0.5 |
| Ferrous sulphate | - | - | 0.01 |
| $K_2SO_4$ | - | - | 0.35 |
| Cellulose or Filter paper | - | - | 1.0 |
| Malt extract | - | 30.0 | - |
| Mycological peptone | - | 5.0 | - |
| Agar powder | 3.0 | 15.0 | 12.0 |
| Distilled water(ml) | 1000.0 | 1000.0 | 1000.0 |

**Acetate buffer - pH 5.6**

Acetate buffer (pH 5.6) was prepared by mixing 4.8 ml of 0.2 M acetic acid with 45.2 ml of sodium acetate in a volumetric flask. The mixture was made up to 100 ml with distilled water.

### 2.1.4 Isolation of cellulolytic and lignolytic fungi

Powdered rice bran (1 g) was suspended in 10 ml of sterile aqueous peptone solution (0.1%) and vortex mixed. Thereafter 1 ml aliquots were inoculated on malt agar medium supplemented with minimal medium and 0.1% crystalline cellulose. Thin strips were cut from Nigerian palm wood with a sterile knife and plated on to the agar plates. Plates were incubated at 25, 30 & 37 $^{o}C$ and isolates subcultured on malt extract agar and identification of fungal isolates was confirmed by the International Mycological Institute London, UK.

### 2.1.5 Screening of fungal isolates on surface agar culture

Carbohydrates, pectin and phenolic substrates were added individually (0.1% w/v) to basal minimal medium, autoclaved separately and aseptically mixed with sterile agar solution (2% w/v). The pH was adjusted to 5.5 with 1.5% $K_2HPO_4$ (dibasic) or 1 M $H_2SO_4$ prior to sterilisation. Solid lignin agar containing medium was prepared by dissolving 0.1 g of industrial lignin

(Indulin AT) in 50 ml of 0.4% sodium hydroxide, pH was adjusted to 5.5. The solution was filter sterilised and added to agar (50 ml) previously autoclaved at 121 $^{o}C$ for 15 min with mixing. About 20 ml of each medium was dispensed into plates. Thereafter plates were inoculated with 0.5 $cm^2$ agar blocks of young cultures of test fungi followed by incubation for 5 days. Growth was assessed by visual inspection and graded on a 6 point scale.

### 2.1.6 The microporous bag for solid state fermentation

A novel containment system described earlier by Cuero *et al.* (1985) was employed for solid substrate fermentations. The containment system is an opaque - white microporous film bag (supplied by Van Leer Ltd, UK) formulated from polypropylene materials with submicron pores (< 0.4 µm). The ends of the bag were provided with transparent strips or windows of non-porous polypropylene which permitted regular examination of cultures (Plate 6.1). The microporous film was protected externally by a coarsely perforated polypropylene film. The microporous nature of the bag permitted efficient air (25 $cm^3$ $cm^{-2}$ $min^{-1}$ at 1 atm.) and moisture vapour transmission (500 g $m^{-2}$ $d^{-1}$ at 23 $^{o}C$ and 50% relative humidity), but prevented cross movement of micro-organisms. The bags were autoclaved and usually sealed using a suitable adhesive tape. Bags containing particulate substrate (sample) and inoculum were incubated in Fison Environmental cabinets (Models 280E/MU/R-IND(D)/1982 and

FE/80/H/MP/R-IND/F/1984) equipped with temperature and humidity control systems (Plate 2.1). At certain times bags were incubated in an improvised humidity chamber constructed from an aquarium tank adapted, as above, but with provisions for inlet of air at 98% relative humidity.

### 2.1.7.1 Inoculum preparation for submerged and solid state culture

Young actively growing cultures ( 5 $mm^2$ agar plugs) were inoculated onto sterile starter media supplemented with either 1.7% malt extract or 1% cellulose (for submerged cultures). The flasks were incubated under static condition for 4 - 8 days at either 30 $^oC$ or 37 $^oC$ depending on the organism. Mycelia was fragmented at medium speed for 60 sec in an Ato-mix laboratory blender (M.S.E Ltd, London). Hyphal suspension from 100 ml culture volume (2.5 ml or 1 ml) were used to inoculate culture flasks and microporous bags, respectively. Mycelial biomass for enzyme studies was harvested, washed free of soluble sugars and cellulose particles with sterile distilled water prior to fragmentation.

### 2.1.7.2 Preparation of spore inoculum

*Aspergillus flavus* spores were harvested from malt extract agar slants using 10 ml aliquots of 0.1% (v/v) Tween 80. The slopes were shaken vigorously to dislodge spores and mycelia separated by filtration ( through sterile absorbent cotton wool) into 150 ml sterile


PLATE 2.1a Fison Environmental unit (External view).

PLATE 2.1b Fisons Environmental unit (interior view).

bottles. The filtrate was transferred aseptically into sterile centrifuge tubes, centrifuged at 1430 X g for 20 min. The supernatant was discarded and the pellet, containing spores, washed three times by centrifugation. A known volume of 0.1% Tween 80 was added to the final spore pellet followed by appropriate dilution to obtain a desired spore concentration of $4.5 \times 10^6$ per ml with the aid of a haemocytometer, using the following formula;

$$\frac{n}{T} \times \frac{4}{1} \times \frac{10^6}{1}$$

Where;

n = total number of spores

T = total number of squares (eg B X S)

ie B = number of big squares used (5)

S = number of small squares within a big square (16)

**2.1.7.3 Spore propagules on water agar**

Needle point inoculum of spores was aseptically added to semi-solid suspension of sterile water agar and thoroughly mixed with a flamed loop before use. Vials containing spores could be sterilised and re-used four or five times before discard (Pitt, 1979).

**2.1.7.4 Viability test of fragmented hyphal cells**

Mycelia of test fungi previously grown in minimal media supplemented with 0.1% glucose were fragmented for 10, 30 and 60 sec in a sterile Ato-mix laboratory blender at medium speed. Fragmented mycelia were transferred into

sterile 150 ml bottles, stoppered and stored at 4 $^{o}$C. The effects of period of fragmentation, with or without storage, were assessed by plating out duplicate sets of 0.1 ml of fragmented hyphal suspension on malt extract agar supplemented with 0.1% glucose in minimal media. Plates were incubated for 72 h and viability scored as the degree of mycelial growth by visual inspection.

### 2.1.8 Cultivation condition for enzyme studies

Minimal medium (50 ml) was dispensed into 250 ml Erlenmeyer flasks containing 1% (w/v) Whatman crystalline cellulose. Experimental flasks contained, in addition to 1% cellulose, varying concentrations of 4-methoxy 3-hydroxycinnamic acid (0.05%, 0.1% and 0.5% ; w/v). The pH was adjusted to 5.5 $\pm$ 0.25 prior to sterilisation. The flasks were inoculated and incubated at 30 $^{o}$C for 14 d under static conditions. Samples were collected every second day for enzyme studies. Broth samples were centrifuged at 5000 X g at 4 $^{o}$C for 30 min. The supernatant was buffered to either pH 4.8 with citrate buffer or 5.6 with acetate buffer prior to cellulase and phenol oxidase assays. Mycelial biomass formed was recovered by filtration with Whatman Number 1 filter paper.

### 2.1.9 Solid state fermentations (SSF)

Pulverised spruce wood was moistened with a precise amount of minimal medium in a 2 l flask to a moisture

content of 70%. The flask was sealed to air tight with a rubber bung, lubricated with vaseline and equilibrated at 4 $^{o}$C for 48 h. Equilibrated samples (20 g) were dispensed into the microporous bag sealed with adhesive tape , autoclaved at 121 $^{o}$C for 30 min. On cooling sterile sample bags were inoculated aseptically with mycelial suspension (1 ml) of test fungi. Samples inoculated with *C. versicolor* and *P. sajo - caju* were incubated for 6 weeks at 28.5 $^{o}$C and *Phanaerochaete chrysosporium* at 40 $^{o}$C respectively.

## 2.2 SUBSTRATE AND MEDIA WATER ACTIVITY

### 2.2.1 Water activity and fungal growth

Minimal medium of varying water activities were prepared by addition of glycerol ( Dallyn & Fox 1980). Media were supplemented with malt extract (1.7%), and peptone (0.3%), solidified with 2% Oxoid agar and sterilised by autoclaving at 121 $^{o}$C for 15 min. Autoclaved media was dispensed into sterile plates which were allowed to set and dry slightly to remove any surface water. Water activity of adjusted media was checked using a Dew point protimeter. Water activity of the media together with the desired amount of glycerol needed was calculated by reference to the following formula :

$$\text{Amount of glycerol(g)} = \frac{M \times M_g \times W}{1000}$$

Where :

M = Molality of required water activity

Mg= Molecular weight of glycerol

W = Weight of solvent

Each organism was inoculated on duplicate plates using spore inocula except for *C. versicolor* and *P. sajo-caju* where 4 mm agar plugs were used. Spore inocula were picked up with the aid of a flamed needle, cooled in a vial of soft agar, and stabbed upwards on to the centre of an inverted petri-plate to avoid showering the surface of the medium with spores. Inoculated plates were incubated at a range of temperatures (25°, 30°, 40 °C) in controlled relative humidity cabinets for 10 d. Fungal growth was measured as increase in colony diameter (mm).

### 2.2.2 ; Moisture content of substrate

For this study the amount of water added to the substrate was calculated by reference to the following formula ;

$$\text{Amount of water required} = \frac{W \times (R_1 - R_2)}{100 - R_1}$$

Where;

W = Weight of substrate required (g)

$R_1$ = Required moisture content (%)

$R_2$ = Initial moisture content of substrate (%)

## 2.3 ; BIOCHEMICAL ANALYSES

### 2.3.1 Determination of moisture content

Samples (0.5 - 1.0 g) were placed in previously weighed platinum crucibles, and dried to constant weight at 105 $^{o}C$ for 24 h.

### 2.3.2. Determination of reducing sugar

#### 2.3.2.1 Nelson-somogyi (1952) method

The assay described by Nelson (1944) and Somogyi (1952) was employed for determination of soluble reducing sugars.

In principle Copper II oxide is formed when cupric ions present in Nelson's reagent react with reducing sugars. Under ideal conditions, the amount of $Cu_2O$ produced is proportional to the concentration of reducing sugar in the sample. On addition of an arsenomolybdate reagent, a blue colour is obtained, the intensity of which is measured spectrophotometrically. It must be noted that different sugars have different reducing power, for instance sucrose and glycerol at high concentration have a slight reducing effect (Karkalas, personal communication). Also ferrous iron and other reducing substances interfere with this method.

Reagents

A. Nelson's Reagent "A"; $Na_2CO_3$ 25 g, potassium sodium tartrate 25 g, $NaHCO_3$ 20 g were dissolved in 600 ml distilled water, followed by the addition of 200 g $Na_2SO_4$ under continous stirring with a mechanical stirrer ( J.W.

Towers Co. Ltd, UK). The solution was diluted to 1l and stored at 37 $^{o}$C until required.

B. Nelson's Reagent "B" ; $CuSO_4.5H_2O$ (30 g) was dissolved in distilled water, then 2 drops of concentrated sulphuric acid were added and the mixture made up to 200 ml.

C. Nelson's Reagent; 25 ml reagent A was mixed with 1 ml reagent B. This reagent was prepared freshly for each experiment.

D. Arsenomolybdate Reagent : 25 g ammonium molybdate was dissolved in 450 ml water followed by the addition of 21 ml concentrated sulphuric acid . $Na_2HASO_4.7H_2O$ (3g) was dissolved in 25 ml water and added. The mixture was made up to 500 ml, transferred to a brown bottle and incubated at 37 $^{o}$C for 24 h. The solution was filtered through Whatman glass microfibre filter (GF/A, 7 cm diam). Solutions A and B were filtered.

Procedure ;

A. Standard solutions; containing 40, 80, 120, 160 and 200 $\mu g\ ml^{-1}$ glucose were freshly prepared.

B. Reducing sugar determination ; 1 ml each of blank solution, standard sugar solution (1 ml) and test samples (1 ml) were placed into separate test tubes. 1 ml of Nelson's reagent was added to each tube, vortex mixed and immersed in boiling water. After 20 min the tubes were cooled in running tap water for 5 min, followed by the addition of 1 ml arsenomolybdate reagent. The tubes

were vortex mixed , left to react for 3 min and 7 ml distilled water added and mixed several times by inverting the tubes. Absorbance was measured at 500 nm , and concentration of reducing sugar was determined by reference to the straight line standard.

**2.3.2.2 Dinitrosalicylate solution(s)(DNS) (Englyst & Cummings, 1988)-** 3,5-dinitrosalicylic (3 g) and 8 g NaOH were dissolved separately in 500 ml flasks containing 50 ml and 200 ml distilled water, respectively. Solutions were mixed followed by gradual addition of 150 g sodium,potassium tartrate with occasional shaking. The mixture was quantitatively transferred and made up to 500 ml with distilled water. The solution was stored in capped dark bottle, and kept for 2 d before use. The solution was stable at room temperature for at least 3 months.

Procedure ; DNS solution (1.0 ml) and 1.0 ml of standard glucose solution, was made alkaline with 0.5 ml NaOH (3.9 M) in a test tube, vortex mixed and placed in a boiling water bath for 10 min then cooled in water to room temperature and 10 ml distilled water added. The O.D was measured at 530 nm against a suitable blank. A standard graph was prepared using D-glucose (0.5 to 2.0 mg $ml^{-1}$).

### 2.3.3 Enzymic method for glucose determination - glucose oxidase

Principle; Glucose is oxidised to gluconic acid and hydrogen peroxide in the presence of glucose oxidase at

pH 7. The quantity of hydrogen peroxide produced is proportional to the glucose in the sample. The hydrogen peroxide is further decomposed by peroxidase and a pink colour produced in the presence of phenol and 4-aminophenazone. The intensity of pink colour was measured spectrophotometrically. This method was selective for glucose but invertase and amylase impurities in the glucose oxidase enzyme may interfere under certain conditions. As a result highly purified glucose oxidase enzyme is recommended.

GLUCOSE OXIDASE - PEROXIDASE REAGENT ; The following reagents - 11.5 g $Na_2HPO_4 . 12H_2O$, 2.5 g $KH_2PO_4$, 500 mg phenol, 75 mg 4-aminophenazone, 3500 units glucose oxidase (Sigma G-6125) and 3500 units peroxidase(Sigma P-8125) were dissolved in distilled water and diluted to 500 ml. The mixture was filtered through Whatman glass fibre filter and stored at 4 $^{o}C$ in a dark bottle for 24 h before use. Fresh reagent was prepared each month.

Procedure; Glucose oxidase reagent (5 ml) was added to test tubes containing 1 ml aliquots of sample. The mixtures were vortexed, and placed in a water bath at 35 $^{o}C$ in the dark . After 45 min tubes were cooled to ambient temperature and the absorbance measured at 505 nm. A standard of glucose (20 - 160 $\mu g\ ml^{-1}$) was used to calculate concentrations.

### 2.3.4 Determination of cell wall nitrogen

#### 2.3.4.1 Protein nitrogen (A.O.A.C, 1980);

Principle; Organic compounds were oxidised with hot concentrated sulphuric acid with catalysis by cupric selenite. The boiling temperature of the acid was raised by addition of potassium sulphate. Organic nitrogen was converted to ammonium sulphate which remained in solution. Addition of sodium hydroxide liberated ammonia that was distilled into boric acid solution where it was trapped. Subsequently total ammonia was titrated with standard hydrochloric acid using a suitable indicator. This method did not permit the conversion of nitrates and nitrites into ammonia, and as a result they are not determined.

Procedure; A Pulverised sample (100 - 200 mg) was weighed on cigarette paper and transferred into a digestion tube. $K_2SO_4$ (3.5 g), $CuSeO_3$ (0.1 g) and 5 ml concentrated sulphuric acid were added and the contents of the tube were gently mixed. The tube was incubated in a pre-heated (450 - 500 $^{o}C$) digestion block and digestion was complete after 30 min. The tubes were allowed to cool to ambient temperature prior to addition of 50 ml distilled water. NaOH (50 ml of 40%) was added to each tube followed by distillation into 250 ml conical flasks containing 25 ml boric acid. About 125 ml of distillate was collected and titrated with standard hydrochloric acid (0.05 M) until

the colour of the distillate matched that of the control. The percent nitrogen was calculated by reference to the formula ;

$$\% \text{ N} = \frac{(\text{ml HCl titre}) \times 0.7 \times 100}{(\text{wt. of sample(g)}) (1000)}$$

Conversion factor of % nitrogen to crude protein = 6.25

**2.3.4.2 Determination of ammoniacal nitrogen at low concentration ;**

Reagents ;

A. Alkaline sodium hypochlorite: NaOH (25 g) was dissolved in 500 ml distilled water, 9 ml sodium hypochlorite (BDH, GPR=10-14% available Cl) added. The mixture was made upto 1l with water and stored in brown bottle at 5 $^{o}$C. The solution was stable for at least 1 month.

B. Phenol reagent : phenol(50 g) and 0.25 g of sodium nitroprusside were dissolved in water and made up to 1l in volumetric flask. The solution was stable for 1 month.

C. 0.005 M $H_2SO_4$ ; Concentrated $H_2SO_4$ (280 µl) was mixed with 800 ml water and diluted to 1 l.

D. Stock ammonium sulphate solution; $(NH_4)_2SO_4$ (1.179 g) was dissolved in water and made up to 100 ml in a volumetric flask (11.79 mg $ml^{-1}$ = 2.5 mg N).

E. Standard Nitrogen solution; standard solutions containing 20 to 100 µg N /100 ml were prepared from a stock of 20 ug N $ml^{-1}$. Each standard solution also

contained 10 ml of 0.005 M $H_2SO_4$. A reference solution was prepared containing 10 ml of 0.005 M $H_2SO_4$ diluted to 100 ml with distilled water.

Procedure ;(1) 10 ml of each solution (2 to 10 µg N $ml^{-1}$) was transferred into a series of 20 ml Pyrex tubes fitted with screw caps. Hypochlorite reagent (3 ml) was added and mixed followed by addition of 3 ml phenol reagent. The 16 ml reaction mixture was thoroughly mixed by repeated inversions and the tubes left for 30 min for colour development. The colour was stable for at least 1 h. A reference of 2 - 10 µg N $ml^{-1}$ was used for calibration.

2. Similarly 80 ml of distillate of test sample from Kjeldahl digest, collected into a 100 ml conical flask containing 10 ml 0.005 M $H_2SO_4$ was quantitatively transferred into 100 ml volumetric flask and made up to volume. Aliquots(10 ml) of the diluted digest were treated as in step 1.

3. The absorbance at 635 nm was read and nitrogen concentration was calculated by reference to the standard curve.

### 2.3.5 Fibre determination

Total carbohydrates in feedstuffs can be divided into two groups: (a) Metabolisable carbohydrates - including simple sugars such as glucose, fructose, sucrose, lactose, but also dextrins, and starch.

(b) Unavailable carbohydrate or dietary fibre including

pectic substances, hemicelluloses, celluloses, gums, algal polysaccharides and lignins. The term dietary fibre was initially used to define skeletal remains of plant cell walls resistant to digestion by the enzymes of the human gut (Trowell, 1972). Lignin, although not a carbohydrate, is classified in this group.

Until recently, the term crude fibre has been widely used to describe the residue obtained by boiling feedstuffs in succession with 1.25% $H_2SO_4$ and 1.25% NaOH. This treatment dissolves starch, pectins, hemicelluloses, protein and some of the lignin. The residue is predominantly cellulose. However, depending on the origin of the sample, variable amounts of cellulose are solubilised by this method. For this reason and because it underestimates the bulk of unavailable carbohydrate, the method is no longer favoured. The acid detergent fibre (ADF) method of Van Soest (1963) has gained considerable popularity as being simple and rapid. The sample is boiled with cetyl trimethyl ammonium bromide (CTAB) in 0.5 M $H_2SO_4$. In this protocol the acid hydrolyses starch, pectins and hemicelluloses, while CTAB solubilises protein. The residue consists of cellulose and lignin although small amounts of resistant hemicelluloses and protein may be present as contaminants. Inorganic matter is also present in ADF especially in samples rich in silica such as grasses and rice hulls.

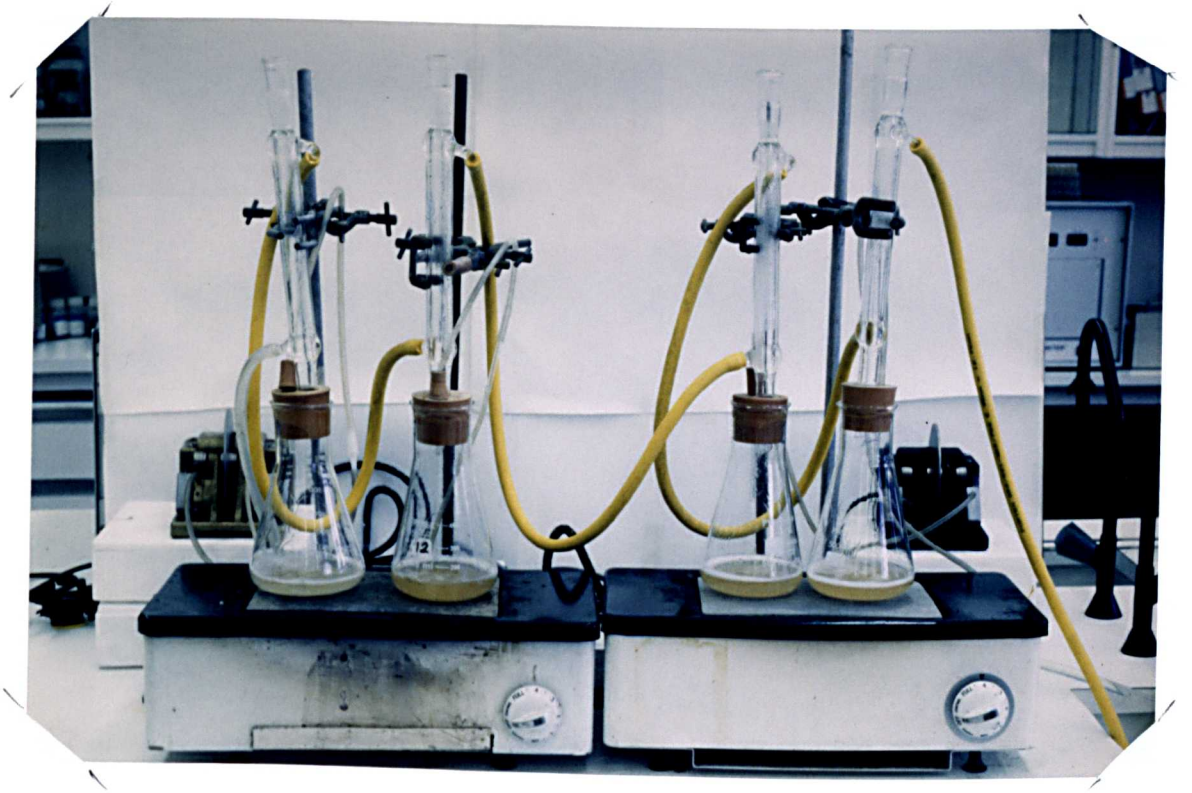


PLATE 2.2 Reflux system for acid and neutral detergent fibre analyses.

The neutral detergent fibre method (NDF) also developed by Van Soest (1963) is based on the use of sodium lauryl sulphate (SLS) in neutral buffer to solubilise the protein, pectin and some of the starch (Morrison, personal communication). Estimation of the residue is a good measure of unavailable carbohydrate in leafy vegetables and forages, but the method is not applicable to cereal grains as starch is not hydrolysed. This difficulty can be overcome by the use of amylolytic enzymes (Southgate, 1976).

### 2.3.5.1 Acid detergent fibre (ADF) (Association of Official Analytical Chemists, 1984)

Reagent; CTAB (20 g) was dissolved in 0.5 M $H_2SO_4$ and diluted to 1000 ml in a volumetric flask.

**Apparatus;**

a. Refluxing apparatus (Plate 2.2), hot plate, air pump, conical flasks (500 ml) and glass crucibles porosity 2.

Procedure; Homogenised (0.2 - 0.5 g) sample was weighed into a flask and 100 ml acid detergent solution added. The flask was fitted into a reflux condenser and heated to boiling for 10 min. Heating was adjusted to a steady but not too vigorous boiling and continued under reflux for 60 min. The air pump was used to pass air through a tube in the stopper, to control foaming. The hot solution containing insoluble residue was filtered through the porosity 2 crucible under gentle suction. Each flask was rinsed with 2 aliquots of 200 ml hot water and washings

poured quantitatively into crucibles.

The residue was finally dried under vacuum and washed with 50 ml acetone. Crucibles containing residue were dried overnight at 100 $^{o}$C, cooled in a desiccator and weighed.

$$\% \text{ ADF} = \frac{[(\text{Wt of crucible + Fibre}) - (\text{Wt of crucible})] \times 100}{\text{Dry weight of sample}}$$

**2.3.5.2 Acid detergent lignin** (ADL) (A.O.A.C 1984).

Reagents ; 72 % $H_2SO_4$ was prepared by gradual addition of 1200 g $H_2SO_4$ to 440ml distilled water in a 1 l volumetric flask cooled in ice in a water bath. This solution was stored at 4 $^{o}$C.

Procedure ; The crucible containing acid detergent fibre (2.3.5.1) was placed in a 250 ml flask for support. The contents were covered with cold 72% sulphuric acid, stirred at intervals and the crucible was refilled with 72% $H_2SO_4$. After 5 h the contents were filtered under vacuum and washed with hot water until free from acid. The crucible was dried at 100 $^{o}$C overnight, cooled in a desiccator and weighed (W1). The crucible was further ignited in a muffle furnace at 550 $^{o}$C for 5 h, transferred to a desiccator, cooled and re-weighed ($W_2$).

$$\% \text{ ADL} = \frac{W_1 - W_2}{\text{wt. of sample}}$$

### 2.3.5.3 Neutral detergent fibre (NDF)

Reagent ; Neutral detergent solution was prepared by dissolving 18.61 g EDTA $Na_2$ and 6.81 g $Na_2B_4O_7.10H_2O$ in hot water in a beaker. 30 g sodium lauryl sulphate and 10 ml 2-ethoxyethanol were added followed by 11.5 g $Na_2HPO_4.12H_2O$, dissolved in 200 ml water. The 900 ml solution was made up to 1 l with distilled water.

Procedure ; The method used was similar to that employed for acid detergent fibre determination, except that 100 ml neutral detergent solution was added to the reflux flask containing 0.2 - 0.5 g sample and 0.5 g sodium sulphite. The mixture was refluxed as in ADF, filtered , washed with hot water and acetone and dried overnight at 100 $^{o}$C. Crucibles were cooled in a desiccator and weighed.

$$\% \text{ NDF} = \frac{[(\text{Wt.of crucible + Fibre}) - \text{Wt. of crucible}] \times 100}{\text{Wt. of sample.}}$$

### 2.3.5.4 Cellulose and hemicellulose determination by detergent method ;

(i) CELLULOSE = ADF - ADL

(ii) HEMICELLULOSE = NDF - ADF.

2.3.6 **Klason lignin**(Effland, 1977)

**Treatment of sample** ; Test samples were extracted in a Soxhlet apparatus for 3 h with ethanol (95 %) - Diethylether mixture (1:2 v/v) . The residue was dried overnight at 70 $^{o}$C and stored in an air-tight container.

Procedure ; (a). $H_2SO_4$ (72 %) 4 ml was added to 200 -300 mg extracted residue in a test tube, dispersed by vortex mixing. The mixture was placed in a water bath at 35 $^{o}$C, with occasional stirring. After 75 min. the solution was quantitatively transferred to 250 ml Erlenmeyer flask and diluted with 100 ml distilled water. Secondary hydrolysis was carried out in an autoclave by steaming at 120 $^{o}$C for 1 h .

(b). The solution was filtered hot with aid of scintered glass crucible porosity 2. The residue was thoroughly washed with hot water, dried overnight at 100 $^{o}$C , cooled in a desiccator and weighed ($W_1$). Ashing was carried out at 550 $^{o}$C for 5 h, and the crucible cooled and re-weighed ($W_2$). Klason lignin was calculated as percent of extracted, oven dried sample.

$$\% \text{ Klason lignin} = \frac{W_1 - W_2}{\text{Wt. of sample}}$$

## 2.3.7 Cellulose & non soluble polysaccharide (NSP) determination

Distilled water (5 ml) was added to 90 - 100 mg dried sample in a test tube which was then left in a beaker of boiling water on a hot plate for 1 h with occasional mixing. After cooling and centrifugation, the supernatant was removed and discarded. The residue was washed once with 5 ml distilled water and then dried with acetone.

**Acid hydrolysis of polysaccharide ;** sample residue dried in acetone was hydrolysed as described in 2.3.6. (b) The solution was diluted to 250 ml after secondary hydrolysis filtered through Whatman No.1 filter paper.

**2.3.7.1 Colorimetric determination of non soluble polysaccharide (NSP);** 1 ml acid hydrolysates of sample was analysed for total sugars using the DNS method (2.3.2.2). Values for total insoluble polysaccharide were calculated by reference to sugar standards in addition to the formula below;

$$\frac{CT \times VT \times 100 \times 0.9}{WT}$$

Where;

CT = mg sample calculated from standard curve.

VT = volume of solution.

WT = weight of sample (mg).

### 2.3.7.2 Colorimetric determination of cellulose

Similar procedures for analyses and calculation of NSP were adopted for cellulose determinations except that the only monosaccharide in acid hydrolysates quantified was glucose using the glucose oxidase method (2.3.2).

### 2.3.7.3 Hemicellulose determination

NSP - CELLULOSE = HEMICELLULOSE

**EXPRESSION OF RESULTS** ; Some losses of monosaccharides occur during primary and secondary hydrolyses of non soluble polysaccharides (NSP). In order to compensate for such losses, NSP values obtained were multiplied by a factor of 1.18 (This study). Similarly there can be incomplete hydrolysis with poor recovery of glucose contained in celluloses as demonstrated by Figure 3.1 This was corrected by applying a factor of 1.52 to cellulose values as determined by calculation (Fig. 3.1).

### 2.3.7.4 Determination of neutral sugars by gas liquid chromatography (GLC)

Reagents;

(a) Acetate buffer (0.2 M, pH 5.2)

(b) Ethanol (85 %)

(c) Dimethyl sulphoxide (DMSO)

(d) Sulphuric acid (72 % or 12 M)

(e) Ammonium hydroxide (3 M & 12 M)

(f) Internal standard - Allose solution (1 mg allose $ml^{-1}$), this solution was stable at $4^{o}C$ for at least 2-days.

(g) Potassium hydroxide (7.5 M)

(h) Sodium borohydride; 200 mg $NaBH_4$ dissolved in 4 ml of 3 M ammonium hydroxide. This was prepared freshly .

(i) Enzyme solution ; A mixture of 0.1 ml pancreatic amylase (Boehringer 102814) and 0.07 ml of pullulanase (Boehringer 108944),

(j) Standard sugar solutions ; Sugars (50 mg each) - Rhamnose, arabinose, xylose, mannose, galactose and glucose were weighed into a single 50 ml beakers, dissolved with 25 ml of 1 M $H_2SO_4$, then transferred quantitatively into a 100 ml volumetric flask and made up to mark with 1 M $H_2SO_4$.

Procedure ; Dry pulverised sample (30 - 50 mg) was weighed into a 50 ml test tube. Rice bran samples were defatted and enzymatically treated to hydrolyse starch prior to acid hydrolysis of polysaccharide. However, spruce and birch sawdusts were hydrolysed directly with acid after removal of solubles.

(b) **Defatting and drying of sample (rice bran)** ; Acetone (40 ml) was added to samples in test tubes mixed with glass rod for 30 min, and then centrifuged. Supernatants were removed using a Pasteur pipette without disturbing the pellet. This residue was dried by placing tubes on a hot plate at 65 - 70 $^{o}C$.

(c) **Starch hydrolysis (rice bran)** ; Dimethyl sulphoxide

(2 ml) was added to test-tubes containing defatted sample, capped and mixed for exactly 2 min on a magnetic stirrer. The tube was immersed in boiling water with occasional mixing for 1 h, then cooled and 8 ml of 0.2 M sodium acetate buffer (pH 5.2) was added with vortex mixing. α- Amylase solution (0.1 ml) was added followed by 0.07 ml of pullulanase. The solution was vortex mixed, capped and incubated at 42 $^{o}$C for 16 h. Absolute ethanol (40 ml) was added, with mixing and the tube left at ambient temperature for 1 h, and centrifuged at 2640 X g for 10 min. The supernatant was removed by aspiration and the residue washed with 40 ml of 85% ethanol, then with acetone (40 ml). Residual acetone was evaporated on a hot plate.

**(d) Removal of solubles (spruce & birch sawdust) :** Distilled water (40 ml ) was added to a tube containing the sample, which was capped and placed in boiling water for 1 h. The tube was then cooled, centrifuged and supernatant discarded. The residue was washed with 40 ml of 85% absolute ethanol and dried with acetone. **(e) Acid hydrolysis ;** 2 ml of 12M $H_2SO_4$ were added to the dried residue, dispersed by vortex mixing, capped and incubated at 35 $^{o}$C with occasional mixing. After 75 min, 22 ml of distilled water was added, mixing was performed several times by inversion. Hydrolysis was effected in an oven at 100 $^{o}$C for 3 h. The tube was then cooled and the contents filtered.

**(f) Derivatisation of neutral sugars as alditol acetates**

Internal standard solution (1 mg allose $ml^{-1}$ : 0.5 ml) was added to 3 ml of cooled hydrolysate in a 10 ml test tube followed by the 0.6 ml 12 M ammonium hydroxide, with vortex mixing. Freshly prepared $NaBH_4$ in 3 M $NH_4OH$ (0.4 ml) was added and the tube incubated in a water bath at 40 $^oC$ to carry out reduction of aldoses to alditols. The standard sugar solutions were treated similarly . After 1 h 0.3 ml glacial acetic acid (0.3 ml) was added with vortex mixing and 0.4 ml of the acidified solution transferred into 20 ml test tube with a screw cap. 1-Methyl-imidazole (0.4 ml) and 4 ml acetic anhydride were added, the mixture vortex mixed and left for 10 min. before the addition of 0.5 ml of absolute ethanol. After 5 min., 4 ml of water was added and tubes placed in cold water to aid heat dispersal. The solution was made alkaline with 4 ml of 7.5 M KOH and replaced in the cold water. After 3 min a further 4 ml portion of 4 ml 7.5 M KOH was added. The mixture was repeatedly inverted and then left undisturbed for 40 min. The upper ethyl acetate layer containing the alditol acetate esters was transferred into small conical vials (1 ml capacity) with aid of a 1 ml syringe. A small quantity of anhydrous sodium sulphate was added to remove traces of water. Then the ethyl acetate was transferred into fresh dry vials and the mixture was evaporated to dryness in a stream of $N_2$ to concentrate the sugars. The derivatised sugars were redissolved in 25 ul of ethyl acetate and 2 - 3 µl

solution was used for each GLC injection. The alditol acetate esters could be stored for several days.

**APPARATUS** The sugars were separated by GLC (Pye Series 104, W.G. Pye Ltd, England) equipped with a flame ionisation detector and computing integrator and fitted with a glass column (2.1 m X 2 mm internal diameter) packed with 5 % OV 225 on Chromosorb W.AW, DMCS, 100 -150 mesh. Chromatography was performed isothermally at 240 $^{o}C$. The flow rate of the carrier gas ( $N_2$ ),hydrogen and air were 30, 30, and 500 ml $min^{-1}$ respectively. Sensitivity was achieved with the attenuation of the amplifier set at 1 X $10^3$ .

**Expression of results** : Some losses and incomplete recovery of monosaccharides occurred during acid hydrolysis of polysaccharide. This is corrected for by applying the following factors - rhamnose - 1.98, arabinose - 1.07, xylose - 1.14, mannose - 1.10, galatose - 1.07 ( Englyst & Cummings, 1988) and glucose - 1.52 (present study)

### 2.3.8 Batch culture rumen fermentation

Test samples ( 1.0 - 1.5 g ) were dispensed into 150 ml bottles followed by the addition of 50 ml fresh rumen fluid (7.2.2) previously buffered with artificial saliva (7.2.3) and filtered with 50 $\mu$ mesh nylon bags. Bottles were stoppered with Suba seals (size 33, BDH Ltd ) and flushed simultaneously in batches of 11 with 95% $N_2$ and

5% $CO_2$. Samples were incubated in the dark for 42 - 44 h at 39 $^oC$ in a water bath. At the end of incubations, samples were analysed for carbon dioxide and methane trapped in the gas space.

### 2.3.9. Analyses of volatile acids

Effluent or rumen fluid filtrate (0.9 ml) was acidified with 100 µl of 98% formic acid. The mixture was inverted several times, and stored frozen until use. Aliquots (1 µl) were injected into a gas chromatograph (Perkin Elmer model 8700) equipped with a flame ionisation detector (FID). The flow rate of the carrier gas ($O_2$ free $N_2$ ) was maintained at 55 ml $min^{-1}$ ( 20 psi). The glass column (Length 2 m, internal diameter 1.8 mm ) was packed with GP 10% SP - 1200 + 1% $H_3PO_4$ on (80 - 100 mesh) Chromosorb W.AW. Detector and injector temperatures were 210 $^oC$ and 200 $^oC$, respectively. The oven temperature was initially held at 130 $^oC$ for 2 min , then increased to 150 $^oC$ at a ramp rate of 30 $^oC$ $min^{-1}$. Acetate, propionate, butyrate, iso - butyrate, valerate, iso- valerate and hexanoate (10 mM solutions) were used as external standards(Appendix 1) and treated as above. The concentration of volatile fatty acids in samples was calculated using the formula ;

$$\frac{S_i \quad X \quad Conc.}{Std_i}$$

Where ; $S_i$ = peak area of a particular volatile acid per microlitre injection.

$Std_i$ = peak area of known concentration of standard volatile acid per microlitre injection

Conc. = Concentration of the standard sample(in this case 10 mM).

**2.3.10 Gas analyses**

Several methods have been described for analysing rumen gases such as $O_2$, $CH_4$, $CO_2$, $N_2$ and $H_2$ utilising thermal conductivity detectors and often a single line reference system. Czerkawski and Clapperton (1968) recommended that a basic GC analyser should satisfy the following conditions;

(a) it should be capable of analysing small samples of gas;

(b) it should give a complete separation and allow quantitation of the common gases;

(c) it should have a reproducible injection system capable of dealing with wet gases such as those obtained during the ruminal fermentation;

(d) the time required for each analyses should be relatively short and calculation of results straightforward; and

(e) the basic apparatus should be commercially available and capable of modification to meet individual requirements.

The GC system based on the Perkin Elmer model 8700 used for this study met these requirements.

**Apparatus and condition(s) ;** The GC used was a Perkin Elmer model 8700 equipped with a hot wire detector (HWD) connected to a micro computer. The column used was a CTRI dual column (8700) sole supplied by Altech Associates, UK, and a reference column (2.0 m X 2 mm) supplied by Phase Separations Ltd. The CTRI column was a dual steel column with the following characteristics; (i) An outer column 6ft X 1/4" packed with activated molecular sieve. (ii) An inner column 6ft X 1/8" packed with porapak mixture.

**Analytical procedures;** The injector and detector temperatures were maintained at 200 $^{o}$C and 300 $^{o}$C respectively. The oven temperature was held at just above ambient temperature (28 - 32 $^{o}$C). The carrier gas was Helium and the flow rate was 68.5 ml $min^{-1}$ for both separation and reference columns (2.0 m X 2.0 mm). 1 ml replicate gas samples were injected by use of gas tight Hamilton syringe (model 1005, RNE 1810 ) The inner column resolved carbon dioxide from other gases whereas the outer column separated $O_2$, $N_2$, $CH_4$, and CO (Appendix 2 ). The instrument was calibrated with mixtures of gases of known composition (supplied by Supelchem Ltd, Essex). Quantification of gases was performed using the formula below and results were expressed in umol $ml^{-1}$ gas space($\mu$mol $ml^{-1}$ gas space).

**QUANTIFICATION OF GASES;**

Since detector response of each gas was affected by changes in room temperature and pressure, it was necessary to note ambient temperature and pressure readings at each assay period.

$$\text{X vol. of gas} = \frac{273K \times \text{Pressure(mm)}^{a} \times 1000\ ul}{\text{Temperature(K)} \times 760}$$

$$\text{Y no. of moles standard} = \frac{X}{22.4L} = \text{umoles}$$

$$\text{umoles of gas per ml} = \frac{\text{Sample area} \times Y \times 1^{b}}{\text{Standard area}}$$

Where a = Multiply pressure reading in inches by 25.4 to get value in mm

b = convert 1000 µl to ml

# SECTION 3

## 3 THE COMPOSITIONS OF LIGNOCELLULOSIC SUBSTRATES

### 3.1 Preamble

Chemical composition of woods varies between species and also within the same species, as observed in aspen wood, where significant variation in composition was recorded in relation to site, climate, age and other factors (Wayman & Parekh, 1990). Furthermore trees, will contain tissues of varied ages that will differ in composition. Thus analyses of compositions of lignocelluloses should be treated with some care.

Early studies on wood composition were based upon the findings of Klason that the polysaccharides dissolved readily at room temperature in 12 M (72%) $H_2SO_4$ leaving a darkened but recoverable residue of lignin (Effland, 1977). Saccharide compositions of carbohydrates could then be quantified by analyses of the filtrate. Since these early studies, more sophisticated methods of analysis have been developed for lignocelluloses (Van Soest, 1963, 1965; Effland, 1977; Englyst & Cummings, 1988). In particular the methods developed by Klason ignored the distinctive chemistry of woody species such as larch or certain tropical trees which contain significant proportions of components that behave neither as lignins nor polysaccharides. Thus choice of an analytical method should enable optimisation of analyses of lignocelluloses of study.

It is known there are important differences in the chemical composition of deciduous and coniferous woods especially with respect to non-cellulosic polysaccharides and lignin content (Wayman & Parekh, 1990). Significant differences may be observed within a genus (Timell, 1967). Lignin contents varied from 18.9% in birch to 32.5% in hemlock. Similarly xylose residues varied from 5.3% in hemlock to 26.4% in birch. Mannose residues are present in minor quantities in angiosperm woods (1.8 - 2.4%) and at much higher levels in conifers (8.0 - 12.1%)(Wayman & Parekh, 1990). However, other minor residues such as galactose, arabinose, uronic anhydride and ash do not show significant group differences. Differences in composition may influence the suitability of wood species for any bioconversion process.

In this study substrates for fungal bioconversions were selected on the basis of the degree of lignification together with the ruminal digestibility of their carbohydrate, which will reflect the ability of rumen microbes to gain access to and depolymerise the polysaccharides.

## 3.2 Characterisation of test substrates

### 3.2.1 Enzymic methods for starch determination

Reagents;

(a) Glucose oxidase - peroxidase reagent

(b) Bacteria $\alpha$-amylase (Sigma A-3403) solution from *Bacillus licheniformis*

(c) Amyloglucosidase (Boeringer 208469) solution from *Aspergillus niger*

(d) Glucose (Sigma G-500)

Procedure ; To a Pulverised sample (50 - 100 mg) in a test tube, 0.2 ml $\alpha$- amylase solution was added with 2 ml distilled water. The tube was capped, mixed and a further 6 ml water added. Caps were replaced and the contents mixed by repeated inversion and incubated in a water bath at 85 $^{o}$C for 30 min. The tube was cooled to ambient, contents quantitatively transferred to a 100 ml volumetric flask and made up to volume with distilled water. Thereafter 1 ml was transferred into another tube with addition of 1 ml amyloglucosidase solution, then immersed in a water bath at 60$^{o}$C. After 30 min, 8 ml water was added, and contents were mixed by repeated inversions. Finally hydrolysates was filtered through glass microfibre filters (GF/A 7cm diam.). Replicate samples of standard glucose solutions and an enzyme blank were prepared. Glucose released was determined using the glucose oxidase reagent. The quantity of starch in test sample was calculated using the formula below;

$$\text{Starch content}(\%) = \frac{(A_s - A_b) \times 10 \times 0.9}{F \times \text{mg sample}}$$

Where $A_s$ = Absorbance of test sample

$A_b$ = Absorbance of enzyme blank

0.9 = factor for conversion of glucose to starch

F = Mean absorbance of standard.

### 3.2.2 Determination of free sugar in rice bran

Reagents ;

(a) 0.1 M Sodium citrate buffer (pH 4.6)

(b) Amyloglucosidase solution (2 mg $ml^{-1}$)

(c) Glucose oxidase reagent

Procedure ; Distilled water( 4 ml ) was added to 50 - 100 mg in a test tube followed by 1 ml citrate buffer. The test tube was capped, vortex mixed for 1 min and filtered through a glass microfibre filter or by centrifugation at 2640 X g for 30 min. Supernatant or filtrate (1 ml) was transferred into another test tube with addition of 1 ml amyloglucosidase solution. The mixture was incubated at 60 $^{o}C$ for 30 min. The reaction was then stopped , by cooling to room temperature and the tube contents diluted by adding 3 ml water and filtered with Whatman microfibre filter paper. Aliquots (1 ml) were used for glucose determination by the glucose oxidase method. Similarly duplicate samples of glucose standard (100 mg/litre) were prepared. Percentage of free

sugars calculated as follows;

$$\% = \frac{(A - A') \times 2.5 \times 100}{F \times \text{mg sample}}$$

Where A = Absorbance of test sample

A′ = Absorbance of enzyme blank

F = Mean absorbance value of glucose standard.

### 3.2.3 Determination of ash

Sample (0.1 - 0.5 g) was placed in platinum crucible, ashed at 550 $^{o}$C overnight, cooled in a dessicator and weighed. Rice bran samples were ashed at 700 $^{o}$C because of their high silica content.

### 3.2.4 Crude enzyme digestibility (Jones & Hayward 1973, 1975)

Reagent;

(a) Cellulase (BDH 39074 ) from *Trichoderma viride* ( 0.8% w/v) in citrate buffer pH 4.8).

(b) Pepsin A ( BDH 39032 ) 0.2% (w/v) in 0.05 M HCL.

Procedure ; Samples (0.1 - 0.3 g) were weighed quantitatively into glass crucibles (porosity 2) with rubber stoppers underneath. Pepsin (15 ml of 0.2% in 0.05 M HCl) was added, stoppered on top with perforated foam bung, incubated at 50$^{o}$C in a water bath. After 24 h, the pepsin solution was filtered off and the residue rinsed with distilled water and replaced with a fresh cellulase enzyme solution for a further 24 h incubation. The enzyme

solution was removed and the residue in the crucible washed thoroughly with water, dried and weighed. The weight loss was expressed as percent solubility of dry matter (digestibility = dig.).

$$\% \text{ Dig.} = \frac{(\text{Dry wt of sample} - \text{wt of dry residue}) \times 100}{\text{Dry weight of sample}}$$

### 3.3 RESULTS ; Compositions of lignocellulose

Compositions of the rice bran, and spruce and birch sawdusts used in this study are presented in Table 3.1. Lignin contents were determined by two methods : the detergent technique (A.O.A.C., 1984) and Klason method (Effland, 1977). Estimations of acid insoluble lignin (ADL) were observed to be lower than values for Klason lignin. Rice bran had a low lignin content, approximately 3% as ADL. Cellulose and hemicellulose were the major polysaccharides in both spruce and birch sawdust whereas starch and hemicelluloses dominated the carbohydrates in rice bran. The detergent method (ADF - ADL) appeared to give higher estimates of cellulose contents of spruce and birch sawdust than techniques utilising glucose oxidase in colorimetric methods. However, GLC quantification of monosaccharides yielded similar values to the colorimetric assay. Both initial substrate concentration and time of hydrolysis influenced the estimation of cellulose by the glucose oxidase method. After a 5 hr hydrolysis with 12 M $H_2SO_4$, a 99% recovery of glucose

TABLE 3.1 : CHEMICAL COMPOSITION OF TEST SUBSTRATES (% dry wt).

| | RICE BRAN | SPRUCE SAWDUST | BIRCH SAWDUST |
|---|---|---|---|
| ADF | 10.34 ± 0.74 | 78.99 ± 0.32 | 64.60 ± 0.26 |
| NDF | 25.68 ± 2.08 | 93.61 ± 0.14 | 87.46 ± 0.05 |
| Klason lignin | ND | 28.70 ± 0.85 | 16.48 ± 0.10 |
| ADL | 2.98 ± 0.14 | 27.62 ± 0.05 | 14.32 ± 0.18 |
| NSP(colorimetric) | ND | 69.63 ± 3.12 | 68.68 ± 1.2 |
| Cellulose(ADF - ADL) | 7.36 | 51.38 | 50.27 |
| Cellulose(colorimetric,CP) | ND | 43.21 ± 0.20 | 38.47 ± 0.4 |
| Cellulose(GLC) | 7.82 | 47.54 | 37.12 |
| Hemicellulose(NDF - ADF) | 15.34 | 14.61 | 22.87 |
| Hemicellulose(NSP - CP) | ND | 26.39 | 30.21 |
| Hemicellulose(GLC) | 20.27 | 20.75 | 26.21 |
| Rhamnose | 0.73 ± 0.24 | 0.38 ± 0.02 | 0.32 ± 0.11 |
| Arabinose | 6.94 ± 0.24 | 1.2 ± 0.04 | 1.4 ± 0.05 |
| Xylose | 9.63 ± 0.63 | 5.07 ± 0.4 | 21.32 ± 0.37 |
| Mannose | 0.72 ± 0.04 | 11.94 ± 0.58 | 1.59 ± 0.08 |
| Galactose | 2.25 ± 0.07 | 2.15 ± 0.07 | 1.58 ± 0.30 |
| Glucose | 8.98 ± 0.32 | 53.41 ± 0.24 | 41.7 ± 0.56 |
| Free sugars | 2.1 ± 0.11 | ND | ND |
| Starch | 18.3 ± 0.40 | ND | ND |
| Protein | 16.47 ± 0.08 | 0.51 + 0.01 | 1.4 + 0.06 |
| Ether extract | 21.05 ± 0.05 | ND | ND |
| Ash at 550°C | 10.66 ± 0.01 | 1.44 ± 0.24 | 7.37 ± 0.14 |
| Ash at 700°C | 8.93 ± 0.07 | ND | ND |
| *In vitro* digestibility | 50.54 ± 2.40 | 7.87 ± 0.44 | 13.78 ± 0.7 |
| Cellulase digestibility | 68.28 ± 2.26 | 7.21 ± 0.1 | 15.09 ± 0.2 |

ND = not determined

from 100 mg of crystalline cellulose was recorded whereas only 95% of glucose was recovered when <30 mg ∝-cellulose was used (Fig. 3.1). Estimates of the hemicellulose content of the substrates were consistently lower with the detergent method (NDF - ADF) than using alternative methods.

In gas chromatograms of polysaccharide hydrolysates, baseline separation of monosaccharides was obtained (Fig 3.2) under conditions in which the last monosaccharide (glucose) eluted at 10 min. It was clear that mannose (12.00%) was the dominant non-cellulosic monosaccharide in spruce sawdust whereas xylose was dominant in both rice bran (9.63%) and birch sawdust (21.32%).

Protein was present at low concentrations in sawdusts (0.51 - 1.43%), an order of magnitude higher in rice bran (16.47%). Ether extract was 21% of rice bran. Moreover, *in vitro* rumen digestibility assessments of the substrates showed that spruce sawdust (7.87%) was least digestible, with birch sawdust having twice the digestibility (13.78%) and 50.54% the highest value was recorded for rice bran. Using commercial cellulase enzymes higher estimates of digestibility were obtained for both birch sawdust and rice bran.

## 3.4 DISCUSSION

The techniques used in this study have been used previously for estimations of composition of complex substrates (Van Soest, 1967 ; Effland, 1977 ; A.O.A.C, 1984; Englyst & Cummings, 1988). Detergent analyses of

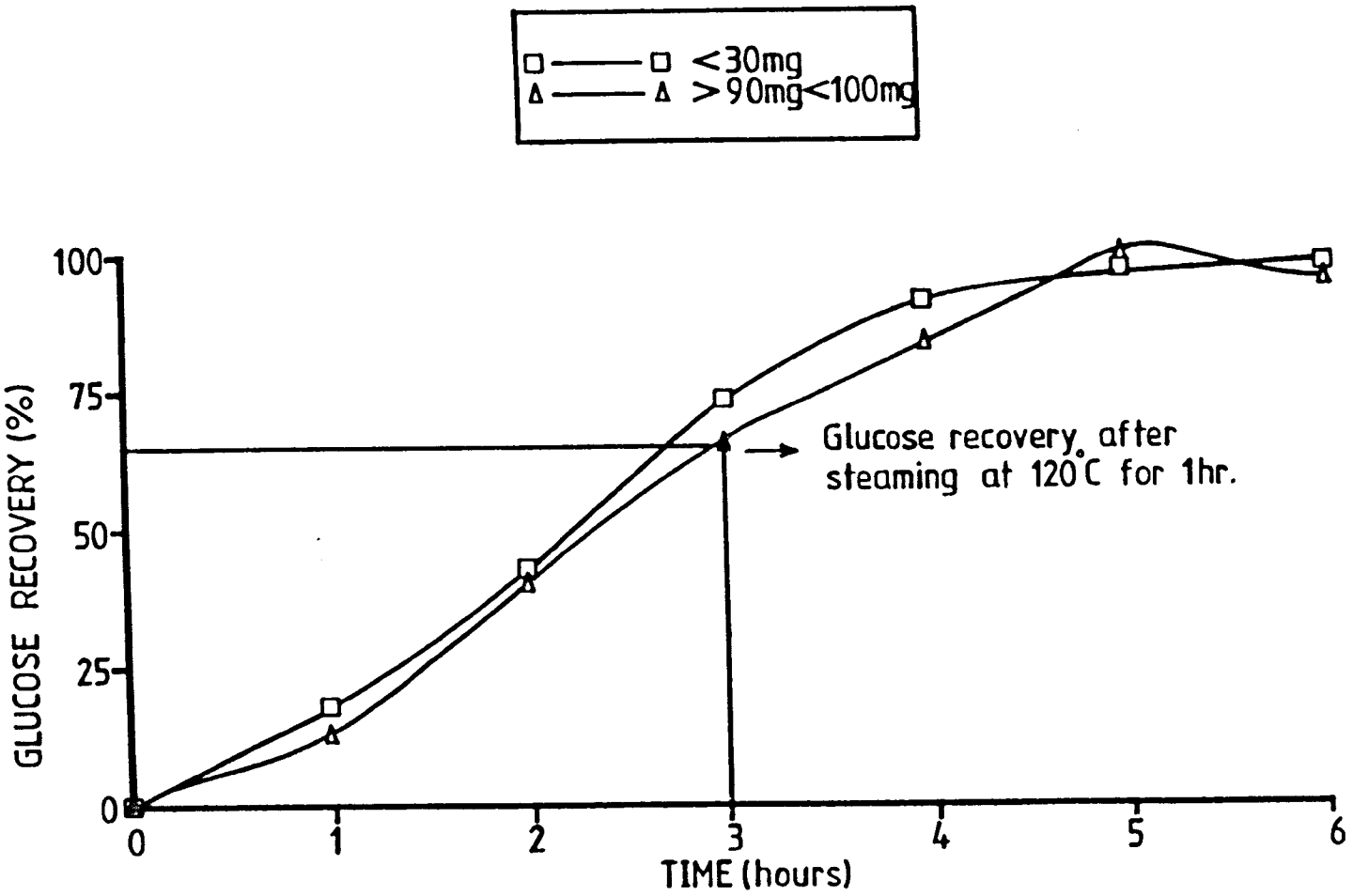
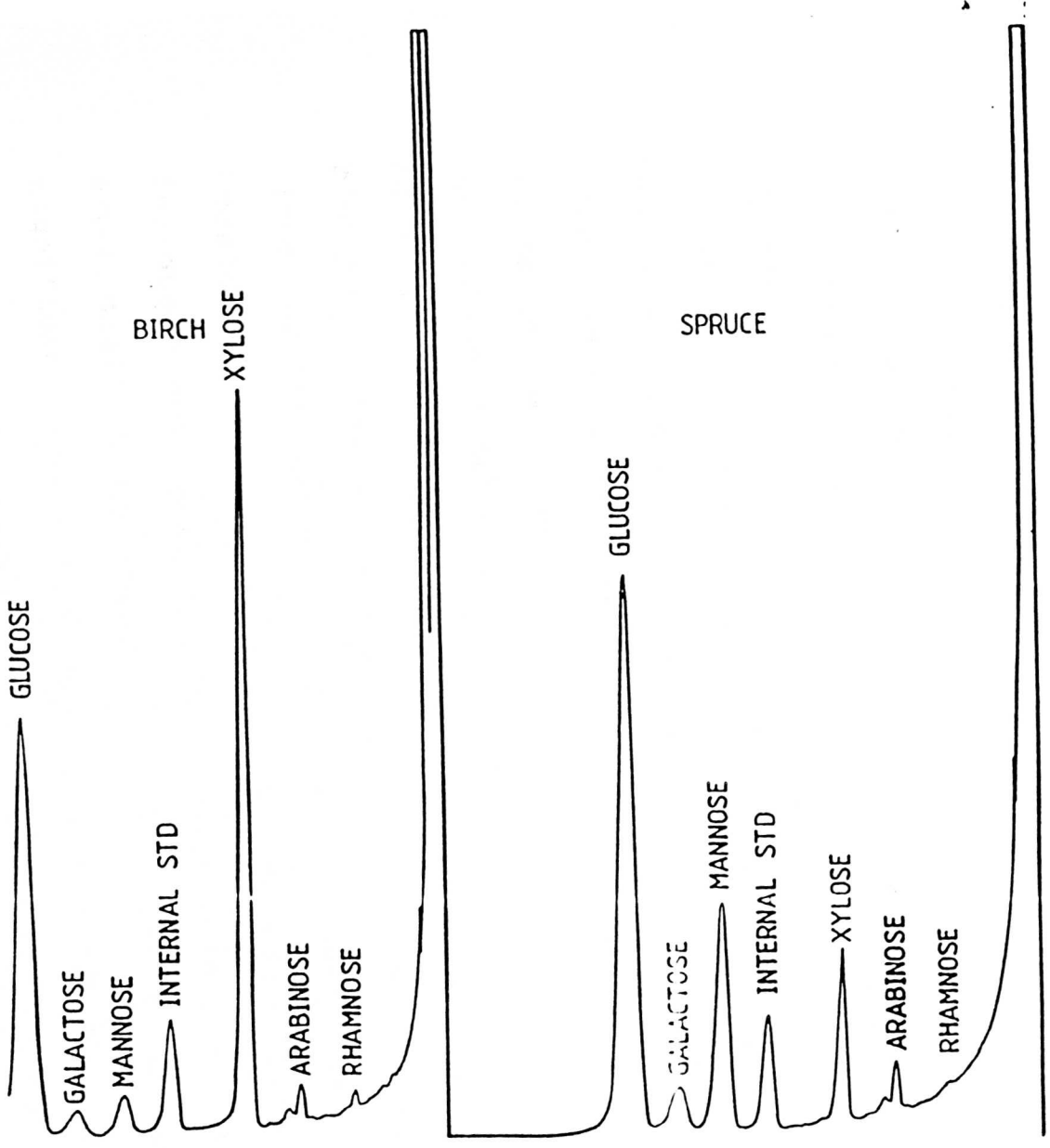



Fig. 3.1 Time course of glucose recovery from cellulose hydrolysis with 12 M $H_2SO_4$ followed by incubation at 100°C.




TIME (sec)

264 - Rhamnose
406 - Arabinose
545 - Xylose
743 - Allose
860 - Mannose
973 - Galactose
1107 - Glucose

FIG. 3·2 TYPICAL CHROMATOGRAM OF ACID HYDROLYSATE OF CELL WALL POLYSACCHARRIDE OF SPRUCE & BIRCH SAWDUST.

lignocellulosics (ADF and NDF) were developed in the early sixties for analyses of high-fibre ruminant feeds and diets (Van Soest, 1967) and have gained acceptance through their simplicity and rapidity. The acid detergent reagent removes starches, pectins, hemicelluloses and proteins leaving a residue principally of lignin and cellulose, while neutral detergent solution solubilises protein and pectic substances.

Estimations of pectins, gums and mucopolysaccharides in detergent assays will vary depending on composition (Robertson & Van Soest, 1981). Certain components - notably gums, pectins, alginates and tannins - dissolve readily in neutral detergent solutions but to varying extents in acid detergent reagents, which may lead to errors in the estimation of hemicellulose using the subtraction of NDF from ADF (Keys *et al.*, 1969; Robbins *et al.*, 1975). The presence of lipids or microbial biomass may cause problems in estimation of detergent fibre contents, since the reagent cetyltrimethyl ammonium bromide is soluble in lipid phases when there is inaequate detergent in the aqueous phase, yielding artefactually higher values for fibre contents. Moreover, significant levels of protein increase the ability of detergent solutions to form soluble complexes. Furthermore, estimations of Klason lignin do not include lignin that dissolves in sulphuric acid, a particular problem in graminaceous materials. Although Effland (1977) reported that such soluble lignin constitute only

a small fraction of lignin in soft woods this could be as much as 3 to 5% of the phenolic material in hard woods suggesting this method was not suitable for analyses of decayed woods.

In this study detergent methods yielded lower estimates of hemicellulose contents of birch and spruce sawdust than colorimetric assays. Similarly, differences were observed in quantifications of cellulose contents of the two substrates. Theander and Aman (1980) have shown that ADF from plant residues and forages may contain significant quantities of protein (1 - 4%) and hemicellulose (7 - 14%) in addition to cellulose and lignin. However they also observed that values for cellulose determined from ADF was markedly higher than those obtained from sugar analyses after acid hydrolyses of cellulose. Morrison (1980) reported that the ADF fraction of fruits, vegetables, legumes, forages and wood can contain up to 15.4% hemicellulose. Thus it can be concluded that the lower estimates of hemicellulose and higher of cellulose contents obtained using detergent techniques in this study are as predicted from published data.

The results obtained compare favourably with the findings of other workers (Timell, 1967; Effland, 1977; Juliano, 1985 ; Englyst and Cumming, 1988). The lignin content of birch sawdust was slightly lower than the minimum of the range for hard woods (18 - 25%). This can be attributed either to the wood not being fully mature

or the presence of impurities such as bark in the sawdust. The lignin content of spruce sawdust is within the range reported by Ladisch (1979) for soft woods (25 - 35%). The content of acid insoluble lignin (ADL) of rice bran, at 2.98%, is within the range quoted as typical of brans by Juliano (1985).

Estimates of polysaccharide composition yielded values similar to those reported by other workers with related substrates (Wayman & Parekh, 1990). These authors concluded that angiosperm, such as birch and maple, and gymnosperm woods (spruce, pine) would have polysaccharide contents in the range 69 - 77% on a dry weight basis. In addition, the hemicellulose composition of rice bran is similar to values reported by Englyst and Cummings (1988) and Juliano (1985) and the monosaccharide composition of the two sawdust substrates (Table 3.1) is in accord with the findings of Timell (1967). The higher estimates of digestibility obtained using crude cellulase enzymes than with simulated rumen fermentation may be attributed to limitations in the ability of this technique to simulate the rumen with respect to contents of anaerobic fungi and protozoa. The lower digestibility of sawdusts than rice bran reflect quantitative and compositional differences in the lignins and phenols that restrict enzymic depolymerisation of the polysaccharides (Paterson, 1989).

SECTION 4

# 4 : THE INFLUENCE OF CELL WALL PHENOLICS AND CARBOHYDRATES ON GROWTH OF FILAMENTOUS FUNGI AND EXTRACELLULAR ENZYME PRODUCTION

## 4.1 Preamble

It is generally considered that the commercial bioconversion of lignocelluloses is limited largely through lack of understanding of the biochemistry and physiology of microbial breakdown of glycolignin, the material that forms a lignin-hemicellulose matrix separating cellulose protofibrils in the interrupted lamella model of the lignified plant cell wall (Young, 1986). However the effective conversion, in Chile, of wood by mixed microbial cultures to produce the animal feed *palo podrido* (Gonzalez *et al.*, 1989) suggests that there is considerable potential in bioconversion processes. Reid (1989a) has concluded that with an increased understanding of the physiology of lignin-degrading fungi further biological processes for enhancing the digestibility of low-value lignocellulosic feed can be developed.

Results obtained, to date, with purified fungal lignin peroxidases in *in vitro* studies have suggested that the value of enzymes in the absence of fungal biomass is limited. However the production of lignin peroxidases and laccases by two basidiomycete fungi *Coriolus versicolor* and *Phanerochaete chrysosporium* has

been extensively studied and it has been shown that these enzymes effect lignin depolymerisation. The physiology of production and secretion of lignin peroxidases by *P.chrysosporium* is well documented and in this organism the production of depolymerising lignin peroxidases is stimulated by limited availability of nitrogen and sulphur in growth media (Glenn & Gold, 1983) although this is not true for other ligninolytic fungi (Leatham & Kirk, 1983).

In contrast, data on physiological regulation and biological role of laccases in ligninolytic fungi is limited. Both inducible and constitutive laccases are produced by *Coriolus versicolor* (Evans *et al.*, 1984) and production of laccases in *Agaricus bisporus* may be causally related to developmental events (Wood, 1980). Although purified laccases have been shown to degrade lignin model compounds (Kwai *et al.*, 1986) and depolymerise polymeric lignin *in vitro* (Evans, 1985) the extent to which this takes place *in vivo* may be limited by oxygen supply. In comparison to other amorphous and semi-crystalline carbohydrates enzymic depolymerisation of cellulose is slow due to the crystalline and insoluble nature of the substrate which arises from the association and interlinking by hydrogen bonding of the β 1,4-glucan chains. Cellulose is also unable to swell and lose its ordered structure with heating, an important aspect of crystalline starch behaviour. However, lignified tissues may, in addition to polymers contain significant

quantities of low molecular weight phenolic compounds such as ferulic or p- hydroxycinnamic acid contributing to bonding between carbohydrates or between hemicelluloses and lignins. Phenolic compounds are also present in locally high concentrations in wood layers immediately under the bark.

The quantitation of ligninolytic activity in fungi has been facilitated by the development of assays based upon decolourisation of polymeric chromophores, an activity associated with lignin oxidation (Glenn & Gold, 1983). However, the correlation between such assays and ability to effect delignification of plant residues has received little attention. In contrast, the measurement of changes in contents of Klason (72% acid insoluble) lignin has been widely used.

The effect of addition of alternate carbohydrate sources on lignolytic activity in fungal fermentations has been studied (Reid, 1989) and addition of glucose, malt extract or xylose has been shown to enhance lignin degradation by certain fungi (Martin & So, 1969; Levonen-Munoz *et al.*, 1983) whereas in solid state fermentation of straw with *Coprinus* supplementation with either molasses or whey reduced lignin depolymerisation (Yadav, 1987).

The objectives in these experiments were to clarify the effect of varying concentrations of the natural cell wall component ferulic acid on production of enzymes central to lignocellulose breakdown. The effect of

varying concentrations of nitrogen and alternate carbon sources on biomass and enzyme production was also clarified. Lignin peroxidase activity in surface cultures was estimated using dye decolourisation, and laccase production was quantified in submerged liquid cultures. In parallel experiments, the effects of nitrogen and alternate carbon source on production of xylanases and total fungal biomass were assessed. A collection of ten lignocellulosic fungi, selected for their ability to grow on wood and cereal residues, was studied. Cellulolytic activities in a subset of five fungi, grown in the presence of differing concentrations of ferulic acid, was analysed in detail to clarify whether this phenolic compound might influence solid-substrate fermentations of natural lignocelluloses.

## 4.2 EXPERIMENTAL - Fungal saccharification of crystalline cellulose

### 4.2.1 Test for detecting phenol oxidases

Preamble; Efforts have been made to correlate phenol oxidase production with the type of decay associated with many species of fungi. Most basidiomycete fungi which deplete both polysaccharide and lignin components of wood give a positive reaction; the brown rot fungi which attack mainly the carbohydrate portion of wood with slight modification of lignin structure evoke a negative reaction (Kirk, 1971; Davidson *et al.*, 1938; Harkin &

Obst, 1973).

A quick diagnostic test normally used for detecting secretion of phenolases by fungi is that described by Bavendamn (1928). The test is considered positive when a brown coloured zone is formed around the fungal mycelium on a suitable agar medium with 0.5% of either tannic acid or gallic acid in Petri dishes. However Lyr (1958) encountered strong inhibition of mycelial growth at 0.5% concentration of tannic or gallic acid. Although reduction in concentration of gallic or tannic acid did not improve the ability of the test to predict rot type (Harkin & Obst 1973). Lyr (1958) further suggested that an extracellular laccase (p- diphenol:oxygen reductase) was responsible for producing a positive Bavendamn test. Previous workers implicated laccase, tyrosinase (o-diphenol:oxygen oxidoreductase) or peroxidase donor(hydrogen peroxide oxidoreductase) as participants in the reaction (Linderberg & Holm, 1952; Higuchi, 1953). However, the use of dye decolourisation assay to screen for lignolytic activity has received widespread acceptance (Glenn & Gold, 1983).

**(A) Bavendamn Reaction** : A modified Bavendamn reaction as described by Harkin and Obst (1973) was employed. A 9 mm agar plug of young growing culture of the test organism was inoculated on to an agar plate with 0.1% syringaldazine or guaiacol in basal mineral medium at pH 5.5 which was incubated at the optimum growth temperature of the organism and examined after 5-days . A

positive test was indicated by either a brown or dark coloured zone around the fungal mycelium.

### 4.2.2 Xylanase assay

Xylanase activity was estimated using a plate assay method as described by Teather and Wood (1982) except that plates contained 0.5% crude oat spelt xylan with or without differing concentrations of ferulic acid (0%, 0.05%, 0.1% & 0.5%). Plates were inoculated and incubated for 7 d . Extent of xylanase production was detected by flooding duplicate plates with congo red solution (1 mg $ml^{-1}$). Congo red stains the unhydrolysed polysaccharide (Bucker, 1985).

### 4.2.3 Detection of cellulases in surface culture

The ability of mycelial fungi to break down insoluble polysaccharides like cellulose has received considerable attention in recent times. Attempts have been made to determine cellulolytic activity in an ecosystem by incorporating intact filter paper or pieces of wood, the extent of degradation being assessed by gravimetric method or weight loss. Such methods may require very long periods of incubation for any meaningful assessment to be made. However, new methods have been developed which overcome some of these shortcomings by using dye labelled insoluble substrates (Smith, 1977; Mahasneth & Stewart, 1980) or by incorporating a soluble cellulose derivative

(carboxymethy-cellulose) which can be precipitated with a detergent to visualise a zone of hydrolysis on an agar plate (Harkin & Anagonastakis, 1977). Most of these techniques still have shortcomings such as lack of sensitivity and restricted range of substrates which can be assessed. Recent methods using dyes such as Congo red which shows a strong interaction with polysaccharides containing β (1-4) linked D-glucopyranosyl units provides a better option (Teather & Wood, 1982). It also has a potential advantage of being adapted to quantify the degree of cellulolytic activity or cellulase production (Bucker, 1985).

Procedure ; The plate screening assay of Hoffman and Wood (1985) was employed for detecting cellulase production. A 9 mm agar plug of the test organism was inoculated on agar plate containing 0.1% cellulose powder as substrate in minimal media. Inoculated plates were incubated for 5 d. The extent of polysaccharide breakdown was estimated by flooding the agar plates with Congo red solution (1 mg $ml^{-1}$).

### 4.2.4 Quantitative enzyme assay

#### 4.2.4.1 Determination of temperature optima for cellulose depolymerisation

Plugs (10 mm) were removed from 90 mm agar plates containing 1% (w/v) Whatman crystalline cellulose in sodium citrate buffer ( pH 4.8 ) and 0.1 ml aliquots of

culture supernatant were placed in the wells formed. After 24 h of incubation at 40°, 50° or 60°C plates were flooded successively with Congo red solution(1 mg $ml^{-1}$) and NaCl (1 M) and diameters of zones of cleared depolymerised cellulose were measured.

#### 4.2.4.2 β -glucosidase

This was estimated using the procedure described by Yamanobe *et al.* (1987) except that activity was assayed by mixing 0.5 ml cellobiose solution (1%), 0.25 ml citrate buffer (0.1 M, pH 4.8) and 0.25 ml enzyme solution. All assays were carried out at 50 °C for 30 min. using enzyme complexes with an optimum at this temperature. Glucose produced was estimated using the glucose oxidase method (Karkalas, 1985).

#### 4.2.4.3 Saccharification of crystalline cellulose

This was determined as glucose produced from hydrolysis of absorbent cotton wool (200 mg) by 3 ml of enzyme solution with 2 ml 0.1 M sodium citrate buffer pH 4.8 at 50 °C for 24 h (Mandels *et al*, 1974) using the glucose oxidase method (Karkalas, 1985).

#### 4.2.4.4 Endo β 1,4 glucanase

This was determined by estimating reducing sugar liberated by enzymes from 0.5 ml aliquots of 1% carboxymethylcellulose (CMC). Activity was assayed in 0.5 ml of 0.1 M citrate buffer(pH 4.8) with a 30 min. incubation by the method of Nelson (1944) and Somogyi

(1952).

#### 4.2.4.5 Initial depolymerising cellulase activity

This was determined as reducing sugar released from filter paper (Whatman no. 1; 2 discs each 5 mm diameter, 22 mg) during a 1 h incubation in 0.5 ml enzyme solution at 50 $^{o}$C in 0.1 M sodium citrate (1 ml) pH 4.8. In each case results were expressed as reducing sugar equivalent to glucose (Mandels *et al.*, 1976).

#### 4.2.4.6 Laccase activity

Laccase activity was estimated using a modification of methods described by Eriksson *et al.* (1983) and Nigam *et al.* (1987) in which syringaldazine is oxidised to a quinone. An assay mixture containing 0.5 ml 0.1% syringaldazine in 99% ethanol and 1.5 ml sodium acetate (0.2 M, pH 5.6 ) was made up to 3 ml with culture supernatant and assays were incubated at 30 $^{o}$C. Enzyme activity was defined as one unit of enzyme that will produce a change in absorbance ($A_{525}$ nm) of 0.001 per min at pH 5.6.

#### 4.2.4.7 Polymeric dye decolourisation (Glenn & Gold, 1983);

Ligninase activity was estimated by measuring zones of clearance of Remazol brilliant blue dye (0.1 mg $ml^{-1}$) produced by growth on agar containing minimal media or omitting 0.2% $NH_4H_2PO_4$ on 0.05%, 0.1% and 0.5% (w/v) ferulic acid and 1% crystalline cellulose, for up to 7

days at 37 $^{o}$C or 30 $^{o}$C. The effect of exogenous nitrogen or carbon sources on dye decolourisation was equally monitored using the same procedure except that distilled water served as the media solution and dye concentration was 1 mg $ml^{-1}$. Total ligninase production (%) was estimated as proportion of the agar that was decolourised during growth (mm) divided by diameter of the agar plate and multiplied by 100.

#### 4.2.4.8 Total Biomass

Total biomass was estimated as a total cell wall nitrogen in the culture residue using the Kjeldahl method of low nitrogen determination( 2.3.4.2). The blue colour developed in the 16 ml reaction mixture was stable for at least 1 h.

## 4.3 RESULTS

### 4.3.1 Growth on lignocellulose-related carbon source

#### 4.3.1.1 Lignin related phenolic compounds

Ten fungi were assessed for their ability to grow on agar media containing a range of lignin-related carbon sources and upon agar in the absence of any carbon source (Table 4.1). Stimulation of growth of *C. versicolor*, *T. harzianum*, *A. terreus*, *S. lignicola* and *P. sajo-caju* was observed when phenolic compounds were present in media. Coumarin and phenol appeared to inhibit growth of most fungi, except *C. versicolor* and *P. sajo-caju*, whereas

TABLE 4.1 GROWTH OF CELLULOLYTIC FUNGI IN THE PRESENCE OF LIGNIN RELATED PHENOLIC COMPOUNDS

| | ORGANISMS | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J |
| Ferulic acid | 2* | 3 | 3 | 4 | 3 | 3 | 3 | 4 | 4 | 3 |
| Catechol | 1 | 0 | 0 | 2 | 3 | 3 | 2 | 3 | 3 | 2 |
| Syringaldehyde | 0 | 1 | 2 | 3 | 3 | 4 | 2 | 3 | 3 | 3 |
| Syringaldazine | 1 | 3 | 4 | 3 | 3 | 5 | 3 | 3 | 3 | 4 |
| Vanillin | 1 | 0 | 0 | 3 | 2 | 4 | 3 | 2 | 3 | ND |
| Cinnamic acid | 1 | 0 | 0 | 1 | 1 | 3 | 1 | 2 | 0 | 1 |
| Vanillic acid | 4 | 3 | 4 | 3 | 4 | 5 | 3 | 3 | 4 | 2 |
| Phenol | 0 | 0 | 0 | 3 | 2 | 0 | 0 | 0 | 0 | 0 |
| Benzoic acid | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Syringic acid | 2 | 2 | 3 | 3 | 3 | 5 | 4 | 3 | 4 | 2 |
| Guaiacol | 1 | 1 | 1 | 3 | 3 | 0 | 3 | 2 | 3 | 2 |
| Coumarin | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Indulin lignin | 3 | 1 | 0 | 3 | 3 | 5 | 4 | 3 | 5 | 2 |
| Poly remazol B | 5 | 0 | 5 | 4 | 3 | 5 | ND | 3 | 5 | 2 |
| Control(Agar) | 3 | 2 | 5 | 2 | 2 | 4 | 5 | 2 | 4 | 0 |

Key to Table;

A = *P. chrysosporium*, B = *C. cellulolyticum*, C = *T. reesei* , D = *C. versicolor*, E = *P. sajo-caju* , F = *T. harzianum* , G = *A. corymbifera* , H = *A. terreus*, I = *A. fumigatus* , J = *S. lignicola*.

* Numerical figures in the Table represent hyphal density after a period of 5 days ( 0 = No growth, 1 = Traces, 2 = Low, 3 = Average, 4 = High, 5 = Very high).

Results are observations on two culture plates

benzoic acid was toxic to all the strains at 0.1%. *Chaetomium cellulolyticum* and *T. reesei* did not grow well in the presence of catechol, vanillin, cinnamic acid, guaiacol or lignin.

During growth on a number of phenolic compounds decolourisation of the media surrounding the nascent mycelia was observed prior to hyphal growth through that region of the agar. This was particularly marked with *P.chrysosporium*, *C. versicolor*, *P. sajo-caju*, and *S. lignicola* (Plates 4.1 - 4). An alternative observation with *T. harzianum*, *A. corymbifera*, *A. fumigatus*(Plate 4.3) and *A. terreus*, was that zones were cleared in parallel with and as a result of colony expansion.

**4.3.1.2 Carbohydrate polymers:**

The response to addition of carbohydrates as carbon sources was varied (Table 4.2) but these compounds appeared generally to stimulate growth. With the exception of *P. sajo-caju* and *A. terreus* polygalacturonic acid (pectic acid) and polygalacturonic acid methyl ester (pectins) stimulated fungal growth. Crystalline cellulose, carboxymethylcellulose and also the natural lignocellulose sawdust stimulated growth as was observed, with all mono- and disaccharides and all fungal species with the exception of *S. lignicola*.

**4.3.2 Production of lignocellulolytic enzymes**

Five strains were selected, on the basis of secretion of depolymerising cellulases or phenol

TABLE 4.2 GROWTH OF CELLULOLYTIC FUNGI IN THE PRESENCE OF CELL WALL RELATED CARBOHYDRATE POLYMERS[c]

| | ORGANISMS | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J |
| Pectin | 5 | 4 | 5 | 4 | 3 | 5 | 4 | 3 | 5 | 2 |
| PGA[a] | 4 | 3 | 5 | 4 | 3 | 5 | 4 | 2 | 4 | 5 |
| Cellulose | 5 | 4 | 5 | 3 | 3 | 5 | 5 | 3 | 4 | 5 |
| CMC[b] | 2 | ND | 5 | 3 | 2 | 5 | 5 | 2 | 4 | 3 |
| Xylose | 5 | 3 | 5 | 4 | 4 | 5 | 5 | 3 | 5 | 2 |
| Fructose | 5 | 3 | 5 | 3 | 3 | 5 | 4 | 4 | 5 | 2 |
| Arabinose | 4 | 4 | 5 | 4 | 4 | 5 | 4 | 3 | 5 | 5 |
| Sucrose | 3 | 4 | 5 | 4 | 4 | 5 | 4 | 3 | 5 | 5 |
| Galactose | 5 | 4 | 5 | 4 | 4 | 5 | 4 | 3 | 5 | 5 |
| Mannose | 5 | 3 | 5 | 3 | 3 | 5 | 4 | 2 | 4 | 2 |
| Glucose | 5 | 3 | 5 | 3 | 3 | 5 | 4 | 2 | 4 | 2 |
| Xylan | 5 | 4 | 5 | 4 | 3 | 5 | 5 | 3 | 5 | 2 |
| Sawdust(spruce) | 5 | 4 | 5 | 4 | 3 | 5 | 5 | 3 | 5 | 3 |
| Control (Agar)[c] | 3 | 2 | 5 | 2 | 2 | 4 | 5 | 2 | 4 | 0 |

Key to Table;

A = *P. chrysosporium*, B = *C. cellulolyticum*, C = *T. reesei* , D = *C. versicolor*, E = *P. sajo-caju* , F = *T. harzianum* , G = *A. corymbifera* , H = *A. terreus*, I = *A. fumigatus* , J = *S. lignicola*.

* Numerical figures in the Table represent hyphal density after a period of 5 days ( 0 = No growth, 1 = Traces, 2 = Low, 3 = Average, 4 = High, 5 = Very high).

[a] PGA = Polygalaturonic acid , [b] CMC = Carboxymethyl-cellulose

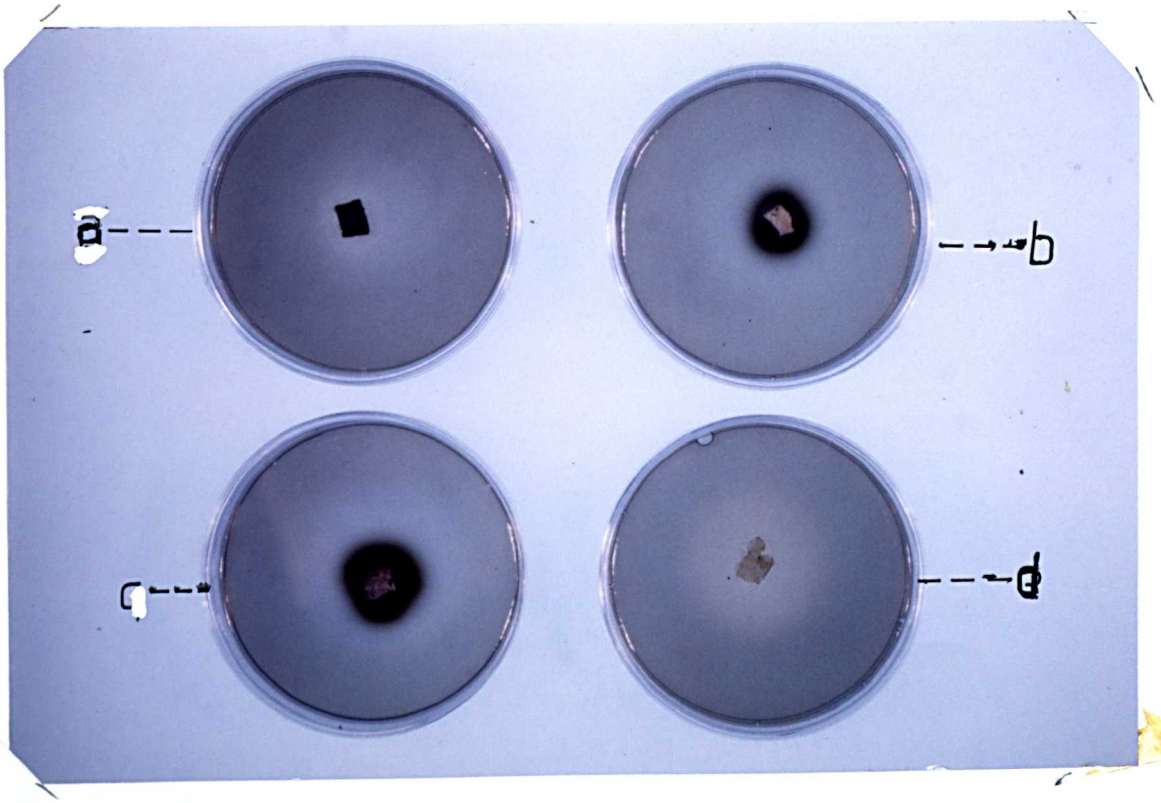
[c] Growth stimulation was observed in the presence of additional carbon source within the first 3-days of incubation, comparatively this became less distinct relative to control cultures after 5-days.

Results are observations on two culture plates

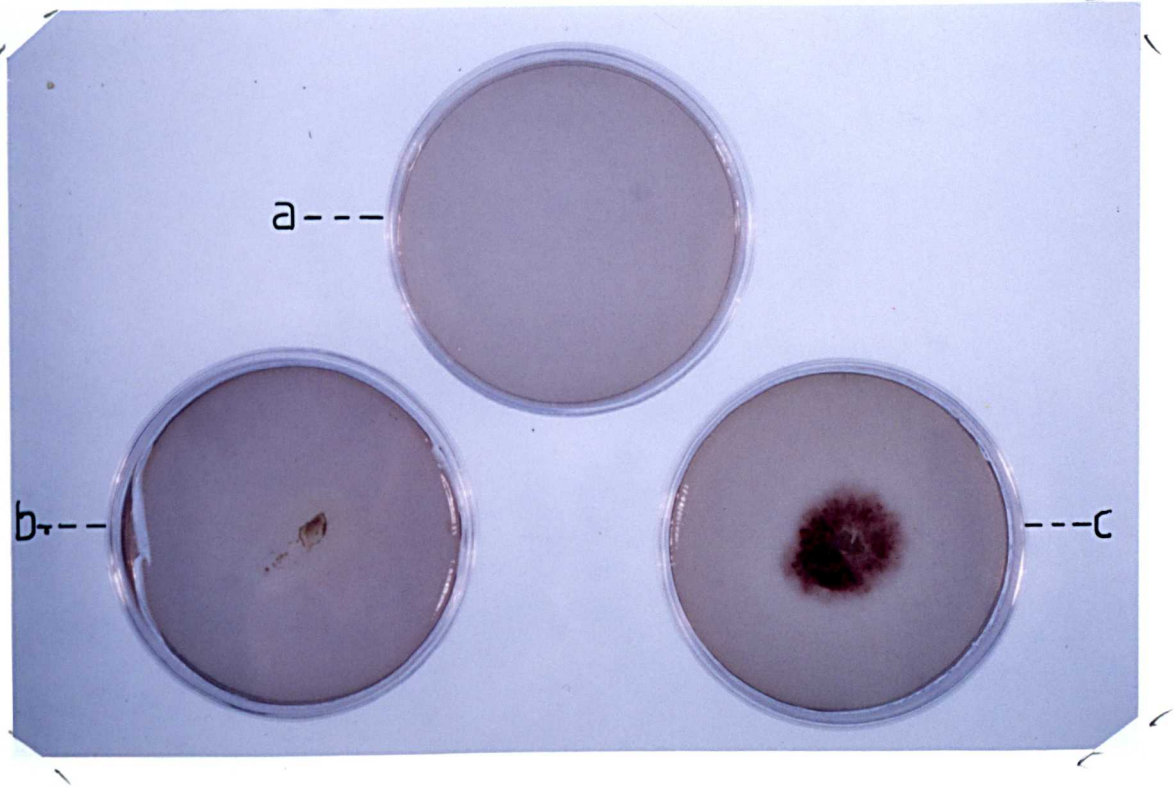
PLATE 4 .1 Coloured zones showing positive Bavendamm reaction by laccase producing strains[1] in catechol medium (a) *S. lignicola* (b) [1]*P. sajo-caju* (c) [1]*C. versicolor* (d) *P. chrysosporium*.

PLATE 4.2 Positive Bavendamm reaction exhibited by *P. chrysosporium* in guaiacol medium. (a) Control (b) *A. terreus* (c) *P. chrysosporium*.

4.1




4.2



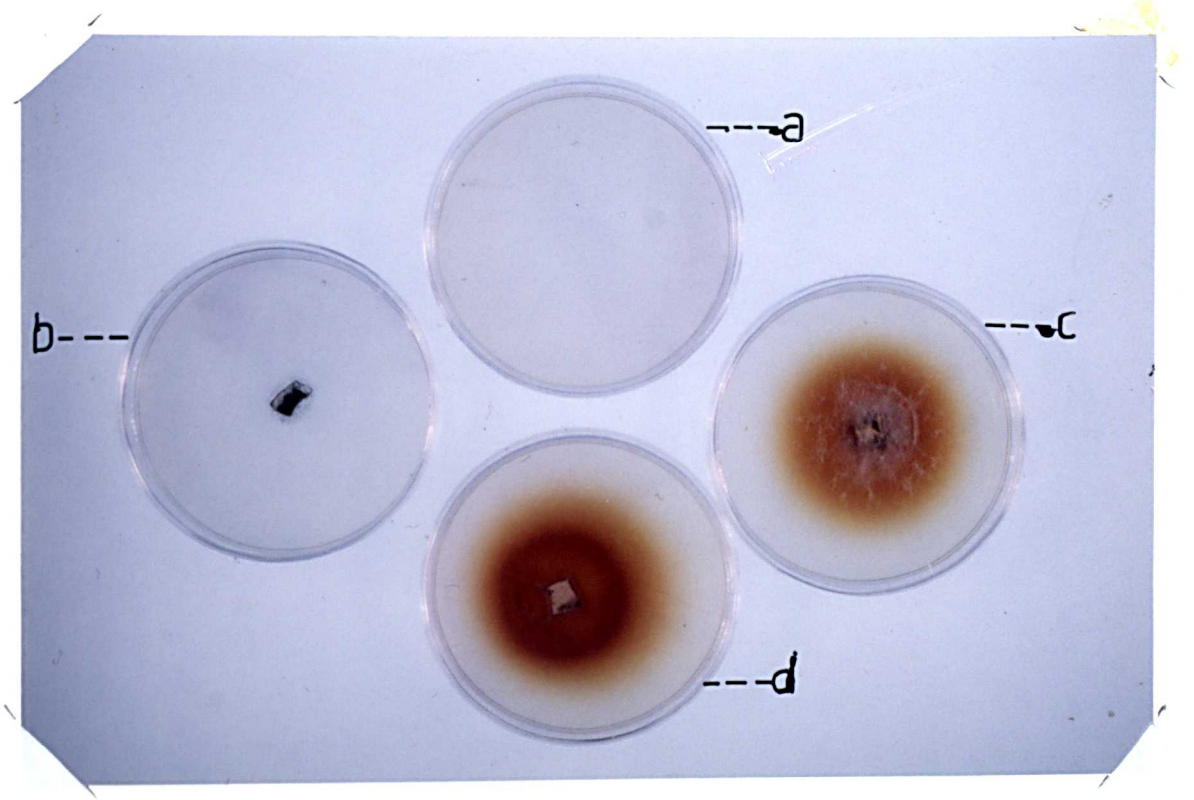



PLATE 4.3 Positive Bavendamn reaction exhibited by phenol oxidase[1] producing strains in vanillin medium (a) control (b) A. fumigatus. (c) [1]C. versicolor. (d) [1]P. sajo-caju.

PLATE 4.4 Polymeric dye decolourization in presence of 1% glucose minimal media agar containing 0.2% $NH_4H_2PO_4$ .(a) *P.chrysosporium* (b) *C.versicolor* (c) *P.sajo-caju* (d) *T.harzianum* (e) *S. lignicola*.

PLATE 4.5 Polymeric dye decolourization in presence of 1% glucose media devoid of $NH_4H_2PO_4$ (a) *P. chrysosporium* (b) *C.versicolor* (c) *P.sajo-caju* (d) *T. harzianum* (e) *Scytallidium lignicola*

PLATE 4.4

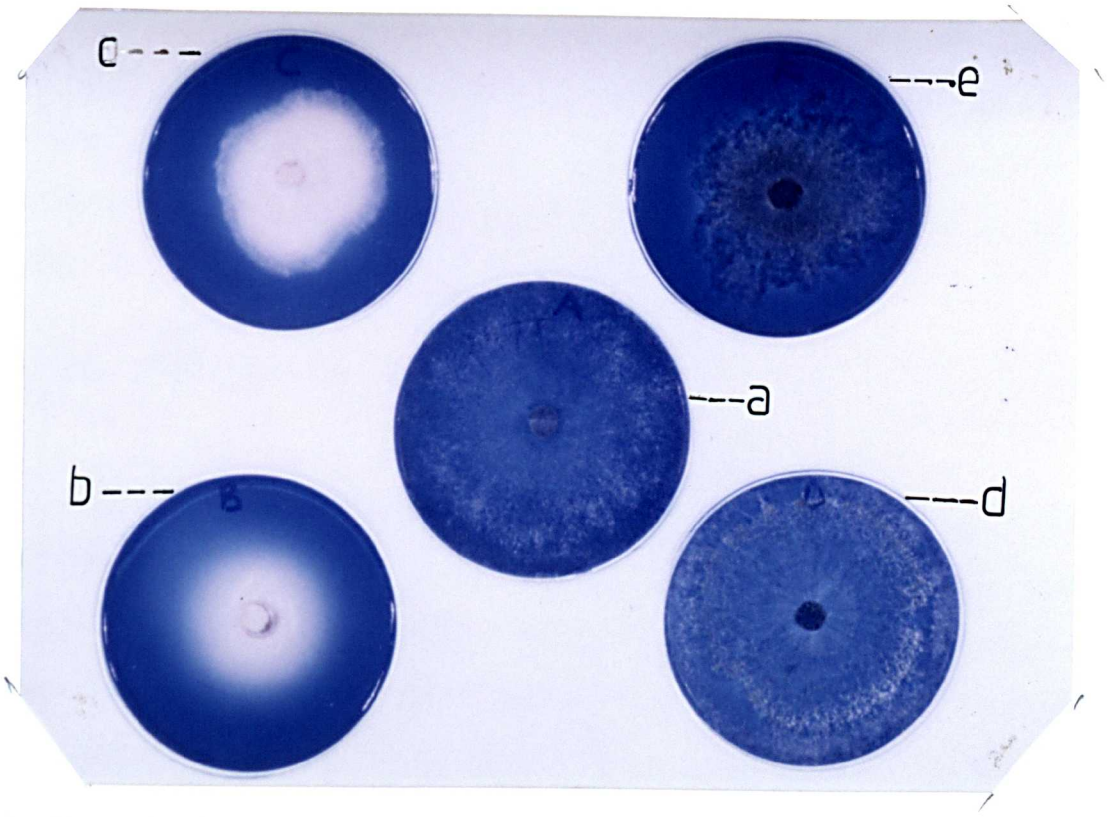
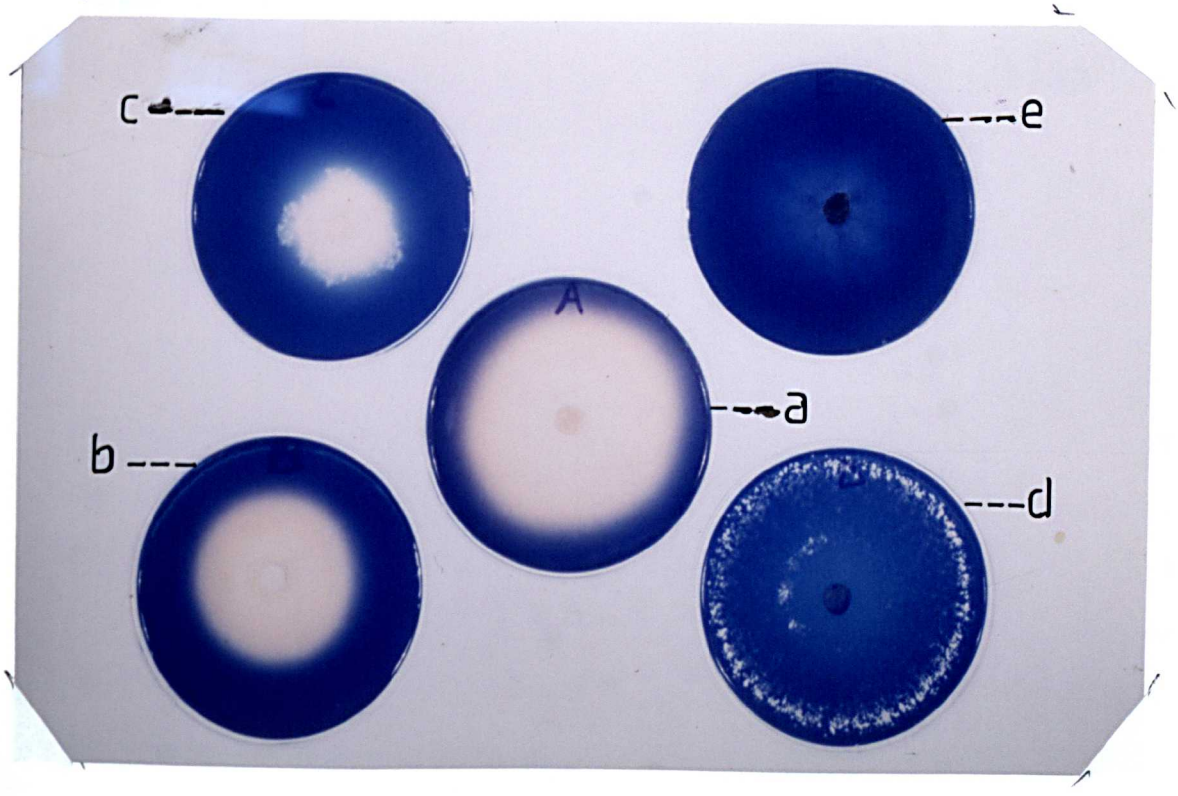



PLATE 4.5



oxidases, for an in detail study of the effect of ferulic acid on activities related to lignocellulose breakdown. These fungi were *C. cellulolyticum*, *T. harzianum*, *P. sajo-caju*, *C. versicolor* and *P. chrysosporium* (Table 4.3).

### 4.3.3. Temperature optima for cellulose breakdown by secreted enzymes

Assays of cellulose depolymerisation, made by estimating areas of zones of clearing of insoluble cellulose in agar overlays (Table 4.4) showed that for each of the 5 strains (*T. harzianum*, *C. cellulolyticum*, *P. chrysosporium*, *P. sajo-caju* and *C. versicolor*) cellulose depolymerisation by extracellular enzymes in culture filtrates was significantly greater at 50° than at either 40° or 60°. Consequently this temperature was used in subsequent assays for cellulolytic enzyme activities.

### 4.3.4 Effects of ferulic acid on cellulolytic enzymes

a. β-glucosidase

Cultures were grown in minimal medium containing 1% cellulose as sole carbon source in the presence of ferulic acid at concentrations of up to 0.5% (w/v). Duplicate cultures were harvested every second day and filtrates assayed for B-glucosidase activity. Enzyme activities for cultures grown in the presence of ferulic acid were compared with parallel control cultures.

TABLE 4.3 PRODUCTION OF LIGNOCELLULOSE DEGRADING ENZYMES

| | ORGANISMS | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J |
| Phenol oxidase | + | - | - | + | + | - | - | - | - | + |
| Cellulases | + | + | + | + | + | + | (+) | + | + | + |
| Ligninases[1] | + | - | - | + | + | - | - | - | - | - |

Key to table;

A = *P. chrysosporium*, B = *C. cellulolyticum*, C = *T. reesei* , D = *C. versicolor*, E = *P. sajo-caju* , F = *T. harzianum* , G = *A. corymbifera* , H = *A. terreus*, I = *A. fumigatus* , J = *S. lignicola*.

+ indicates presence, - indicates absence of enzyme activity.

[1]Ligninases assayed as ability to decolourise polymeric dyes.

TABLE 4.4 TEMPERATURE OPTIMA FOR CELLULOSE DEPOLYMERISATION BY FIVE CELLULOLYTIC FUNGI

| ORGANISM(S) | TEMPERATURE ($^{o}$C) | | |
|---|---|---|---|
| | 40 | 50 | 60 |
| *P. chrysosporium* | 16.5* ± 0.5 | 18.0 ± 0.5 | 14.5 ± 0.00 |
| *C. versicolor* | 13.5 ± 0.25 | 15.0 ± 0.5 | 12.0 ± 0.5 |
| *C. cellulolyticum* | 14.5 ± 0.5 | 15.5 ± 0.5 | 13.5 ± 0.5 |
| *T. harzianum* | 16.5 ± 0.00 | 17.5 ± 1.0 | 12.5 ± 0.5 |
| *P. sajo-caju* | 13.0 ± 0.5 | 14.5 ± 0.25 | 11.5 ± 0.5 |

* Average zones of clearance in mm from two replications.

The results shown in Fig. 4 .1, clearly demonstrate that the presence of ferulic acid in the media reduced the level of β-glucosidase activity in *C. cellulolyticum*, *P. chrysosporium* and *T. harzianum* in culture supernatants. In contrast, 0.05% ferulic acid stimulated B-glucosidase activity between two- and threefold with *C. versicolor* and *P. sajo-caju* whereas at 0.5% the phenolic compound significantly repressed β - glucosidase activity.

b. Saccharification of cellulose

Extracellular enzyme was obtained from cultures grown for up to 14 d on cellulose powder. Glucose produced by each culture supernatant in a 24 h hydrolysis of absorbent cotton wool was estimated using glucose oxidase (Fig. 4.2). Three strains (*T. harzianum*, *C. cellulolyticum* and *P. chrysosporium*) exhibited between two- and four-fold suppression of saccharification in the presence of ferulic acid. The other two strains showed normal levels of saccharification except in the presence of 0.5% ferulic acid, where the activity was suppressed.

Estimations of initial depolymerising activity, typical of hydrolytic cellobiohydrolases, for each culture supernatant, assayed as reducing sugar released from filter paper cellulose during a 60-min incubation, are shown in Fig. 4.3. This enzymic activity appeared to be stimulated by increasing concentrations of ferulic acid in each of the fungi. A maximum 3 -fold stimulation

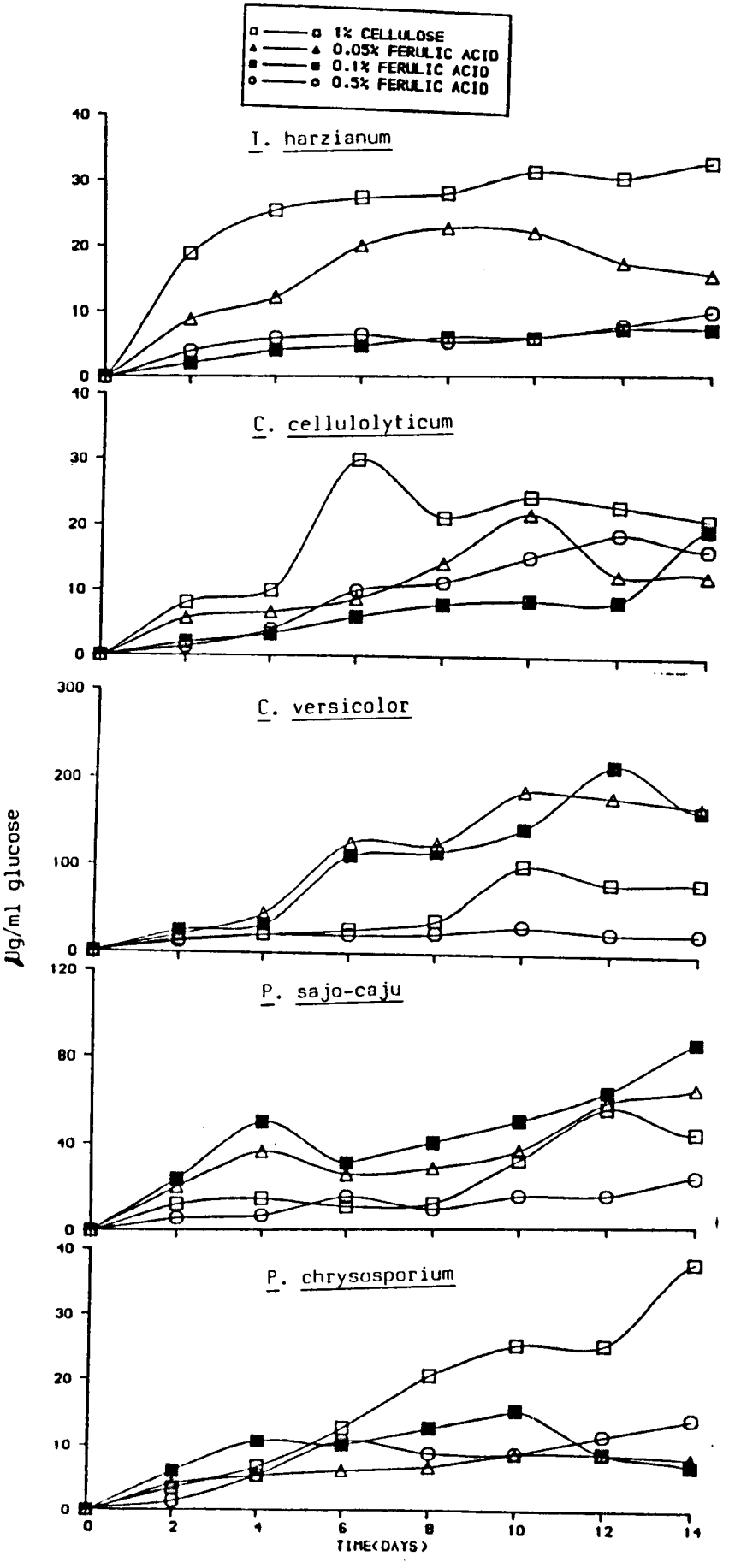



Fig. 4.1 Effect on β-glucosidase activity of addition of ferulic acid to cultures of five cellulolytic fungi growing on cellulose.

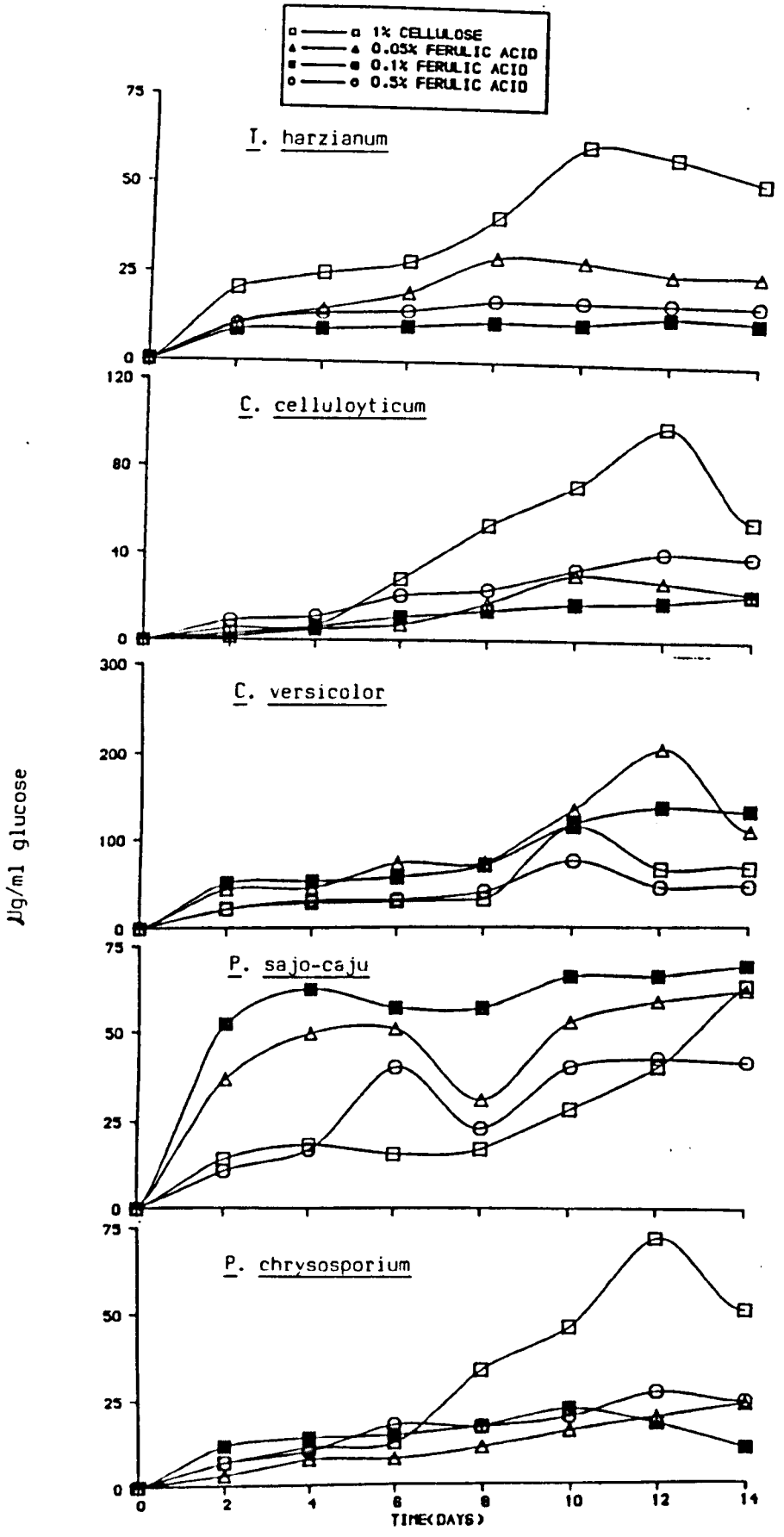



Fig. 4.2 Effect on total cellulose saccharifying enzymes of addition of ferulic acid to cultures of five cellulolytic fungi growing on cellulose.

was observed with *C. versicolor* after 10 days.

Values for endoglucanase activity, determined as carboxymethylcellulose hydrolysis, are shown in Fig. 4.4. Significant inhibition of CMCase activity was observed after the 6th day of growth of *T. harzianum*, *P. chrysosporium* and *C. cellulolyticum* whereas moderate concentrations of ferulic acid (<0.1%) stimulated CMCase activity in *C. versicolor* throughout the period of study. The pattern observed with *P. sajo-caju* was more complex since after 8 days of growth the control showed a twofold stimulation which was not observed in cultures grown with ferulic acid yet the presence of this compound at 0.5% gave a stimulation of CMCase activity throughout the growth period.

d. Production of extracellular xylanases

Growth on oat-spelt xylan (0.5% w/v) in solid media was assessed over a 7-day period and xylanase activities were estimated by congo red - polysaccharide interaction technique. It was observed that with *P. chrysosporium*, *T. harzianum* and *C. cellulolyticum* ferulic acid at 0.5% repressed production of extracellular xylanases whereas only slight inhibition was observed with *C. versicolor* and *P. sajo-caju* (Fig. 4.5). Ferulic acid at 0.1% appeared to significantly repress xylanase production in *T. harzianum*, *P. chrysosporium* and *C. cellulolyticum*.

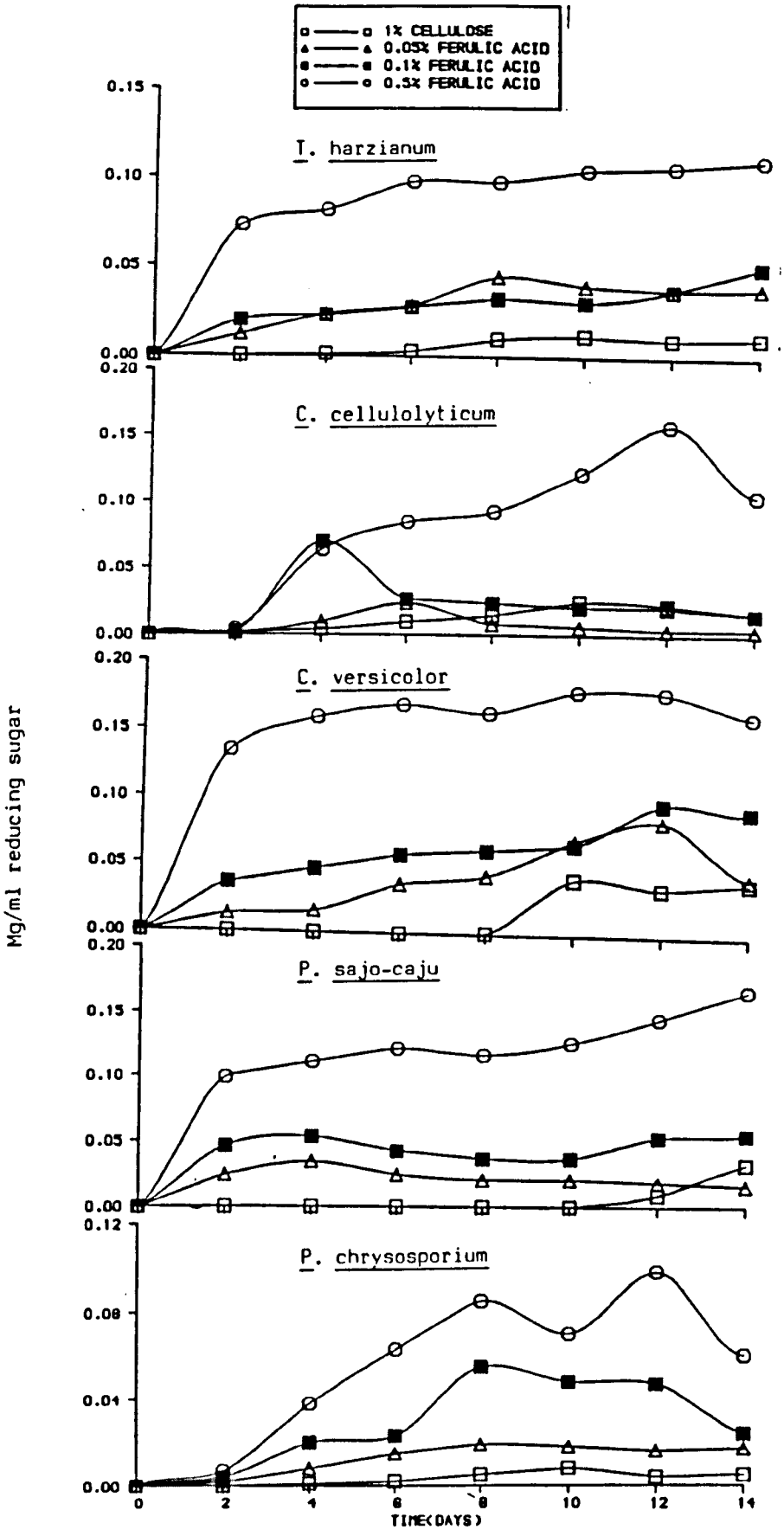



Fig:4.3 Effect on initial crystalline cellulose depolymerising activity of addition of ferulic acid to cultures of five cellulolytic fungi growing on cellulose.

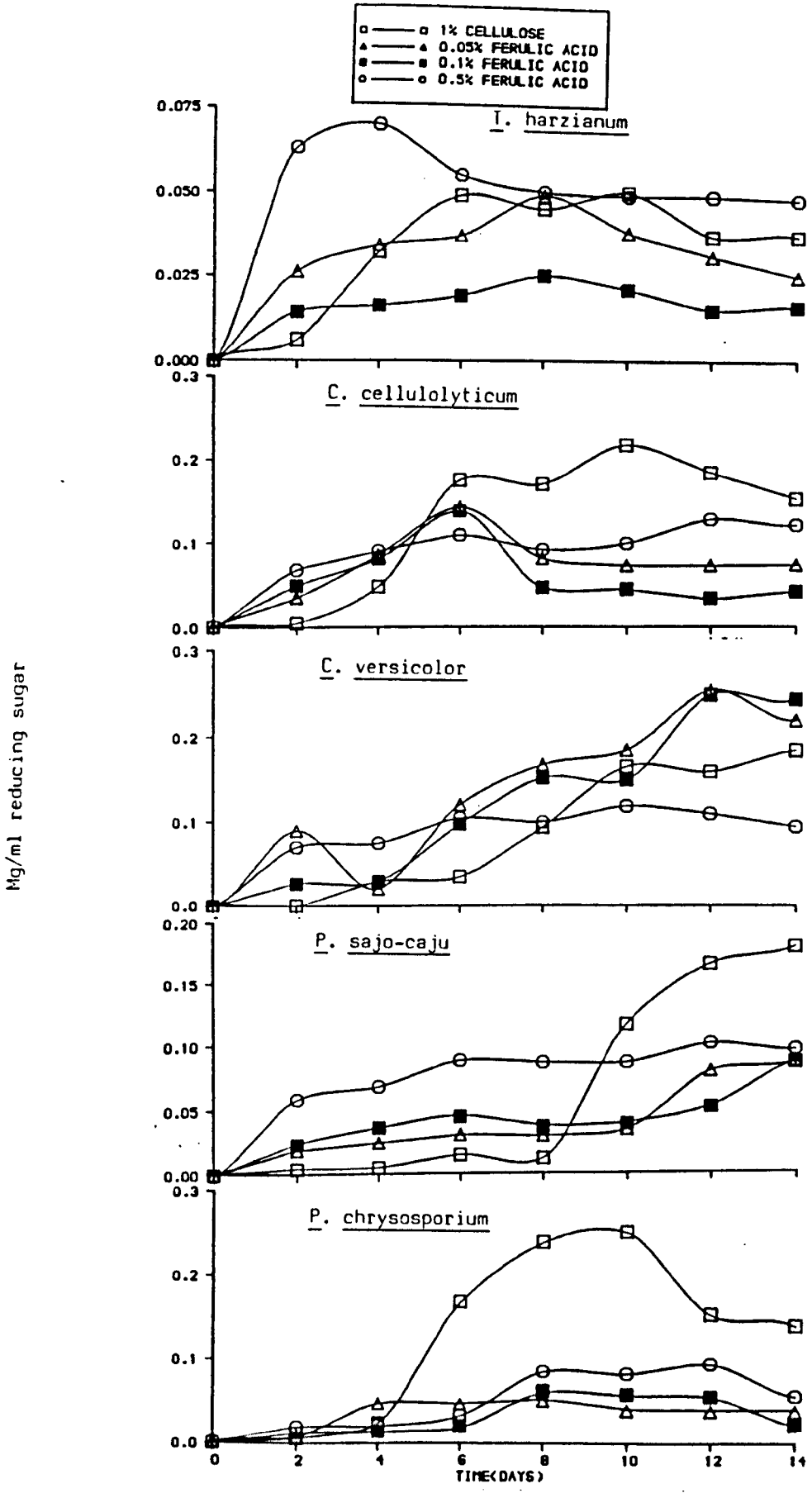



Fig. 4.4 Effect on CMC-hydrolysing (endoglucanase) activity of addition of ferulic acid to cultures of five cellulolytic fungi growing on cellulose.

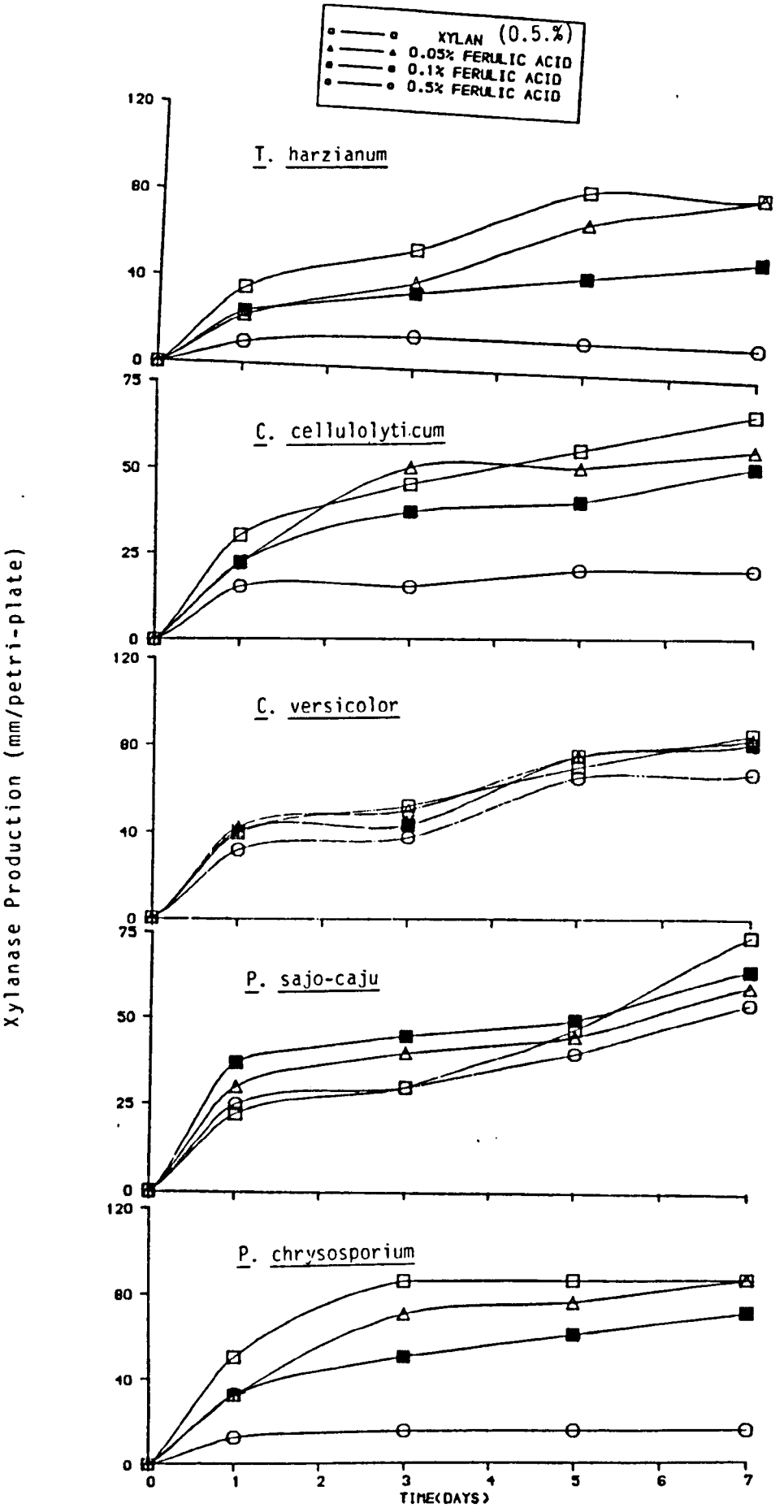



Fig. 4.5 Effect on xylanase production of addition of ferulic acid to cultures of five xylanase secreting fungi growing on xylan.

e. Total soluble sugars

High levels of soluble sugar accumulated in culture supernatants growing in the presence of significant concentrations of ferulic acid (Fig. 4.6). Only trace quantities of reducing sugar were detected in supernatants of control cultures of *P. sajo-caju*, *C. versicolor* and *T. harzianum* grown for up to 8 days. In cultures of these organisms grown in the presence of ferulic acid, reducing sugars accumulated in a direct relationship with increasing phenolic concentration, without any lag phase. In *P. chrysosporium* cultures, following a distinct lag phase, after 8 days of growth cultures containing 0.5% ferulic acid showed a fivefold increase in sugar accumulation over control cultures. In *C. cellulolyticum* sugars accumulated throughout the period of the experiment.

f. Laccase activities

Extracellular laccase activities, assayed as oxidation of syringaldazine, were determined for each organism harvesting cultures every second day over a 14 day period. Only *Pleurotus sajo-caju* and *Coriolus versicolor* were able to produce detectable levels of laccase activity under the experimental conditions studied (Table 4.5). In control cultures, *C. versicolor* secreted appreciable levels of laccase, suggesting constitutive production of the activity, whereas *P. sajo-caju* filtrates exhibited no detectable activity. However,

TABLE 4.5 EFFECT OF FERULIC ACID ON LACCASE PRODUCTION[a] FROM *CORIOLUS VERSICOLOR* AND *PLEUROTUS SAJO-CAJU*

| | INCUBATION PERIOD (DAYS) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 14 |
| **1% cellulose(control)** | | | | | | | |
| *Coriolus versicolor* | 8.5 | 16.7 | 20.4 | 24.7 | 34.7 | 31.7 | 27.3 |
| *Pleurotus sajo-caju* | - | - | - | - | - | - | - |
| **0.05% ferulic acid[b]** | | | | | | | |
| *C. versicolor* | 55.0 | 67.33 | 109.0 | 96.0 | 42.7 | 96.5 | 126.7 |
| *P. sajo-caju* | 1.0 | 6.8 | 37.4 | 68.1 | 124.0 | 165.3 | 166.7 |
| **0.1% ferulic acid[b]** | | | | | | | |
| *C. versicolor* | 36.7 | 83.3 | 157.0 | 87.3 | 44.5 | 44.0 | 73.3 |
| *P. sajo-caju* | 2.0 | 12.0 | 21.0 | 42.0 | 92.0 | 82.0 | 110.0 |
| **0.5% ferulic acid[b]** | | | | | | | |
| *C. versicolor* | - | - | - | - | - | - | - |
| *P. sajo-caju* | - | - | 0.35 | 0.46 | 0.60 | 0.50 | 1.0 |

[a] One unit of laccase activity defined as that which will produce a change in absorbance of 0.001 per min at 525 nm (Enzyme activity is in units).

[b] Experimental flasks contained the phenolic in addition to 1% cellulose in liquid culture.

All values are means of two replications.

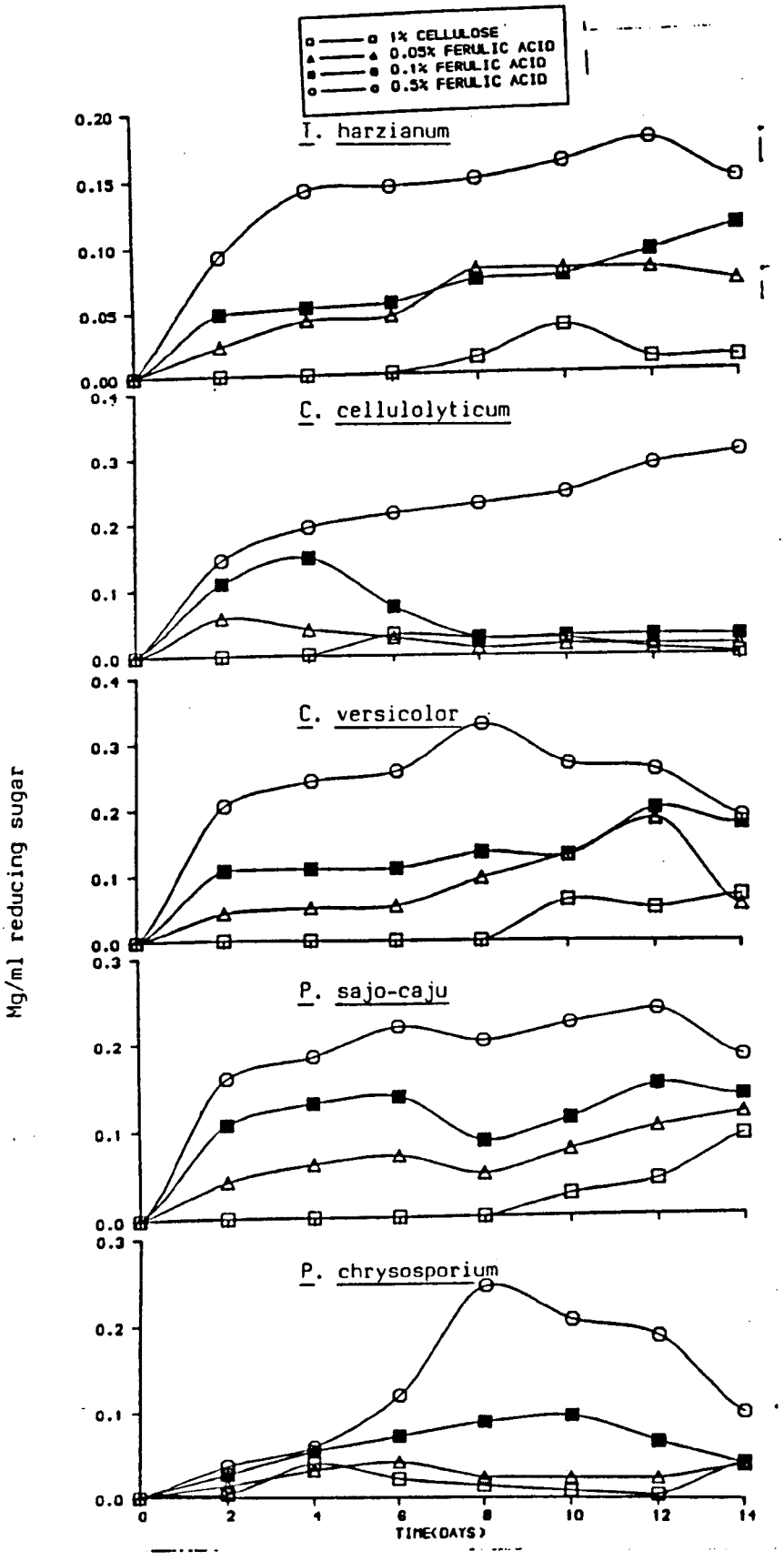



Fig. 4.6 Effect of addition of ferulic acid to cultures of five cellulolytic fungi growing on cellulose: accumulation of sugars in media.

during growth in the presence of 0.05% ferulic acid, *P. sajo-caju* secreted a laccase activity which increased with time, reaching a maximum at 14 days. This maximum was also at 14 days in 0.1% ferulic acid. In contrast, growth with 0.5% ferulic acid yielded laccase activities reduced approximately two orders of magnitude below those observed with 0.1% ferulic acid.

During growth in the presence of 0.05% and 0.1% ferulic acid *C. versicolor* apparently exhibited cycles of activity with a periodicity of approximately 6-8 days. No laccase activity was detected in the presence of 0.5% ferulic acid.

g. Secretion of ligninases

Decolourisation of Remazol brilliant blue R was used as a convenient method of quantifying ligninase activity. Only three of the fungi ; *C. versicolor, P. sajo-caju* and *P. chrysosporium.* showed significant levels of dye decolourisation. In Fig. 4.7 is shown the effect of ferulic acid on ligninase activities over a seven-day period during growth of fungi on minimal medium containing 1% cellulose and 0.2% ammonium dihydrogen phosphate as nitrogen source. Each of the three organisms showed significant levels of ligninase production. Analyses of surface cultures of *C. versicolor* and *P. sajo-caju* over the 7-day period showed that ferulic acid at 0.05 and 0.1% had a slight stimulatory effect on dye decolourisation whereas at 0.5% the

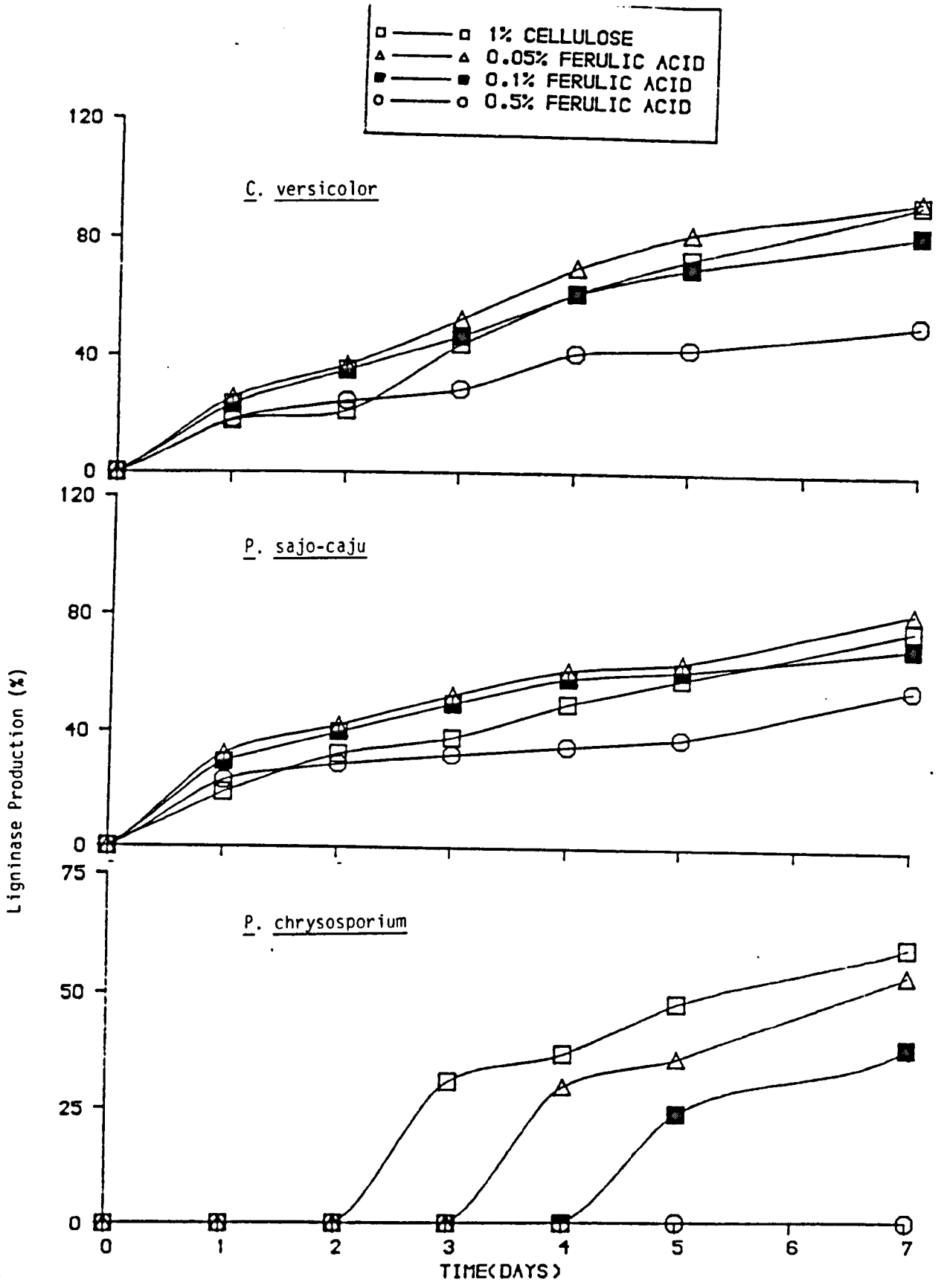



Fig. 4.7 Effect on lignase production of addition of ferulic acid to cultures of three lignolytic fungi growing on cellulose.

phenolic compound markedly inhibited the activity. With *P. chrysosporium*, 0.05 and 0.1% ferulic acid increased the lag phase prior to ligninase secretion, in proportion to concentration. In the presence of 0.5% ferulic acid, ligninase production was completely repressed.

h. Biomass Production

Biomass production, estimated as total protein, was determined every second day over a 14-day period, during growth on crystalline cellulose in the presence of ferulic acid. It was observed that growth of all five fungi was severely repressed by 0.5% ferulic acid (Fig 4.8). In contrast, 0.05% ferulic acid appeared to stimulate growth in *P. sajo-caju*, *C. versicolor* and *T. harzianum*. In *P. chrysosporium* and *C. cellulolyticum* increasing ferulic acid concentration resulted in decreased biomass production.

### 4.3.5 Ligninase production in the presence of alternate carbon sources

The three ligninase secreting organisms were grown on agar plates containing 1 mg $ml^{-1}$ Remazol brilliant blue and either 1% glucose, 1% xylose, 1% D(+) glucosamine or 1% yeast extract (each w/v). The latter two substrates were chosen to simulate autolysis of fungal cells. The progress of dye decolourisation is summarised in Fig. 4.9.

The presence of yeast extract suppressed dye

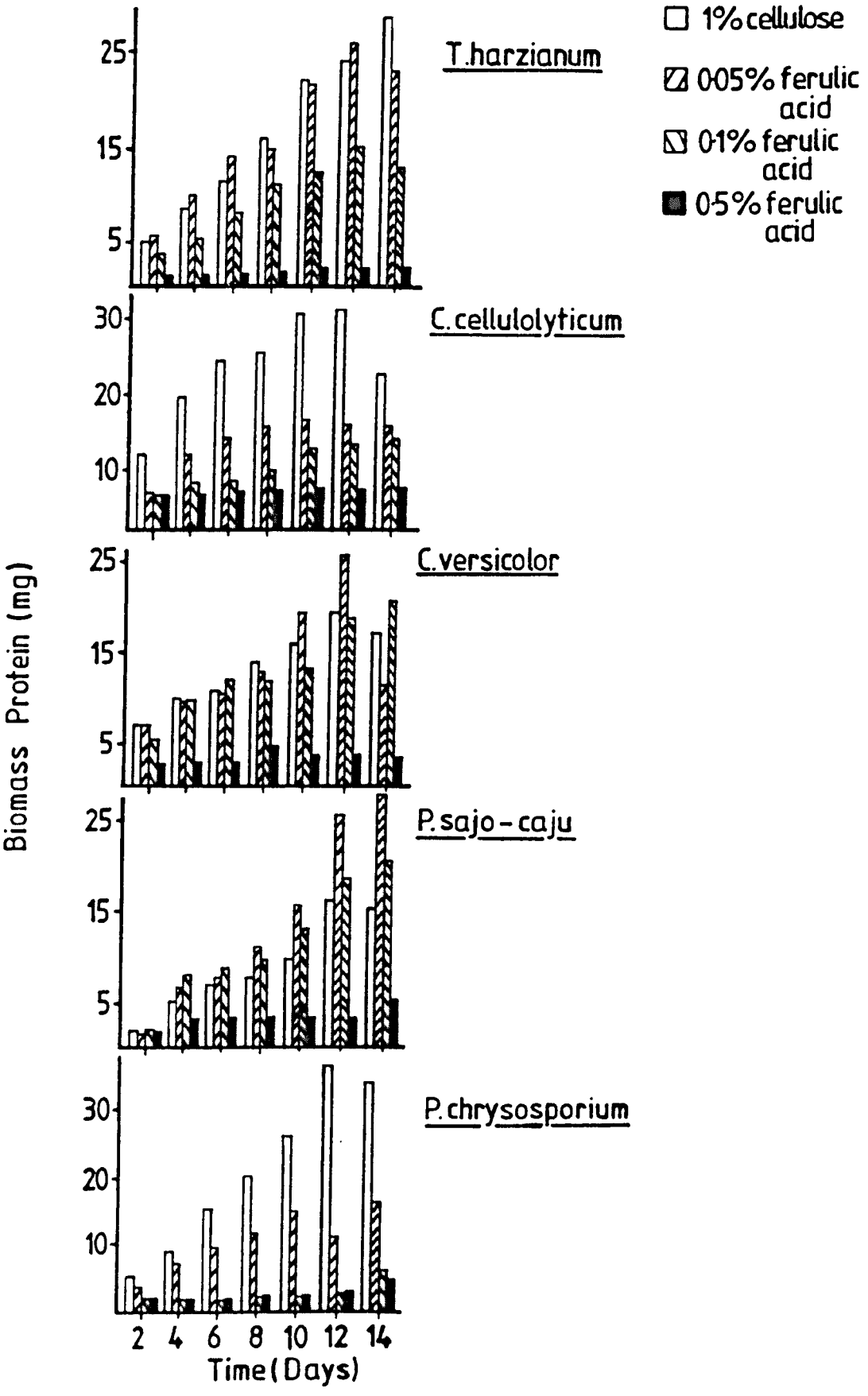



Fig. 4.8 Effect on biomass protein accumulation of addition of ferulic acid to cultures of five cellulolytic fungi growing on cellulose.

decolourisation in each organism, although *C. versicolor* was released from this repression by day 4 of the incubation. Glucosamine totally repressed dye decolourisation by *P. chrysosporium*, had no significant effect on *P. sajo-caju* but a small significant effect on *C. versicolor*. In contrast, neither glucose or xylose, the end-products of cellulose or hemicellulose saccharification appeared to significantly repress dye decolourisation.

### 4.3.6 Secretion of ligninases in the presence of varying concentrations of the nitrogen source $NH_4H_2PO_4$

The experiment was repeated using minimal medium containing 0.2% $NH_4H_2PO_4$ and 0.1 mg $ml^{-1}$ Remazol blue in the presence or absence of cellulose in the medium (Fig. 4.10). The results obtained suggested that ligninases were secreted by *P. sajo-caju* and *C. versicolor* at similar levels whether or not the nitrogen source was added to the media and in the presence of crystalline cellulose. In contrast, with *P. chrysosporium* it was observed that addition of the nitrogen source resulted in a 2-day delay in the onset of ligninase production followed by repression of the level of ligninase activity throughout the period of study (Plate 4.4 -5).

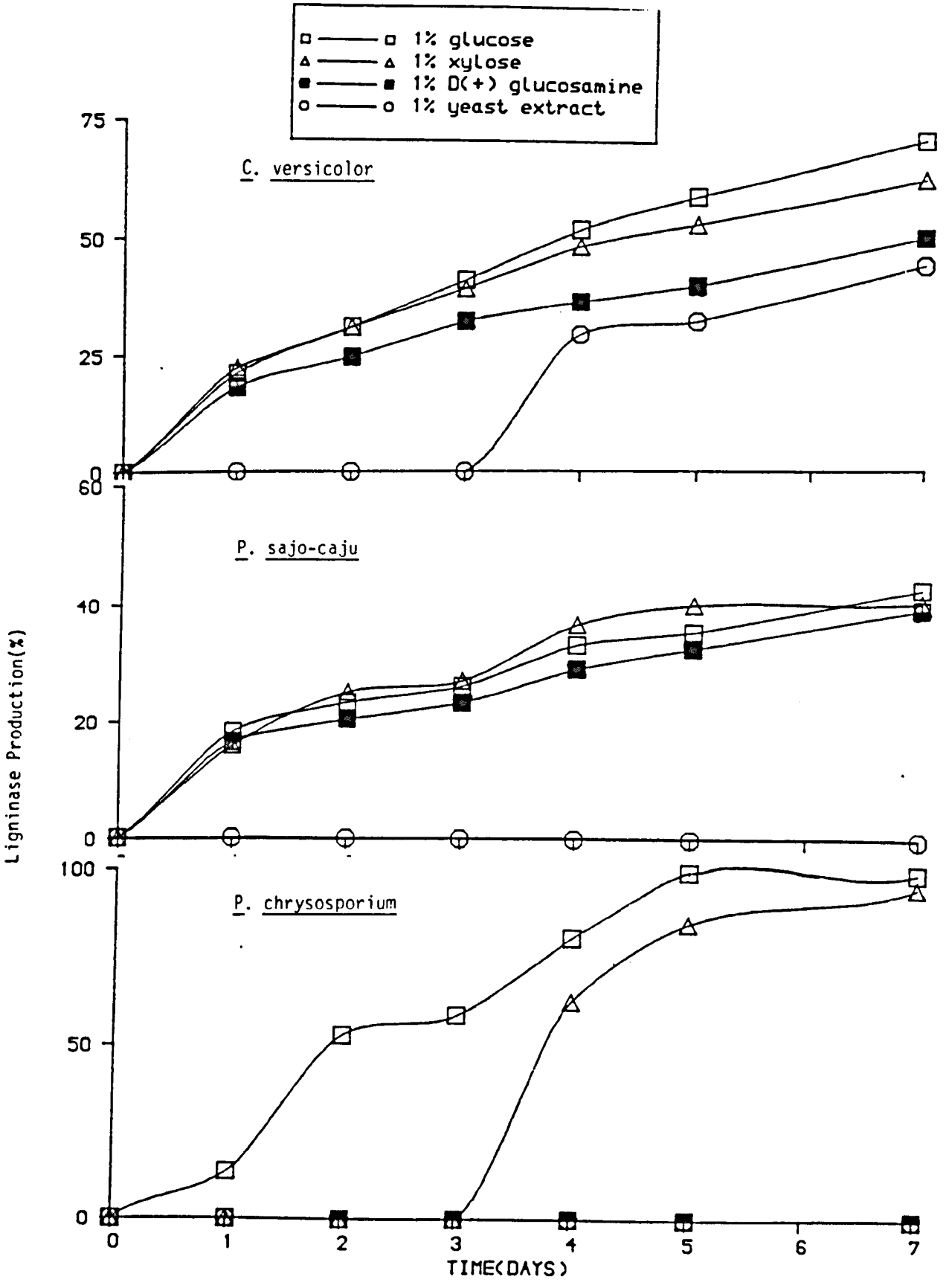



Fig. 4.9 Effect on ligninase production of addition of alternate carbon and nitrogen source to cultures of three lignolytic fungi growing on agar.

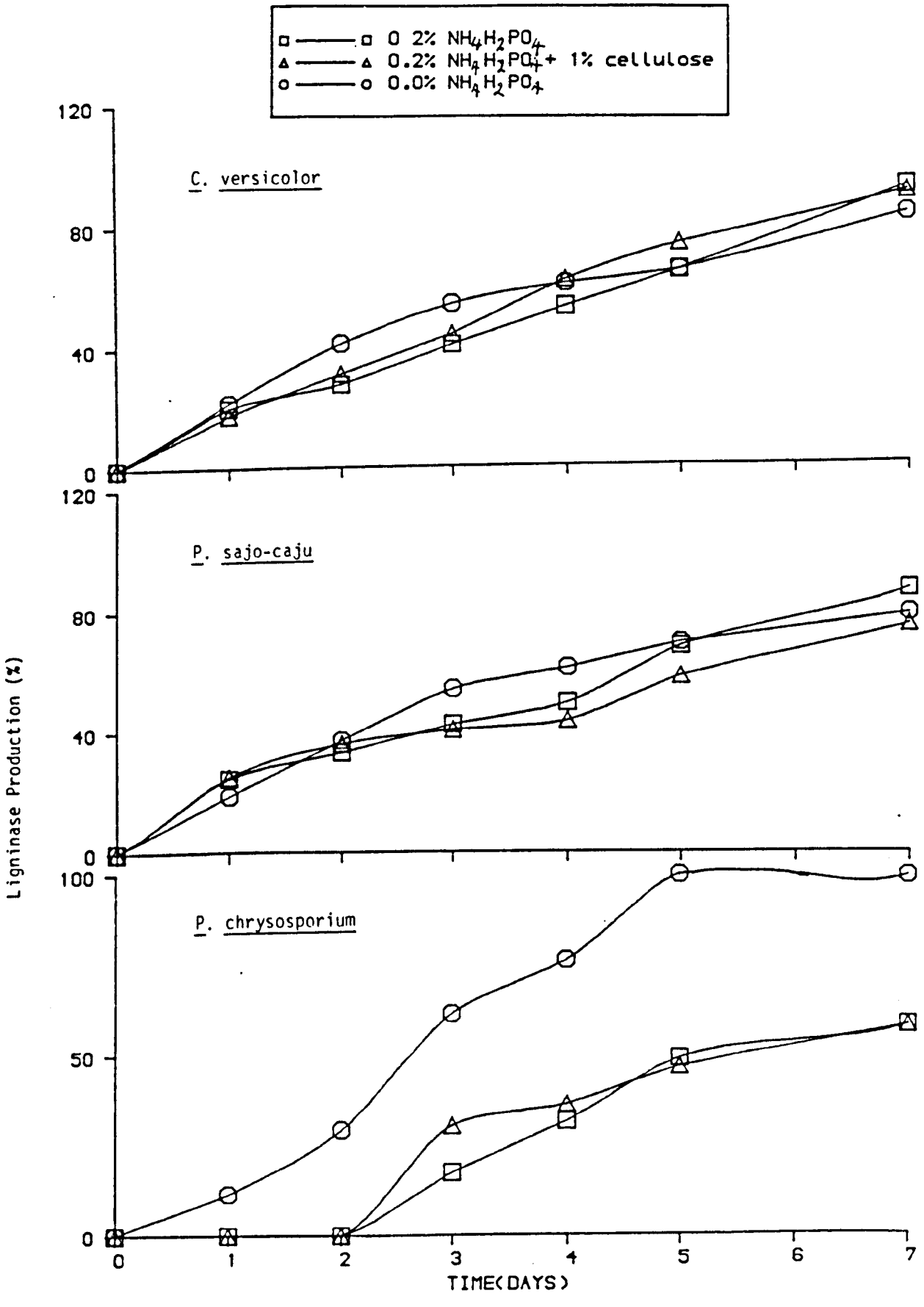



Fig. 4.10 Effect on ligninase production of addition of either cellulose or ammonium di-hydrogen phosphate to cultures of three lignolytic fungi.

## 4.4 : DISCUSSION

The results outlined in this section suggest that fungi differ in their response to concentration of ferulic acid and other phenolic compounds in lignocellulosic plant residues. Other workers (Harkin & Obst, 1973; Dalton & Stirling, 1982; Eriksson *et al.*, 1983) have reported that significant lignin-degrading activity in an organism is not necessarily accompanied by the ability of the organism to take up and utilise lignin breakdown products and related compounds as sole carbon source. Moreover it is clear that many phenolic compounds are toxic to fungi. Field and Lettinga (1989) demonstrated that the initial products of lignin monomer oxidation are toxic whereas polymerised products are non toxic. However, Nazareth and Mavinkurve (1987) screened a range of fungal isolates for their ability to utilise lignin model compounds as sole carbon source and found that benzoic and cinnamic acid, and cinnamaldehyde were extremely toxic to all the fungi whereas p-hydroxybenzoic and ferulic acids and catechol were widely utilised. They also recorded that most of the isolates demonstrated the ability to catabolise guaiacol.

Fungi may be able to grow on agar containing an inhibitory phenolic compound by detoxifying the medium prior to growth (utilising agar as the carbon source), either by breaking down or polymerising the soluble compound, or alternatively fungal hyphae may secrete

enzymes that modify phenolics to yield compounds that can be taken up by the organism. However production of phenol oxidases is not necessary for growth in media containing ferulic acid (Table 4.1). Harkin and Obst (1973) showed that a white rot fungus *Crytoderma yamanoi*, with the ability to degrade the lignin component of wood faster than polysaccharide, gave a negative Bavendamm reaction for extracellular phenol oxidases with syringaldazine. Liwicki *et al.* (1985) isolated and screened several phenol oxidase negative mutants of *P. chrysosporium* for lignin degrading ability and concluded that several mutants showed lignolytic activity similar to that of the wild type. Thus it does not seem that phenol oxidase activity, as detected by the o-anisidine plate test, is necessary for lignin degradation.

The temperature optima for cellulose depolymerisation by crude mixtures of extracellular enzymes in each culture filtrate was 50°, significantly higher than the growth maxima for each organism. Although it could be argued that this is not necessarily the temperature optima for each class of enzyme involved in saccharification, it is clear that breakdown of insoluble crystalline cellulose is the rate-limiting step in this process. Thus this temperature seemed appropriate for further assays of cellulolytic enzymes. Strains were chosen for detailed study on the basis of secretion of depolymerising cellulases and phenol oxidases to provide insight on whether ferulic acid and the products of its

oxidation inhibited, or relieved the inhibition of cellulose depolymerisation.

In the analysis of the effect of the presence of ferulic acid on cellulolytic activities of fungi, a variety of patterns was observed. Although it is possible to assay individual cellulase enzymes using specific chromophore substrates (Claeyssens *et al.*, 1989) it is difficult, and time consuming, to assign activities for a wide range of fungal exo- and endoglucanases simultaneously. Furthermore, since a significant proportion of the enzymes will be retained in biomass and on substrates, quantifying cellulolytic activities in supernatants of fungal cultures, grown on cellulosic substrates, is problematic. However, relative activities can be estimated, assuming that enzymes secreted by different organisms are likely to have similar binding constants. Ander and Eriksson (1977) showed that *P.chrysosporium*, *Phanaerochaete* sp L-1 and *Polyporous dichrous* produced high levels of endo 1,4 B-glucanase in agitated batch culture on cellulose and low levels of phenol oxidase with wood meal. In contrast, *Merulius tremellosus*, *Phlebia radiata* and *Pleurotus ostreatus* produced low levels of endo 1,4 β-glucanase in growth on cellulose and high levels of phenol oxidase activity with wood meal. On the other hand Eriksson (1978) reported that addition of phenolic compounds appeared to repress hydrolytic polysaccharidases in *P. chrysosporium* culture supernatants. Subsequently, Eriksson *et al.* (1983)

reported that addition of exogenous laccase to cultures growing on cellulose, in the presence of vanillic acid, resulted in a stimulation of endo-β-1,4-glucanase activity. Sztejnberg *et al* (1989) investigated the effect of tannins and phenolic extracts from plant roots on the production of cellulase and polygalacturonase(PG) by *Dermatophora necatrix* and concluded that both tannins and phenols decreased cellulase production *in vitro* whereas polygalacturonases were inhibited only by tannin extracts.

Spencer and Tower (1989) reported the induction of virulence among strains of *Agrobacterium tumefaciens* by certain phenolic compounds, including ferulic and syringic acids and monolignols. These authors suggested that the presence of these compounds may trigger the production of bacterial enzymes hydrolysing the plant cell wall.

In this study it was observed that the two fungi which secreted laccases that could oxidise ferulic acid, *P. sajo-caju* and *C. versicolor*, showed stimulation of both depolymerising cellulase (Fig. 4.3) and β-glucosidase (Fig. 4.1) activities. In contrast, fungi unable to secrete laccases exhibited repression of B-glucosidase, total glucanase and endo-glucanases production (except T. harzianum) whilst initial cellulose depolymerisation activities appeared to be stimulated. A similar observation was reported for *Trametes versicolor* by Muller *et al.* (1988) who concluded that

cellobionolactone from oxidative attack on carbohydrate was responsible for this stimulation. It can be concluded that it is such relationships between hydrolytic and oxidative activities that may be important in lignocellulose breakdown. In *P.chrysosporium* a range of cellobiose-quinone oxidoreductases and cellobiose oxidases are induced during growth on lignocelluloses and similar oxidative cellulolytic enzymes may be secreted by other ligninolytic fungi.

Quantification of sugars in the culture supernatants suggested that, in the five fungi studied soluble sugars accumulated in parallel with increasing ferulic acid concentrations. In all five organisms, the highest concentrations of ferulic acid was inhibitory to growth whereas at 0.05% no significant accumulation of sugars over controls was observed.

It was clear that under normal conditions the extracellular glycolignin-degrading enzymic activities increased linearly with growth. An exception was laccase production in submerged culture of the two organisms *Pleurotus sajo-caju* and *Coriolus versicolor* which showed a cyclical pattern of activity. Such physiological patterns were not observed for ligninase production in surface culture, although cyclical production of ligninases in liquid culture has been observed in *P.chrysosporium* (Janshekar & Fiethter, 1988). Smith *et al.* (1989) have reported that during growth of *Agaricus bisporus* in solid substrate culture, cyclical production

of laccase at various depths below the casing layer was observed. In *Aspergillus nidulans*, the correlation between laccase production and morphogenesis, particularly sporulation, has been extensively studied (Law & Timberlake, 1980).

Patterns of laccase production differed markedly between *C. versicolor* and *P. sajo-caju*. It has been shown that *C. versicolor* secretes both constitutive and inducible laccases (Fahraeus *et al.*, 1958; Evans *et al.*, 1984). In this study, enhancement of laccase activity above a basal level was observed in parallel with increasing level of ferulic acid in liquid culture. Thus constitutive production of laccase is enhanced by induction of further laccase activity by ferulic acid. However, at the highest concentration of ferulic acid, laccase activity was absent and production of biomass inhibited, presumably through toxicity of the phenolic compound. Such toxicity may either cause specific repression of laccase synthesis since no detectable extracellular laccase activity was observed in liquid cultures containing 0.5% ferulic acid or a more general repression of metabolic activities. However laccase activity in *P. sajo-caju* was induced by ferulic acid at 0.05% and 0.1% and repressed at 0.5% and no constitutive enzyme activity was detected in the absence of the phenolic inducer.

Dye decolourisation through peroxidase activity in fungi growing in surface culture on agar plates has been

reported to be mediated by Mn3+ ions produced by ligninase action (Glenn & Gold, 1985). It is now known that a minimum of two classes of lignin peroxidase are involved in lignin depolymerisation. Both ligninases, which contain single protohaem IX prosthetic groups (Palmer *et al.*, 1987), and manganese-dependent peroxidases, with protoporphyrin IX prosthetic groups (Paszczynski *et al.*, 1986) are observed in *P.chrysosporium* culture filtrates. Laccases have been shown to be oxidised both by $O_2$ and $H_2O_2$ (Reinhammar, 1984). Both laccases and ligninases are secreted by *C.versicolor* during attack on lignins (Evans *et al.*, 1984) but only ligninases appear in culture filtrates of *P.chrysosporium*. In these studies it was not possible to distinguish between the different classes of ligninolytic enzymes using dye decolourisation. However, it is possible that differences in physiological role may be slight since both enzymes oxidise phenolic substrates and the basidiomycete *Agaricus bisporus* has been shown to mineralise lignin and secretes laccases, but has not been reported to secrete enzymes similar to the extracellular ligninases of *P. chrysosporium*. The balance between activities may be shifted by concentrations of oxygen and hydrogen peroxide available for enzyme reoxidation reactions (Evans, 1985). Thus it would be predicted that differences would be observed with the same organism under differing conditions such as submerged liquid and solid surface culture.

In solid surface culture, ferulic acid in media was observed to repress ligninase production by *P. chrysosporium* as assessed by dye decolourisation (Fig 4.7). The presence of ferulic acid at 0.5% resulted in total inhibition of ligninase activity whereas at lower concentrations the onset of lignolytic activity was delayed in relation to ferulic acid concentration. In contrast, the other two lignolytic fungi exhibited stimulation of ligninase activity at both 0.05% and 0.1% ferulic acid, with only moderate repression at the higher concentration. Glenn and Gold (1983) reported that a variety of inhibitors of lignin degradation including thiourea, azide and 4-O-methylisoeugenol also repressed polymeric dye decolourisation. They also observed that the pleiotropic 104-2 mutant of *P. chrysosporium* which lacked phenol oxidase and lignolytic activity was not able to decolourise dyes, whereas a phenotypic ligninolytic revertant strain 424-2 had regained this capacity.

The absence of any inhibition of xylanase production by *C. versicolor* and *P. sajo-caju*, in the presence of ferulic acid, may be attributed to the detoxification action of the extracellular laccase with these strains. Analysis of biomass production in the five organisms studied, showed growth was inhibited with increasing ferulic acid concentration. Three organisms (*P. sajo-caju*, *C. versicolor* and *T. harzianum*) showed enhanced biomass production in the presence of 0.05% ferulic acid.

In the other two organisms, *P. chrysosporium* and *C. cellulolyticum* ferulic acid was inhibitory to growth at all concentrations.

# SECTION 5

## 5 : CHARACTERISATION OF ENVIRONMENTAL FACTORS LIMITING GROWTH OF FUNGI ON SURFACE AGAR CULTURE

### 5.1 Preamble

Environmental factors such as pH, moisture content or water activity, temperature and type of substrate determine, in part, the rate of growth and biomass production by filamentous fungi in any ecosystem.

Magan and Lacey (1984 a & b ) observed that pH can affect metabolic processes including sporulation and morphogenesis (Gottlieb, 1978). Von Schelhorn (1950) reported that *Aspergillus* grew at lower water activities at pH 7 than at either pH 3 or 5. However, Pitt (1975) concluded that pH values of between 4 and 6.5 were equally suitable for growth of a number of moulds at low water activities.

Water activity is strongly correlated with fungal growth. Pitt (1979) concluded that growth of most unicellular organisms ceases at water activities below 0.90. Similarly, enzyme activities are influenced by water activity. Lack of free water in a substrate will limit both range of micro-organisms and rate of growth. Pitt (1979) suggested that ascomycetous fungi are the only moulds widely able to grow below 0.90 $a_w$.

The objectives were to simulate the conditions of solid substrate cultures on lignocelluloses and study the

growth of the test fungi under varying experimental conditions.

## 5.2 : RESULTS

### 5.2.1 Effect of pH on fungal growth

Analyses of growth of the five test fungi in surface agar culture at a range of pH values showed that optimal pH for growth of *P. chrysosporium*, *C. versicolor*, *P. sajo-caju* and *T. harzianum* was between 4.5 - 5.5 whereas *C. cellulolyticum* grew best between pH 5.5 and 6.5 (Table 5.1). At pH values lower than 4.5, growth of *C. versicolor*, *P. sajo-caju* and *C. cellulolyticum* was retarded while *T. harzianum* and *P. chrysosporium* were still able to grow normally.

### 5.2.2 Effect of pH on biomass production

At pH values near neutrality, biomass production was repressed except for *P. chrysosporium*, *T. harzianum* and *C. cellulolyticum*. Altering the pH from 7.0 to 5.0 enhanced mycelial weights in *P. chrysosporium* and *C. versicolor* (Table 5.2). At pH 6.5, increases were recorded for the other three strains.

### 5.2.3 Effects of water activity and temperature on fungal growth

Growth of fungi increased in parallel with water activity (Table 5.3). At 0.98 $a_w$, temperature had a

Table 5.2 EFFECT OF pH ON MYCELIAL WEIGHT(mg)[a]

| pH[b] | FUNGI | | | | |
|---|---|---|---|---|---|
| | *Phanaerochaete chrysosporium* | *Coriolus versicolor* | *Pleurotus sajo-caju* | *Chaetomium cellulolyticum* | *Trichoderma harzianum* |
| 7.0 | 7[c] | 0 | 0 | 30 | 61 |
| 6.5 | 98 | 35 | 14 | 48 | 114 |
| 6.0 | 187 | 58 | 5 | 43 | 82 |
| 5.5 | 189 | 59 | 5 | 42 | 77 |
| 5.0 | 199 | 72 | 6 | 40 | 75 |
| 4.5 | 157 | 70 | 6 | 40 | 73 |
| 4.0 | 151 | 58 | 6.5 | 26 | 70 |
| 3.5 | 190 | 33 | ND | 14 | 70 |

All values are means of two replcations

[a]Incubated for five days.

[b] pH was adjusted using 0.2M sodium phosphate or citrate phosphate buffer.

[c]Mycelial weight; cultures on agar were steamed filtered with scintered glass crucible (porosity 1) and dried for 24 h at 75° C.

Table 5.3 EFFECT OF WATER ACTIVITY AND TEMPERATURE ON FUNGAL GROWTH(mm) - HYPHAL EXTENSION.

| Temp. (°C) | ORGANISM[a] *Phanaerochaete chrysosporium* | *Coriolus versicolor* | *Pleurotus sajo-caju* | *Chaetomium cellulolyticum* | *Trichoderma harzianum* |
|---|---|---|---|---|---|
| $0.90a_w$ | | | | | |
| 25 | 0 | 0 | 0 | ND | ND |
| 30 | 4 | 0 | 0 | 3 | 4 |
| 40 | 7 | 0 | 0 | 0 | 0 |
| $0.95a_w$ | | | | | |
| 25 | 5 | 6 | 0 | 3.5 | 21.75 |
| 30 | 5 | 7 | 0 | 6 | 28 |
| 40 | 7 | 0 | 0 | 11 | 0 |
| $0.98a_w$ | | | | | |
| 25 | 38 | 56.5 | 16 | 33 | >85 |
| 30 | 54 | 56 | 15 | 51 | >85 |
| 40 | 78 | 0 | 0 | >85 | 0 |

All values are average of two replications

[a]Incubated for 10 days.

marked influence on growth rate. The optimal temperature for growth of *P. chrysosporium* and *C. cellulolyticum* was found to be 40°C whereas no marked differences were found with the other three strains at either 25°C or 30°C. Repeating this experiment with malt extract lignin cellulose agar growth yields of *P. chrysosporium*, *C. cellulolyticum* and *P. sajo-caju* were found to be slightly reduced (Table 5.4).

## 5.3 DISCUSSION

The five fungi were found to require a high moisture content for growth and were intolerant of water activities below 0.95. This study did not reveal marked differences in growth of *P. sajo-caju* and *C. versicolor* at 25 °C or 30 °C whereas *P. chrysosporium* and *C. cellulolyticum* grew better at temperatures above 30 °C. However each organism appeared to have a wide pH optimum for growth.

Use of solid agar media facilitated rapid evaluation of responses of organisms to differences in culture conditions. However, it is often argued that surface culture represents an imperfect simulation of solid state fermentations. Despite this, this strategy has been widely used especially in the analyses of interactions between field and storage fungi and in the study of mixed fungal communities on decaying woods (Carruthers & Rayner, 1979; Magan & Lacey, 1984b). Consequently it can

Table 5.4 GROWTH(mm)[a] OF TEST ORGANISM IN PRESENCE OF MALT EXTRACT LIGNIN CELLULOSE AGAR AT 0.98 WATER ACTIVITY.

| | ORGANISM[b] | | | | |
|---|---|---|---|---|---|
| Temp. (°C) | *Phanaerochaete chrysosporium* | *Coriolus versicolor* | *Pleurotus sajo-caju* | *Chaetomium cellulolyticum* | *Trichoderma harzianum* |
| 25 | 27 | 44 | 10 | 37 | >85 |
| 30 | 45 | 56 | 12 | 47 | >85 |
| 40 | 75 | 0 | 0 | 80 | 0 |

[a] Growth measured as hyphal extension.

[b] Incubated for 10 days

be argued that these results are likely to give some insight on the growth of fungi in solid substrate fermentations.