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

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VOLUME 2

SECTIONS 6 - 9, REFERENCES & APPENDIX

SECTION 6

6: SOLID SUBSTRATE FERMENTATION OF LIGNOCELLULOSES

6.1 Preamble

A range of basidiomycete fungi is known to be able to depolymerise lignin in lignocellulosics. Initial soluble products can then be further metabolised by extracellular enzymes or microbial communities to yield simple compounds such as sugars and phenolic compounds that can be taken up and utilised by the fungal mycelia (Buswell & Odier, 1987; Giovannozi-Sermanni *et al.*, 1989). However, normally such activities will take place in low moisture solid substrate conditions, since excess water will limit oxygen transfer. Thus solid substrate fermentations have obvious advantages over other bioconversion processes (Reid, 1989).

To elucidate whether fungi might co-operate in effecting solubilisation of natural lignocelluloses, test fungi were either grown separately or in pairs on lignocellulosic substrates. In particular it was considered that synergy between lignolytic organisms might enhance the rate of delignifications and enhancement of *in vitro* digestibility.

6.2 EXPERIMENTAL - Solid state fermentation

6.2.1 Screening for delignification of native lignocelluloses

Spruce sawdust, hay straw, barley straw , rice bran and birch sawdust were moistened with minimal medium to a moisture content of 70% , 65%, 70%, 37.5% ($0.98_a w$) and 70% respectively. Moistened substrates were equilibrated at 4 ^oC and fermented with test fungi for 14 days except where otherwise indicated.

6.2.2 Mineralization of spruce sawdust in presence of either 100% air or oxygen

Moistened pulverised spruce sawdust (4 g ; 70% M.C) was dispensed into separate 30 ml bottles, half stoppered and autoclaved at 121 ^oC for 30 min. On cooling, samples were inoculated with 1 ml of suspension of fragmented mycelia and the bottles were aseptically sealed with sterile suba seals (size 45). Inoculated bottles were subjected to the following treatments;

(1) A duplicate set of culture bottles was flushed for 1 min with sterile 100% O_2 and incubated until analysed.
(2) A second set of duplicate culture bottles was flushed every week for 1 min with 100% O_2 with exception of weeks 5 and 7.

(3) A third duplicate batch of cultures was flushed once for 1 min with sterile air with aid of an air pump followed by incubation until analysed.
(4) A fourth duplicate batch was flushed every week for 1 min with sterile air except in weeks 5 and 7.
Flushed culture bottles were made air tight by covering the top side of suba seals with silicone rubber (RS Components Ltd, Belgium ; RS 555 - 588) prior to incubation. Samples were analysed weekly for changes in CO_2 output and O_2 utilisation.

6.2.3 Supplementation with differing concentrations of ferulic acid

Spruce sawdust was moistened with minimal medium containing varying concentrations (0.05%, 0.1%, 0.5% w/v) of ferulic acid and equilibrated. This lignocellulose was fermented for 14 d.

6.2.4 Supplementation with exogenous carbon and nitrogen sources

Spruce sawdust was supplemented with 1% (w/v) concentrations of either monosaccharides (glucose, mannose, xylose, arabinose, or galactose), disaccharides (lactose, sucrose or maltose), simple nitrogen sources (ammonium dihydrogen phosphate or D- glucosamine) or complex nitrogen sources (yeast extract, mycological peptone or malt extract) in distilled water equilibrated

as described above and fermented for 42 d.

6.2.5 Supplementation with complex carbon sources

Spruce sawdust was supplemented with 5 - 30% concentrations (w/w) of rice bran, cow feed concentrate obtained from Hannah Research Institute Ayr (maize grain-10%, Palmers fish meal-10%, sugar beet- 5%, barley - 70%, megalac(p-fat) -2.5% and minerals-2.5%) and birch sawdust, moistened with basal minimal medium prior to fungal fermentation for 42 d.

6.2.6 Application of dual/multiple fungal cultures

6.2.6.1 Simulation and study of fungal interactions on surface culture

Interaction experiments were aimed towards simulating the conditions in native lignocellulosic materials. The fungi were grown alone or in pairs on ; (a) Malt extract , cellulose (0.1%) , industrial lignin (0.1%) agar , with or without water activity adjustment. (b) Spruce sawdust (1%) minimal media agar. Agar discs rich in mycelia of young cultures or water agar containing spores of test fungi were placed on the surface of agar at a distance of 30 mm from each inoculum disc (Carruthers & Rayner, 1979; Magan & Lacey, 1984a & b). The nature of mycelial interaction in culture was recorded (Appendix 3)

6.2.6.2 Interaction in solid state cultures

Fragmented mycelial suspension (1 ml each) from compatible strains were inoculated into sterile microporous bags containing 20 g each of spruce sawdust previously moistened with minimal media to a moisture content of 70%. Inoculated samples were incubated as described above at 28.5 ^{o}C.

6.2.7 Discolouration in study of delignified substrate

Discolourisation of fermented lignocellulosics from the original milk - white coloration (in case of spruce sawdust) to a reddish brown pigmentation was used as a qualitative estimation of the progress of delignification. Extent of discolouration was assessed by visual inspection and results reported based on a 5-point scale.

6.2.8 Determination of pH

Changes in pH during solid state fermentation were determined according to the method of Zadrazil and Brunnert (1980). Substrate (1 g) was suspended and thoroughly mixed with 20 ml distilled water. After 15 min the pH was measured with a glass electrode using an EIL pH meter 7010 (Electronic Instruments, Ltd, Kent).

6.2.9 Soluble sugar

Distilled water (10 ml) was added into test tubes containing 100 - 200 mg sample. Tubes were capped, placed in briskly boiling water for 60 min then cooled over running water. The contents were then filtered with Whatman filter paper and 1 ml aliquots of filtrate were used for soluble sugar assay by Somogyi (1952) method. Total reducing sugar solubilised was reported as mg g^{-1} substrate.

6.2.10 Total solubles

The optical density of filtrate from the boiled sample in section 6.2.9 was measured at 530 nm using a spectrophotometer. The soluble product includes lignin and polysaccharide degradation products and results are reported in O.D units g^{-1} substrate.

6.2.11 Total biomass

The biomass content of complex substrates was determined as total cell-wall nitrogen using the Kjeldahl method (section 2.3.4.1).

6.2.12 Application of cell free enzyme extract for saccharification of spruce sawdust

Cell -free enzyme extract of test fungi grown on minimal medium containing 1% cellulose were obtained by centrifuging cultures at 5000 X g for 30 min. The enzyme extract was buffered with 25% 0.1M citrate buffer (pH

4.8). Substrate enzyme mixture was made up in the following ratios 1 : 5 (1 part substrate : 5 parts buffered enzyme) or 1 : 10 . Treated substrates were incubated at 50^{o}C for 48 h. Mixtures of cell free enzyme extract of the test fungi were treated similarly. Control cultures were inactivated by boiling for 1 h prior to incubation.

6.3 : RESULTS

6.3.1 Viability of hyphal cells

An initial objective was to study the effects of fragmentation and storage at 4 ^{o}C on viability of fungal inocula. Results obtained (Table 6.1) showed that fragmentation of mycelia at half speed in a Waring blender for 60 seconds in the absence of storage had no effect on viability of *P. chrysosporium*, *C. versicolor*, *P. sajo-caju*. However, cold storage appeared to delay the onset of hyphal growth during the first 24 h although normal growth was observed subsequently.

6.3.2 Delignification and enhancement of digestibility of native lignocelluloses

6.3.2.1 Breakdown of lignin

The five fungi selected previously were screened for ability to delignify and enhance rumen digestibility (section 2.3.8 & 7.2) of selected lignocellulosics (Tables 6.2 and 6.3). Each organism was found to be able

TABLE 6.1 EFFECT OF BLENDING AND STORAGE AT 4 ^{o}C ON VIABILITY OF HYPHAL CELLS.

Period of fragmentation (seconds)	incubation time(h)	Period of storage at 4 ^{o}C (days) 0	7	14	28	42	56
			P.chrysosporium				
10	24	+++	+++	+++	++	+	-
	48	+++++	+++++	+++++	++++	++++	++++
	72	+++++	+++++	+++++	+++++	+++++	+++++
30	24	+++	+++	+++	+	+	-
	48	+++++	+++++	+++++	++++	++++	++++
	72	+++++	+++++	+++++	+++++	+++++	++++
60	24	+++	+++	+	+	+	-
	48	+++++	+++++	+++++	++++	++++	++++
	72	+++++	+++++	+++++	+++++	+++++	++++
			C.versicolor				
10	24	+	+	+	+	+	+
	48	+++	+++	+++	+++	+++	++
	72	++++	+++	++++	++++	++++	+++
30	24	+	+	+	+	+	+
	48	++	+++	+++	+++	+++	++
	72	++++	++++	++++	++++	++++	+++
60	24	+	+	+	+	-	+
	48	++	++	+++	+++	++	++
	72	++++	++++	++++	++++	++++	+++
			P.sajo-caju				
10	24	+	-	-	-	-	-
	48	++	+	+	+	+	+
	72	++	++	++	++	++	++
30	24	+	-	-	-	-	-
	48	++	+	+	+	+	-
	72	+++	+++	+++	++	++	+
60	24	+	+	-	-	-	-
	48	++	+	+	+	+	-
	72	+++	+++	+++	++	++	+

- =no growth, += traces, ++= low, +++=average, ++++= high, +++++= very high

TABLE 6.2 EFFECT OF FUNGAL TREATMENT ON LIGNIN (%) AND POLYSACCHARIDE CONTENT (%) OF LIGNOCELLULOSIC MATERIALS

ORGANISM(S)	SUBSTRATE(S)				
	HAY	BARLEY STRAW	SPRUCE SAWDUST	BIRCH[a] SAWDUST	RICE BRAN
Control					
NDF	58.06	83.89	90.04	nd	nd
ADL	4.15	7.22	27.26	13.55	3.14
CP	23.41	33.41	41.98	29.90	16.32[c]
NCP	15.67	24.60	20.56	24.40	nd
[b]Soluble sugar	3.85	0.533	0.121	nd	nd
C.versicolor					
NDF	57.75	67.42	88.51	nd	nd
ADL	3.97	5.71	26.98	10.44	2.76
CP	31.01	29.29	42.17	27.59	14.21[c]
NCP	14.11	18.56	10.49	16.69	nd
Soluble sugar	2.04	2.27	0.223	nd	nd
P.sajo-caju					
NDF	60.38	81.33	87.57	nd	nd
ADL	4.86	9.02	27.42	13.38	2.86
CP	29.14	32.22	38.61	26.19	12.12[c]
NCP	21.31	22.56	16.17	24.32	nd
Soluble sugar	4.07	0.548	0.161	nd	nd
P.chrysosporium					
NDF	58.24	63.15	86.36	nd	nd
ADL	5.45	5.86	26.67	11.67	4.31
CP	25.14	27.30	39.00	24.15	13.50
NCP	18.11	22.07	13.66	21.25	nd
Soluble sugar	2.16	2.85	0.299	nd	nd
T.harzianum					
NDF	67.40	73.82	93.07	nd	nd
ADL	4.59	8.26	27.15	nd	nd
CP	33.26	27.01	41.10	nd	nd
NCP	19.56	16.96	16.07	nd	nd
Soluble sugar	1.30	0.88	0.438	nd	nd
C.cellulolyticum					
NDF	63.41	76.57	93.07	nd	nd
ADL	7.06	10.91	27.08	nd	nd
CP	30.16	31.42	37.30	nd	nd
NCP	12.18	22.80	12.07	nd	nd
Soluble sugar	1.63	0.918	0.161	nd	nd

All values are average of at least two determinations

[a] Inoculated birch was incubated for six weeks. nd = not determined

[b]Soluble sugar = mg/g, [c] Polysaccharide containing α and β glucose residues

NCP= hemicellulose, CP= cellulose, ADL = lignin, nd = not determined.

to utilise the substrates as evidenced by colonisation and depletion of polymer components. Only *C. versicolor* and *P. chrysosporium* were able to effect moderate reductions in lignin contents of barley straw whereas growth of other fungi led to apparent increases in ADL values. Except with *C. versicolor*, increases in ADL were also observed following fungal fermentations of hay. In contrast, with spruce sawdust, each of the strains, with the exception of *P. sajo-caju* reduced slightly ADL values: *T. harzianum* and *C. cellulolyticum* effected less degradation than the other fungi. Depolymerisation of lignin and polysaccharides in birch sawdust and rice bran by three strains - *C. versicolor*, *P. sajo-caju* and *P. chrysosporium* - was quantified, each strain reduced ADL in birch sawdust but produced no marked effect on lignin content of rice bran.

6.3.2.2 Accumulation of soluble sugars

Soluble sugars accumulated in fermented barley straws with highest concentrations observed with *C. versicolor* and *P. chrysosporium* and least with *P. sajo-caju*. In contrast, with hay, with the exception of *P. sajo-caju*, less soluble sugar was observed in fermented lignocelluloses than was present in controls. In all fermented spruce sawdusts increases were observed in soluble sugar contents (Table 6.2).

6.3.2.3 Breakdown of polysaccharides

All five fungi reduced polysaccharide contents of barley straw. Cellulose (33.4%) and hemicellulose (24.6%) contents of barley straw were reduced to 27.0% (19% loss) and 20.0% (31% loss), respectively, by *T. harzianum*. Polysaccharides in straw were reduced least by *C. cellulolyticum*. With all five strains apparent increases in cellulose and hemicellulose were observed with hay. Reductions in cellulose and hemicellulose contents of spruce sawdust were observed after fungal treatment, with *P. sajo-caju* having the most limited effect, a 21% reduction in hemicellulose content.

There were no marked differences between the cellulose content of spruce sawdust treated with *C. versicolor* and *T. harzianum* and untreated sawdust. *C. versicolor*, *P. sajo-caju* and *P. chrysosporium* significantly attacked both cellulose and hemicellulose in birch wood with *C. versicolor* effecting an 8.0%?loss in cellulose (29.9 to 27.6%) and a greater, 31.6%, decrease in hemicellulose (24.4 to 16.7%). On the other hand *P. chrysosporium* preferentially attacked birch cellulose (19.2% loss) over hemicellulose (12.9% loss). All three strains exhibited moderate attack on polysaccharides in rice bran (12 to 26% loss).

6.3.2.4 Changes in rumen digestibility

Estimations of changes in *in vitro* rumen

TABLE 6.3 EFFECT OF FUNGAL TREATMENT ON *IN VITRO* RUMINAL FERMENTATION PERFORMANCE OF TEST SUBSTRATES

SUBSTRATES	ORGANISMS					
	CONTROL	*Coriolus versicolor*	*Pleurotus sajo-caju*	*Phanaerochaete chrysosporium*	*Trichoderma harzianum*	*Chaetomium cellulolyticum*
HAY						
pH	6.25	6.65	6.45	6.50	6.80	6.75
[a]CO2	10.07	10.73	13.21	14.01	10.05	8.95
[a]CH4	0.017	0.020	0.004	0.005	0.010	0.007
DIG(%)	42.56	42.49	37.46	36.38	29.65	32.15
BARLEY STRAW						
pH	7.00	6.75	6.95	6.70	7.00	7.05
[a]CO2	6.16	8.69	6.65	9.31	5.48	5.65
[a]CH4	0.018	0.01	0.019	0.001	0.012	0.01
DIG(%)	31.6	32.21	14.99	35.83	21.29	12.55
SPRUCE						
pH	7.30	7.30	7.25	7.30	7.30	7.30
[a]CO2	3.20	4.09	3.62	4.09	3.98	3.53
[a]CH4	0.019	0.019	0.001	0.016	0.02	0.03
DIG(%)	7.75	11.13	9.4	6.30	nd	nd
[b]**BIRCH**						
pH	7.05	6.70	6.90	6.45	nd	nd
[a]CO2	9.20	10.84	9.74	13.35	nd	nd
[a]CH4	0.55	0.46	0.87	1.51	nd	nd
DIG(%)	13.79	22.57	18.04	32.24	nd	nd
[b]**RICE BRAN**						
pH	5.95[c]	6.15	6.15	6.55c	nd	nd
[a]CO2	14.98c	16.45	19.18	11.81c	nd	nd
[a]CH4	0.54	0.87	0.14	0.42	nd	nd
DIG(%)	43.07	51.23	44.73	47.82	nd	nd

All values are means of at least two replicates.
[a]Rumen gas measured as micromole g^{-1} ml^{-1} gas space
[b]Rumen fluid used for this assay was collected from an animal fed on Mastertek
[c]Incubated for 24hr
nd = not determined

digestibility of lignocellulosics effected through fungal fermentation are summarised in Table 6.3. Fermented barley straw, treated with *P. chrysosporium* or *C. versicolor* reduced the simulated rumen pH from 7.0 to 6.70. This was not observed with other fermented barley straws. Outputs of rumen carbon dioxide were higher with barley straw fermented by *C. versicolor*, *P. sajo-caju* and *P. chrysosporium* than with control straw; treatment with *T. harzianum* and *C. cellulolyticum* reduced CO_2 output. No marked differences in methane production could be demonstrated between straws but *T. harzianum*, *C. cellulolyticum* and *P. sajo-caju* appeared to decrease digestibility of straw, whereas *P. chrysosporium* and *C. versicolor* enhanced digestibility. Results obtained with hay were more difficult to interpret. Whereas control hay yielded a rumen pH of 6.25, fermented hays gave somewhat higher pH values. Only fermentation with *C. versicolor*,*P. sajo-caju* or *P. chrysosporium* enhanced the rumen output of carbon dioxide of fermented hay. None of the fungi enhanced *in vitro* rumen digestibility of hay.

With spruce sawdust, all fungal fermentations yielded rumen pHs slightly above 7.0. Moderate increases in rumen carbon dioxide output were observed with all fermented sawdust. Increases in digestibility were observed for spruce fermented by *C. versicolor* and *P. sajo-caju* whereas *P. chrysosporium* reduced digestibility. Fermented birch sawdust reduced rumen pH below 7.0 and increased *in vitro* rumen carbon dioxide output. Methane

outputs were increased by fermentations except with *C. versicolor* which reduced CH_4 below levels obtained with unfermented sawdust. With fermented bran samples rumen pH values were slightly above those in controls (5.95). Rumen carbon dioxide evolution from bran treated with *P. chrysosporium* was however significantly less than that obtained from unfermented controls. Fermentations yielded no marked improvement in digestibility of rice bran.

To summarise, it can be concluded that some of the fermentations enhanced *in vitro* rumen digestibilities.

6.3.3 Effects of ferulic acid on enhancement of digestibility

Spruce sawdust was supplemented with ferulic acid in solid substrate fermentations to determine if the presence of this compound would influence delignification. The results obtained are shown in Table 6.4. At 0.05% ferulic acid delignification was slightly enhanced with *C. versicolor*, *P. chrysosporium* and *P. sajo-caju* but at 0.5% fermentations yielded less marked decreases in ADL values. Changes in NDF with fermentation were more varied except that increases were observed with *C. versicolor* and *P. sajo-caju* and decreases with other fungi.

There was an inverse relationship between total polysaccharide content (NSP) and ferulic acid in sawdust fermentations with either *C. versicolor* or *C.*

cellulolyticum. More NSP was lost at 0.05% - 0.1% ferulic acid than at 0.5% when wood was fermented with *P. chrysosporium* or *P. sajo-caju*. Cellulose breakdown was enhanced in proportion to addition of ferulic acid in wood fermented by *C. versicolor*, and *C. cellulolyticum* but no marked decrease in cellulose content was observed with 0.5% ferulic acid addition to substrates treated with *P. sajo-caju*(Table 6.4).

There was an inverse relationship between rumen production of carbon dioxide in relation to concentration of ferulic acid, except with fermentations by *P. chrysosporium*. Supplementations of ferulic acid did not appear to influence methane output from fermented sawdust (Table 6.5)

6.3.4 The effect of fermentation time on substrates

The relationship between period of fermentation and substrate decomposition are presented in Fig. 6.1. With increasing incubation time enhanced delignification was observed with *C. versicolor*, *P. sajo-caju*, and *P. chrysosporium*. In contrast, attack on polysaccharide fractions (NSP) was markedly greater in the first 14 days of incubation than subsequently (Table 6.19) *In vitro* rumen digestibility of substrates decreased when the fermentation exceeded six weeks (Fig 6.2).

TABLE 6.4 EFFECT OF FERULIC ACID ON LIGNIN AND POLYSACCHARIDE CONTENT (%) OF FERMENTED SPRUCE SAWDUST

SUBSTRATES	ORGANISMS					
	CONTROL	*Coriolus versicolor*	*Pleurotus sajo-caju*	*Phanaerochaete chrysosporium*	*Trichoderma harzianum*	*Chaetomium cellulolyticum*
SPRUCE SAWDUST						
NDF	90.04	88.51	87.57	86.36	92.16	93.07
ADL	27.26	26.98	27.42	26.67	27.15	27.08
NSP[a]	62.54	52.66	54.78	52.66	57.17	49.37
CP	41.98	42.17	38.61	39.00	41.10	37.30
S.sugar[b]	0.121	0.223	0.161	0.299	0.438	0.161
SPRUCE SAWDUST + 0.05% FERULIC ACID						
NDF	92.50	93.21	91.52	88.41	91.37	92.76
ADL	26.37	25.60	25.47	24.38	27.16	26.59
NSP	62.54	54.31	53.74	50.45	58.01	57.41
CP	41.98	40.71	42.69	39.85	39.38	43.70
S.sugar	0.152	0.282	0.200	0.400	0.280	0.225
SPRUCE SAWDUST + 0.1% FERULIC ACID						
NDF	93.49	93.66	94.11	88.91	91.22	92.95
ADL	26.37	25.74	26.52	23.87	25.14	26.16
NSP	62.54	53.23	50.45	51.03	54.79	55.36
CP	41.98	37.71	37.18	36.15	37.46	40.28
S.sugar	0.120	0.253	0.178	0.424	0.345	0.171
SPRUCE SAWDUST + 0.5% FERULIC ACID						
NDF	93.03	92.74	93.59	87.21	90.81	93.01
ADL	26.37	26.99	26.55	23.99	25.40	26.53
NSP	62.54	47.84	63.21	55.36	55.36	49.37
CP	41.98	37.33	41.89	38.59	38.59	36.39
S.sugar	0.179	0.214	0.207	0.530	0.346	0.253

All values were averages of at least two determinations.

[a] NSP = non starch polysaccharide.

[b] S.sugar = soluble sugar measured as mg g^{-1}

TABLE 6.5 RUMINAL FERMENTATION CHARACTERISTICS OF TREATED SPRUCE SAWDUST WITH OR WITHOUT FERULIC ACID ADDITION.

SUBSTRATES	ORGANISMS					
	CONTROL	*Coriolus versicolor*	*Pleurotus sajo-caju*	*Phanaerochaete chrysosporium*	*Trichoderma harzianum*	*Chaetomium cellulolyticum*
SPRUCE SAWDUST						
[a]CO_2	3.20	4.09	3.62	4.09	3.98	3.53
[a]CH_4	0.019	0.019	0.001	0.016	0.02	0.03
SAWDUST + 0.05% ferulic acid						
[a]CO_2	3.35	3.8	3.68	4.17	3.91	3.46
[a]CH_4	0.021	0.016	0.017	6.4×10^{-3}	0.018	0.019
SAWDUST + 0.1% ferulic acid						
CO_2	3.23	3.56	3.55	4.16	3.83	3.28
CH_4	0.01	8.2×10^{-3}	0.015	0.015	8.2×10^{-3}	0.04
SAWDUST + 0.5% ferulic acid						
CO_2	3.15	3.67	3.59	4.38	3.47	3.58
CH_4	9.1×10^{-3}	traces	0.01	0.013	0.019	3.64×10^{-3}

All values are means of at least two replications

[a]Rumen gases measured as micromole/ml gas space/g

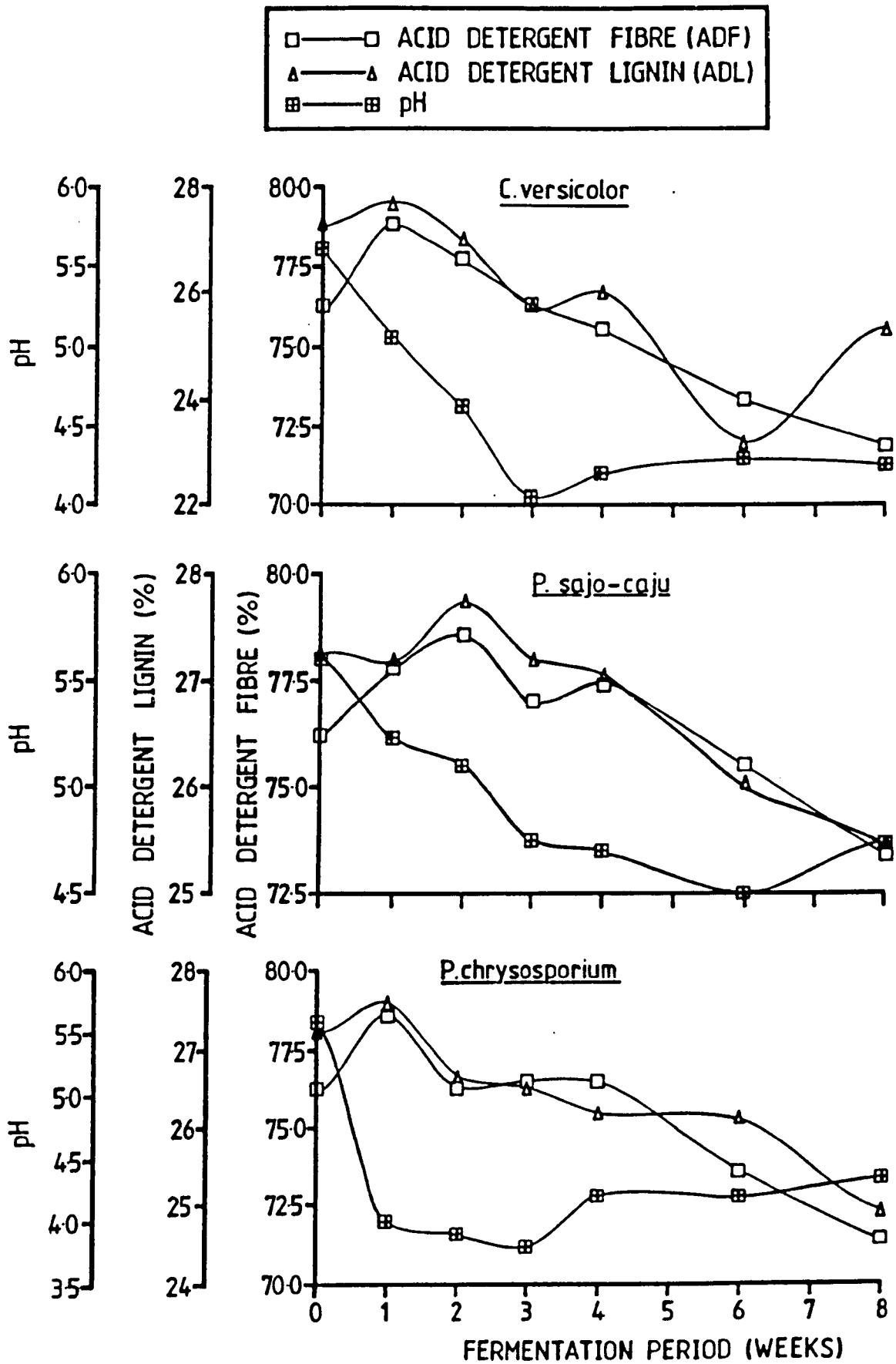


Fig. 6.1 The effect of fermentation time on degradation of spruce lignin.

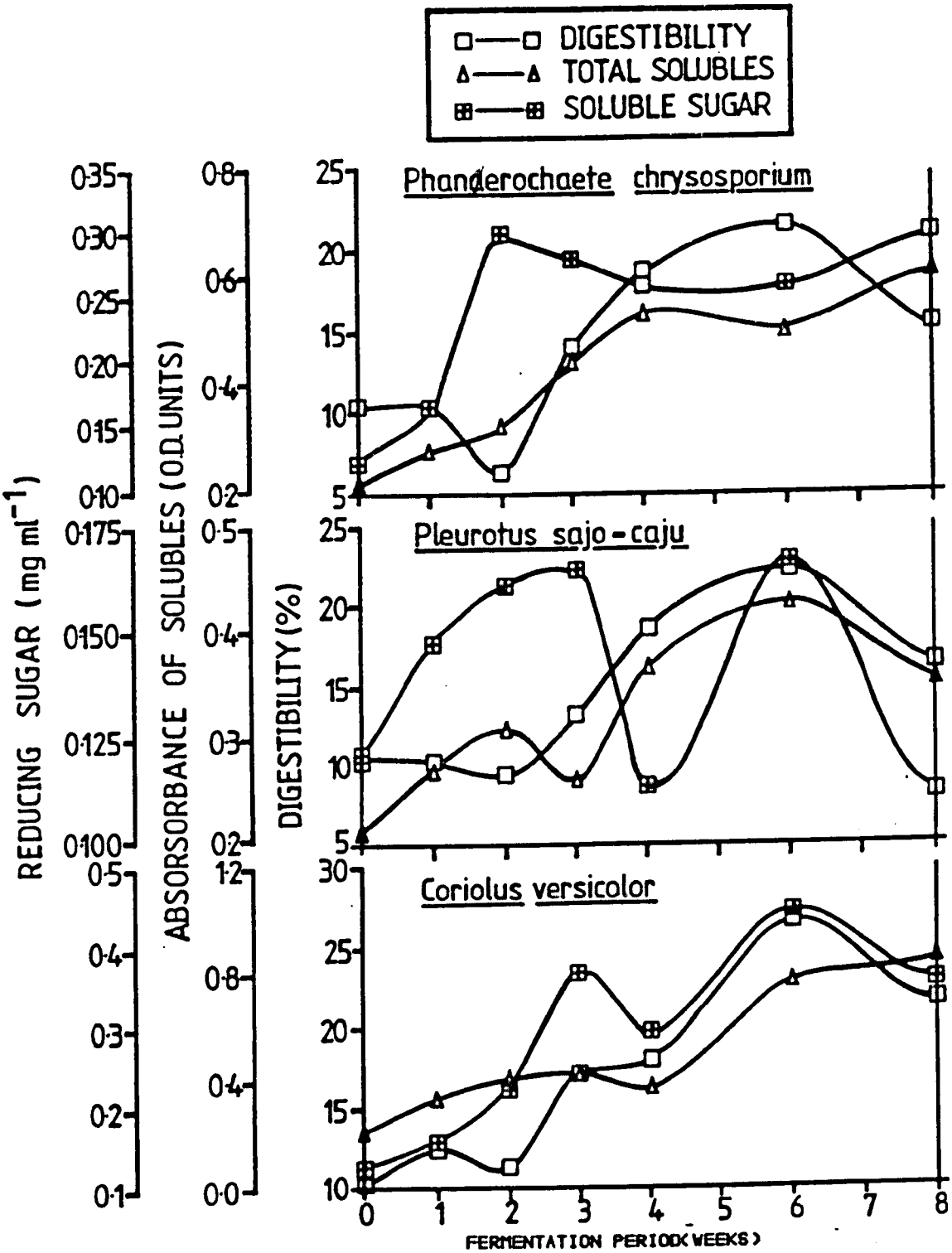


Fig. 6.2 The relationship between *in vitro* rumen digestibility of fermented spruce sawdust and accumulation of solubles.

6.3.5 Influence of particle size on substrate degradation

Neither lignin depolymerisation nor substrate digestibility were markedly influenced by particle size when fermentations were performed with particles fractioned to <1.50 mm , and <500 microns and ballmilled sawdust. Particles of <500 microns and after ball milling had slightly increased and the larger particles markedly decreased digestibility. The larger particles appeared to exhibit slower and more variable fungal growth than the two smallest classes of particle. The smallest, ball-milled, fractions, with reduced air spaces between particles, developed a high density of aerial mycelium (Table 6.6).

6.3.6 Influence of temperature and water activity

The influence of temperature and moisture content on extent of lignin depolymerisation and *in vitro* rumen digestibility is summarised in Table 6.7. Maximal lignin breakdown was observed with *C. versicolor* and *P. chrysosporium* at 28.5 and 30^{o}C respectively. One organism, *P. chrysosporium*, showed an inverse correlation between incubation temperature and decreases in lignin content whereas the other two strains displayed a direct correlation between increasing temperature and extent of delignification except at temperatures above 30 ^{o}C .

Changes in *in vitro* digestibility of the fermented substrate did not show a coherent pattern. While enhanced

TABLE 6.6 EFFECT OF SUBSTRATE PARTICLE SIZE ON LIGNIN DEGRADATION AND *IN VITRO* RUMEN DIGESTIBILITY(%)

PARTICLE SIZE	CONTROL	ORGANISMS *Coriolus versicolor*	*Pleurotus sajo-caju*	*Phanaerochaete chrysosporium*
<1.5mm> 0.5mm				
ADF	77.53 ± 0.18*	76.35 ± 0.89	78.38 ± 0.89	78.03 ± 0.21
ADL	27.35 ± 0.1	24.68 ± 0.08	26.55 ± 0.4	26.26 ± 0.29
DIG.[a]	4.65 ± 0.39	10.24 ± 0.74	- 0.00	8.48 ± 0.62
< 500 microns				
ADF	75.87 ± 0.54	74.22 ± 0.15	75.59 ± 0.7	73.67 ± 0.04
ADL	27.26 ± 0.06	23.21 ± 0.15	26.03 ± 0.14	26.13 ± 0.09
DIG.	8.53 ± 0.78	26.68 ± 0.65	22.22 ± 0.04	21.68 ± 1.51
Ball millled				
ADF	73.34 ± 0.94	69.81 ± 0.3	72.16 ± 0.35	75.95 ± 0.6
ADL	26.53 ± 0.4	28.36 ± 0.24	28.96 ± 0.4	28.31 ± 0.12
DIG.	21.16 ± 1.25	30.88 ± 3.52	15.32 ± 2.5	38.80 ± 1.0

* All values (%) are average of two separate determinations.

[a]DIG = Digestibility

digestibility values were obtained for substrates inoculated with *P. chrysosporium* at 30 ^{o}C and 40 ^{o}C, at 37 ^{o}C digestibility was reduced. Generally highly colonised substrates, especially with *C. versicolor* at 25 ^{o}C, had markedly reduced digestibilities except with *P. sajo-caju*, where heavy colonisation did not lead to marked differences in digestibility at either 25 ^{o}C or 28.5 ^{o}C.

The influence of moisture content on the delignification process and rumen digestibility of treated substrates is summarised in Table 6.7. The optimum moisture content for enhancing digestibility of spruce sawdust was 70%. Although all three fungi grew fairly well at the different water activities, a moisture content of 80% was optimal for lignin depolymerisation by *P. chrysosporium*, whereas for the other two fungi the optimum was 70%. This water activity was optimal for enhancement of rumen digestibility with all three fungi. However, with *P. chrysosporium* moisture level had no apparent influence on digestibility. Although minimal differences in digestibility were obtained between 55% and 70% moisture, with the other two fungi, at 80% a marked reduction was observed

6.3.7 Fermentations of spruce sawdust

6.3.7.1. Changes in pH

Within the first week of fermentation by *P. chrysosporium* the pH of the spruce substrate had

TABLE 6.7 EFFECT OF TEMPERATURE AND MOISTURE CONTENT ON LIGNIN DECOMPOSITION AND SUBSTRATE DIGESTIBILITY(%).

CONDITIONS		CONTROL	ORGANISMS *Coriolus versicolor*	*Pleurotus sajo-caju*	*Phanaerochaete chrysosporium*
TEMP.(^{o}C)					
25	ADL	27.26	25.57	26.95	nd
	DIG[d]	8.88	15.0	22.98	nd
28.5	ADL	27.26	23.21	26.09	23.21[a]
	DIG[d]	8.88	26.68	22.22	21.47[a]
37	ADL	27.25	NG	NG	[b]25.6 26.13[c]
	DIG[d]	8.88	nd	nd	[b]16.5 21.68[c]
MOISTURE CONTENT					
55%	ADL	27.45	23.86	26.38	26.61
	DIG[d]	8.53	24.78	21.88	20.83
70%	ADL	27.26	23.21	26.03	26.13
	DIG[d]	8.88	26.68	22.22	21.68
80%	ADL	26.05	25.14	25.41	23.53
	DIG[d]	9.46	17.24	14.11	20.50

All values are average of at least two separate determinations

NG = no growth, nd= not determined.

[a]Incubated at 30^{o}C [b] at 37^{o}C and [c] at 40^{o}C .

[d] Digestibility

decreased from 5.6 to 4.0 and subsequently pH fluctuated within the range 4.1 + 0.2. In contrast, *C. versicolor* and *P. sajo-caju* the drop in pH was more gradual in the first two weeks of the fermentation (Fig. 6.1) and this was followed by a decline to between 4.25 and 4.75 on the eighth week. A examination of the results displayed in Fig 6.1 showed that a considerable reduction in lignin content occurred between pH 3.9 - 4.4 whereas minimal decreases in polysaccharide were observed in this pH range (Table 6.19b).

6.3.7.2 Changes in total soluble and insoluble saccharide contents

With each of the three fungi a cyclical pattern of soluble sugar accumulation was observed. Maximal reducing sugar (0.448 mg ml^{-1} g^{-1}) was observed in samples treated with *C. versicolor* and the least with *P. sajo-caju* (0.167 mg ml^{-1} g^{-1})(Fig. 6.2). Similar results were obtained for total solubles, including lignin and polysaccharide degradation products, which accumulated during incubation. There was also a significant correlation between accumulation of solubles and *in vitro* rumen digestibility of substrates and increases in accumulation of solubles coincided with increased delignification. On the other hand, the relationship between total NSP degraded and reducing sugar accumulation was variable (Tables 6.19 & 21).

6.3.7.3 Changes in cellulose and hemicellulose contents.

Degradation of polysaccharide components by all three fungi varied considerably (Table 6.19). With each organism initial attack was primarily upon hemicelluloses, depleting this polysaccharide, but subsequently preferential attack on cellulose was observed. Neither degradation was proportional to period of fermentation.

6.3.7.4 Changes in neutral sugars of the fermented material

Residual monosaccharides in carbohydrate differed between the three fungi (Table 6.8) rhamnose was substantially depleted by *P. chrysosporium* whereas mannose was preferentially metabolised by *C. versicolor*. Similarly glucose appeared to be the substrate of choice for *P. sajo-caju* with a 21.8% depletion.

6.3.7.5 Changes in protein contents with fermentations

Microbial biomass was estimated as total protein nitrogen and for each organism there was a linear increase in protein over a six week period, followed by a steep decline in protein in the final week. Maximal microbial biomass was formed by *C. versicolor* (Fig 6.3).

6.3.8 The influence of autoclaving

The influence of autoclaving sawdust containing 70% moisture was examined (Table 6.9). Autoclaving and

TABLE 6.8 EFFECT OF FUNGAL FERMENTATIONS OF SPRUCE SAWDUST ON NEUTRAL SUGARS(%).

SUGARS	FUNGI[a]			
	Control	*P.chrysosporium*	*C.versicolor*	*P.sajo-caju*
Rhamnose	0.10 ± 0.00[b]	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.00
% loss	-	30.0	10.0	20.0
Arabinose	1.02 ± 0.02	0.90 ± 0.01	0.79 ± 0.02	0.93 ± 0.02
% loss	-	11.76	22.55	7.84
Xylose	5.23 ± 0.08	4.72 ± 0.01	4.51 ± 0.02	4.81 ± 0.01
% loss	-	9.7	13.77	8.04
Mannose	10.34 ± 0.06	8.94 ± 0.04	6.74 ± 0.14	9.01 ± 0.01
% loss	-	13.54	34.82	12.86
Galactose	2.43 ± 0.02	1.87 ± 0.03	1.99 ± 0.10	2.22 ± 0.01
% loss	-	22.84	17.70	8.64
Glucose	41.55 ± 0.5	32.09 ± 0.19	27.54 ± 0.22	32.49 ± 0.01
% loss		22.77	34.25	21.78

[a] All values are for experiments terminated on the sixth week.

[b] Results are average of at least two determinations

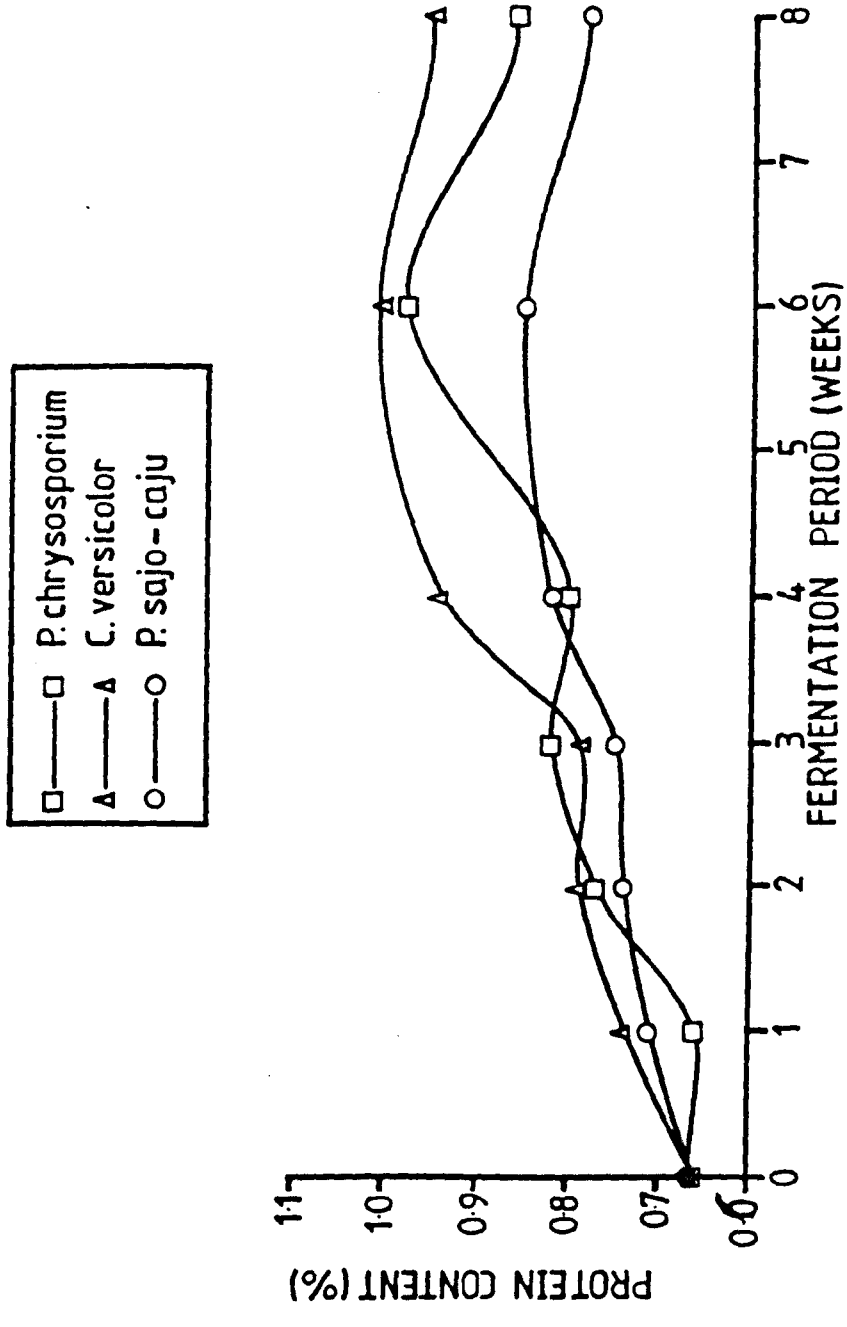


Fig. 6.3 Protein enrichment of spruce sawdust by fungal fermentation.

TABLE 6.9 EFFECT OF AUTOCLAVING WITH OR WITHOUT FUNGAL INOCULATION ON LIGNIN DEGRADATION AND DIGESTIBILITY.

PARAMETER	FUNGI			
	Control	*C.versicolor*	*P.sajo-caju*	*P.chrysosporium*
Autoclaved + fermented				
ADF(%)	77.90	79.06	78.15	76.26
ADL(%)	26.85	26.75	27.15	24.37
Total solubles [a]	0.184	0.282	0.234	0.448
Soluble sugar[b]	0.133	0.190	0.135	0.435
RUMEN CO_2[c]	4.39	4.17	3.86	4.59
Methane[c]	0.027	0.04	0.03	0.03
Digestibility(%)	5.76	9.36	4.23	13.97
Not autoclaved + fermented				
ADF	74.09	75.77	76.23	77.24
ADL	25.09	25.66	25.18	25.98
Total solubles	0.322	0.591	0.596	0.417
Soluble sugar	0.301	0.487	0.510	0.364
RUMEN CO_2[a]	4.65	4.97	4.95	4.88
Methane	0.03	0.011	0.018	0.02
Digestibility	8.96	11.00	12.03	10.36

Control not autoclaved without inoculum and incubation, ADL = 27.6%

All values are means of two replications. a= O.D units, b= mg g^{-1}

[c] Carbon dioxide production in batch cultures incubated for 42 - 44h.

Rumen gases measured as μmol ml^{-1}gas space.

incubation appeared to increase the apparent lignin contents of sawdust. There were visible signs of discolouration of control non sterile sawdusts following incubation. Autoclaving also reduced rumen digestibility and rumen output of carbon dioxide and methane. Inoculation of non sterile sawdust with *C. versicolor*, *P. sajo-caju* or *P. chrysosporium* did not reduce lignin contents but there was enhanced output of rumen gases in the absence of autoclaving and slightly enhanced *in vitro* rumen digestibility.

Autoclaving sawdust prior to inoculation favoured the delignification by all fungi with the exception of *P. sajo-caju*. Moreover, with this exception, autoclaved, fermented woods displayed increases in digestibility, enhanced output of methane and reduction in carbon dioxide production (except *P.chrysosporium*) as compared to controls. Comparing these results with those in Table 6.6 indicates that distilled water gave higher rates of delignification by *P. chrysosporium* than minimal salt medium. However, for the other two fungi the presence of minimal salts enhanced lignin depolymerisation.

Moreover, from comparison of ADL values it is clear that autoclaving reduced the extent of delignification and yields of total solubles and reducing sugars.

6.3.9 The relationship between oxygen consumption and carbon dioxide production during delignification

Oxygen concentration had an effect upon fungal delignification (Fig. 6.4: Table 6.10). In oxygen

TABLE 6.10 MINERALIZATION OF SPRUCE SAWDUST IN PRESENCE OF EITHER OXYGEN OR AIR ATMOSPHERE IN CULTURES FLUSHED ONCE FOR THE ENTIRE ASSAY PERIOD.

PERIOD(WEEKS)	ORGANISMS			
	Control	*C.versicolor*	*P.sajo-caju*	*P.chrysosporium*
		100% O_2 ATMOSPHERE		
WEEK 2				
Oxygen unutilised	[a]36.83	3.4	13.22	5.62
CO_2 produced	0.4	33.63	20.32	16.05
WEEK 6				
Oxygen unutilised	37.0	6.24	6.70	8.30
CO_2 produced	0.60	14.99	13.37	3.15
		100% AIR ATMOSPHERE		
WEEK 2				
Oxygen unutilised	8.82	0.96	0.99	1.45
CO_2 produced	0.12	8.62	5.41	6.37
WEEK 6				
Oxygen unutilised	9.34	2.24	2.65	2.80
CO_2 produced	0.13	3.40	2.53	1.72

All values are means of at least two duplicate culture bottles

[a] values reported in µmol/4g/ml gas space.

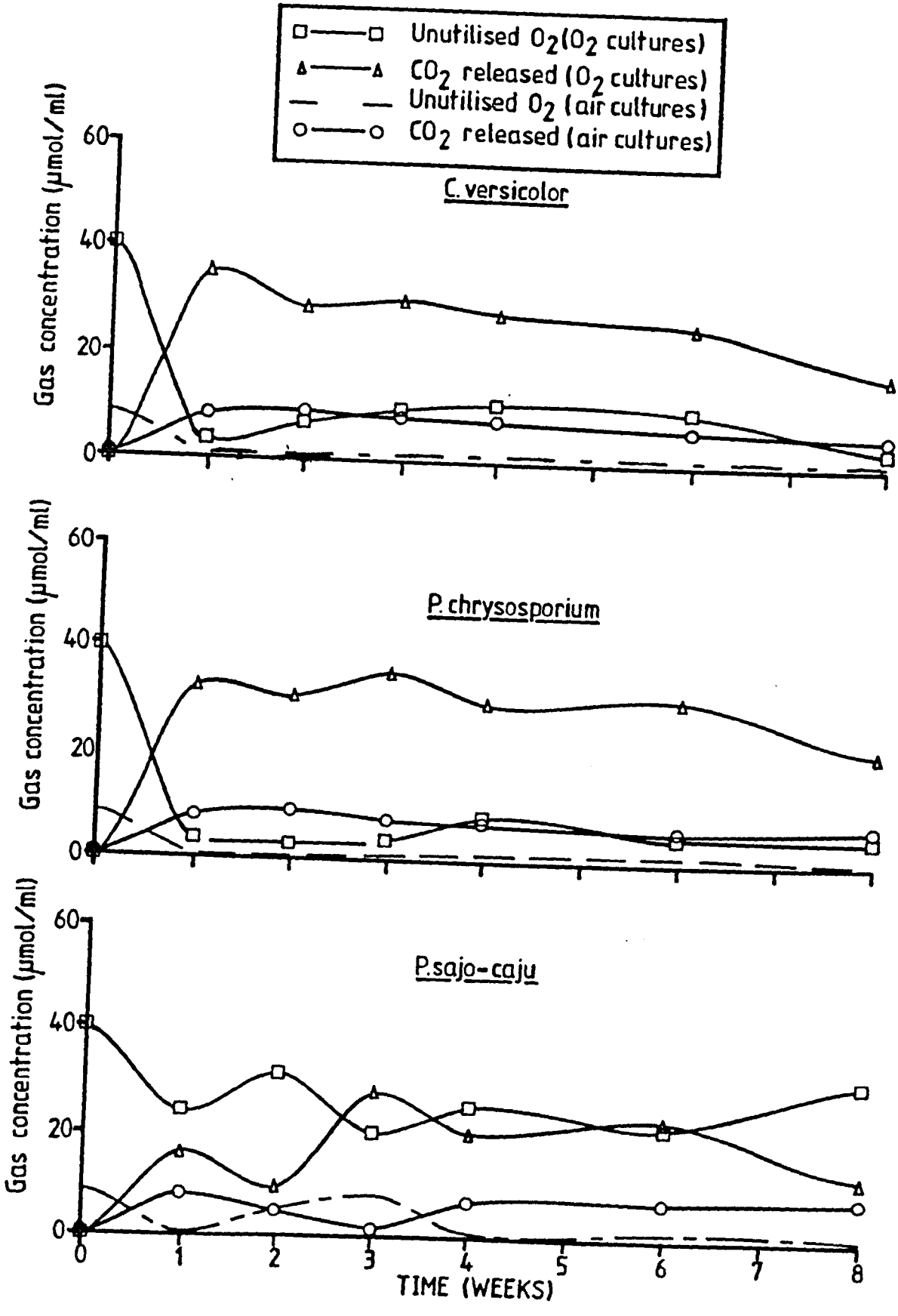


Fig. 6.4 The influence of 100% oxygen or air atmosphere on mineralization of spruce sawdust.

cultures, the evolution of carbon dioxide through mineralisation of sawdust was found to fluctuate during the experiment. In each of the fungi, carbon dioxide release was rapid in the first three weeks of fermentation then decreased subsequently. Production was lower with *P. sajo-caju* than with the other strains. Release of carbon dioxide was 60 - 80% higher in oxygen than in air atmosphere. Carbon dioxide output was stimulated in both oxygen and air cultures when intermittent flushing (Fig. 6.4) was carried out, in comparison with results obtained at the termination of fermentations, from single flushings (Table 6.10).

6.3.10 The effect of carbohydrate supplements on delignification

The presence of certain mono- and disaccharides influenced fungal delignification (Table 6.11). After six weeks of fermentation in the presence of 0.5% or 1% xylose, galactose or arabinose, the extent of delignification by *P. sajo-caju* and *C. versicolor* were repressed below those observed with controls growing in the absence of exogenous sugars. Mannose, or galactose , at 1% suppressed delignification by *P. chrysosporium*. The highest amount of lignin depletion were observed with *C. versicolor* in the presence of 3% mannose. However, good correlation was observed between percentage of lignin loss and the concentration of the three major cell wall monosaccharides (glucose, mannose and xylose) at the

TABLE 6.11 EFFECT OF SUPPLEMENTATIONS WITH MONOSACCHARIDES AND DISACCHARIDES ON DELIGNIFICATION .

SUGARS	ORGANISMS		
	C.versicolor	*P.sajo-caju*	*P.chrysosporium*
Water alone			
% lignin loss	0.37	-1.12	9.24
% NSP loss	8.4	5.65	13.72
Glucose			
0.5%			
% lignin loss	8.02	2.89	2.59
1.0%			
% lignin loss	5.89	nd	9.79
% NSP loss	-1.37	nd	0.71
3%			
% lignin loss	11.68	nd	5.23
Xylose			
0.5%			
% lignin loss	-0.94	-1.98	4.15
1.0%			
% lignin loss	-4.43	-5.83	7.12
% NSP loss	12.53	6.61	10.73
3%			
% lignin loss	0.86	2.39	7.42
Mannose			
0.5%			
% lignin loss	6.62	3.48	-2.60
1.0%			
%lignin loss	0.86	2.0	1.41
% NSP loss	7.12	21.54	16.48
3.0%			
% lignin loss	12.73	-3.53	3.23
Galactose(1%)			
% lignin loss	-3.37	-2.11	-0.50
%NSP loss	2.95	11.85	12.22
Arabinose(1%)			
% lignin loss	-2.23	nd	1.82
% NSP loss	22.05	27.62	27.68
Sucrose(1%)			
% lignin loss	6.80	4.77	7.68
% NSP loss	27.62	15.51	19.13
Maltose(1%)			
% lignin loss	7.77	2.92	5.80
% NSP loss	7.12	10.93	11.03
Lactose(1%)			
% lignin loss	3.65	4.65	0.26
D(+) glucosamine(1%)			
% lignin loss	0.87	5.54	-0.83
% NSP loss	21.19	16.47	13.72

levels tested. Addition of the disaccharides maltose, lactose, and sucrose resulted in only slight stimulation of lignin degradation by *C. versicolor* and *P. chrysosporium*. In the latter organism and *P. sajo-caju*, sucrose stimulated lignin breakdown more than either lactose or maltose. Addition of D(+) glucosamine repressed lignin degradation by *P. chrysosporium* and *C. versicolor* but had no adverse effect on delignification by *P. sajo-caju*.

The fungi degraded 5 - 30% of cell wall polysaccharides in the presence of each sugar, with the exception of glucose. Moreover, in no strain was attack on cell wall polysaccharides adversely affected by the addition of D(+) glucosamine.

Analysing the performance of the fermented carbohydrate-supplemented sawdust in the *in vitro* rumen gave varied results (Table 6.12). Supplementation of *C. versicolor* fermentations with xylose, maltose, or glucosamine reduced digestibility. Minimal increases were observed when the substrate was supplemented with either mannose, sucrose or lactose. Evolution of carbon dioxide was, in some cases, lower than in controls when xylose, mannose, galactose, sucrose, maltose or glucosamine were added to substrates. Methane output was slightly higher than controls when galactose, sucrose or lactose was added to *C. versicolor* fermentations.

Fermentation with *P. sajo-caju* enhanced

TABLE 6.12 IN VITRO RUMINAL FERMENTATION CHARACTERISTICS OF SPRUCE SAWDUST SUPPLEMENTED WITH SUGARS BEFORE FUNGAL FERMENTATION

SUGARS	ORGANISMS			
	Control	*C.versicolor*	*P.sajo-caju*	*P.chrysosporium*
Water alone				
CO_2	3.51 ± 0.06	4.17 ± 0.015	3.86 ± 0.1	4.59 ± 0.25
CH_4	0.004	0.004	0.002	0.002
DIG	5.76 ± 0.33	9.36 ± 0.64	4.23 ± 0.4	13.97 ± 3.42
Xylose (1.0%)				
CO_2	4.26 ± 0.12	4.03 ± 0.08	3.68 ± 0.10	4.86 ± 0.15
CH_4	0.03	0.02	0.05	0.04
DIG	16.49 ± 1.25	15.40 ± 2.62	18.70 ± 1.4	15.53 ± 2.12
Mannose(1.0%)				
pH	7.20	7.20	7.20	7.20
CO_2	5.15 ± 0.13	4.34 ± 0.14	4.83 ± 0.30	5.08 ± 0.20
CH_4	0.010	0.007	0.011	0.011
DIG	8.23 ± 0.5	8.6 ± 1.0	19.8 ± 0.35	17.40 ± 0.4
Galactose(1%)				
pH	7.10	7.20	7.20	7.25
CO_2	5.31 ± 0.04	4.43 ± 0.12	4.04 ± 0.05	4.27 ± 0.16
CH_4	0.004	0.012	0.005	0.06
DIG	12.34 ± 1.52	19.44 ± 1.23	15.39 ± 0.75	15.63 ± 1.04
Sucrose(1%)				
pH	6.80	7.10	7.20	7.10
CO_2	11.62 ± 0.12	6.32 ± 0.010	5.89 ± 0.07	7.82 ± 0.05
CH_4	0.08	0.16	0.16	0.23
DIG	12.56 ± 1.57	12.65 ± 4.72	13.36 ± 2.14	11.00 ± 0.41
Maltose(1%)				
pH	7.15	7.20	7.15	7.15
CO_2	5.21 ± 0.01	4.74 ± 0.25	4.67 ± 0.20	5.08 ± 0.10
CH_4	0.004	traces	0.03	0.01
DIG	7.45 ± 1.75	6.12 ± 0.42	4.74 ± 0.52	15.15 ± 3.35
Lactose(1%)				
pH	7.15	7.25	7.20	7.20
CO_2	4.60 ± 0.31	4.98 ± 0.3	4.68 ± 0.31	4.25 ± 0.03
CH_4	0.005	0.01	0.007	0.007
DIG	15.3 ± 0.25	16.16 ± 1.11	13.54 ± 0.92	14.55 ± 0.3
D(+) glucosamine(1%)				
pH	7.10	7.25	7.15	7.25
CO_2	5.01 ± 0.05	4.17 ± 0.10	4.42 ± 0.12	4.34 ± 0.08
CH_4	0.005	0.004	0.002	0.02
DIG	16.13 ± 0.54	14.29 ± 1.6	7.59 ± 1.1	18.3 ± 2.2

All values are means of at least two determinations
[a] Gas concentration reported as micromole ml^{-1} gas space g^{-1} sawdust.
DIG. = digestibility (%)

digestibility of substrates when xylose, mannose, galactose or sucrose were added, whereas supplementation with maltose, lactose or glucosamine reduced digestibility below values observed in controls. In contrast, carbon dioxide evolution was enhanced by addition of lactose but methane production was found to be higher when xylose, sucrose, maltose or lactose were added.

Fermentation with *P. chrysosporium* enhanced digestion in the presence of exogenous xylose, mannose, galactose, maltose or glucosamine. All sugars, with the exception of xylose, reduced carbon dioxide production below control values. However, addition of xylose, mannose, galactose, sucrose, maltose or lactose enhanced methane release from substrates fermented by *P. chrysosporium*.

In all cultures, with the exception of controls containing sucrose, addition of sugars prior to fungal fermentation resulted in the rumen pH remaining slightly above neutrality.

6.3.11 Influence of addition of complex carbohydrates

The effects of supplementing lignocellulose fermentations with 5%, 10%, or 30% rice bran, cow concentrate or birch sawdust on delignifications and depletion of polysaccharide are summarised in Table 6.13. Delignification by *C. versicolor* increased in parallel with addition of rice bran and cow concentrate, whereas

TABLE 6.13 EFFECT OF COMPLEX CARBOHYDRRATE SOURCES ON DELIGNIFICATION AND SAWDUST DIGESTIBILITY(%).

SUBSTRATE		FUNGI		
RICE BRAN	Control	*C.versicolor*	*P.sajo-caju*	*P.chrysosporium*
5%				
% lignin loss	-	34.7	1.5	14.17
DIG.	21.24 ± 0.6	32.81 ± 0.16	25.40 ± 1.0	29.92 ± 1.43
10%				
% lignin loss	-	55.52	12.96	4.37
% NSP loss	-	22.25	18.45	17.56
DIG.	23.58 ± 2.79	46.36 ± 1.6	26.7 ± 0.7	32.68 ± 1.47
30%				
% lignin loss	-	59.81	4.77	-11.11
DIG.	24.08 ± 0.4	47.50 ± 0.95	30.87 ± 0.7	33.52 ± 1.3
COW CONCENTRATE				
5%				
% lignin loss	-	21.16	0.4	-1.20
DIG.	6.9 ± 2.25	23.70 ± 1.48	13.21 ± 1.49	17.40 ± 3.29
10%				
% lignin loss	-	39.73	-0.76	9.18
% NSP loss	-	17.54	11.76	5.87
DIG.	16.17 ± 2.71	35.52 ± 8.42	17.42 ± 2.25	17.82 ± 1.34
30%				
% lignin	-	51.88	-12.19	nd
DIG	23.75 ± 1.96	18.03 ± 0.04	17.10 ± 0.15	nd
BIRCH SAWDUST				
5%				
% lignin loss	-	7.31	-0.31	0.00
DIG.	13.91 ± 1.35	27.5 ± 2.5	11.79 ± 1.1	14.45 ± 5.05
10%				
% lignin loss	-	6.78	-0.12	-3.95
DIG.	15.92 ± 1.31	17.92 ± 1.98	12.24 ± 2.2	13.15 ± 1.47
30%				
% lignin loss	-	14.39	-1.91	2.21
DIG.	20.52 ± 3.26	12.7 ± 0.95	12.55 ± 2.07	19.48 ± 5.26

All values are average of two determinations.

" -" means zero

the other two strains showed no consistent pattern. For instance, delignification by *P. chrysosporium* was repressed with 30% rice bran or 5% cow concentrate whilst with *P. sajo-caju* similar repression was observed with 10 - 30% cow concentrate and 5 - 30% birch sawdust. One fungus, *C. versicolor*, depleted polysaccharides more slowly than it effected delignification whereas the reverse was the case with *P. chrysosporium* and *P. sajo-caju*.

There were enhancements of rumen digestibility when fermentations by *C. versicolor* were supplemented with either rice bran, 5 - 10% cow concentrate or 5 - 10% birch sawdust but reductions with either 30% birch sawdust or >10% cow concentrate (Table 6.14). Similarly *C. versicolor* fermented spruce sawdust showed enhanced rumen carbon dioxide, evolution with 10% supplementation with the three substrates. In all cases methane output was also enhanced over control substrates (Table 6.14).

Modest increases in *in vitro* digestibility were observed with *P. sajo-caju* when 10% cow concentrate or 5 - 30% birch sawdust was added although substrates colonised by this fungus showed reductions in output of rumen carbon dioxide except with 10% birch sawdust. Fermented substrates gave greater evolution of methane than control substrates. Rumen pH with all substrates remained slightly above neutrality.

Rumen digestibility of substrates colonised by *P. chrysosporium* was markedly reduced with birch sawdust but

TABLE 6.14 *IN VITRO* RUMINAL FERMENTATION CHARACTERISTICS OF SPRUCE SAWDUST SUPPLEMENTED WITH COMPLEX CARBOHYDRATE(10%) SOURCES BEFORE FUNGAL TREATMENT

		FUNGI		
	Control	*C.versicolor*	*P.sajo-caju*	*P.chrysosporium*
RICE BRAN(10%)				
pH	7.10	6.85	7.10	7.05
CO_2[a]	7.33 ± 0.07	10.21 ± 0.04	6.40 ± 0.17	8.10 ± 0.08
CH_4[a]	0.10	0.098	0.26	0.22
COW CONCENTRATE(10%)				
pH	7.05	6.75	7.25	6.95
CO_2	7.85 ± 0.07	9.53 ± 0.32	4.86 ± 0.04	8.65 ± 0.13
CH_4	0.15	0.28	0.16	0.23
BIRCH SAWDUST(10%)				
pH	7.20	7.00	7.20	7.15
CO_2	5.59 ± 0.01	7.90 ± 0.01	6.99 ± 0.04	7.67 ± 0.1
CH_4	0.15	0.25	0.27	0.22

a = rumen gases measured as $\mu mol\ ml^{-1}$

enhanced by addition of rice bran or cow concentrate. Methane and carbon dioxide production was significantly higher for fermented substrates than controls.

6.3.12 Effect of exogenous nitrogen sources on delignification

The effect on delignification of addition of ammonium dihydrogen phosphate, yeast extract and mycological peptone to fermentations on delignification is summarised in Table 6.15. Complex nitrogen sources such as yeast extract enhanced delignification for all three strains, whereas a simple nitrogen source ammonium dihydrogen phosphate had a very limited effect. In all three fungi, polysaccharide depletion was stimulated with 1% yeast extract and less so with malt extract.

Yeast extract enhanced *in vitro* digestibility more than mycological peptone with *C. versicolor* or *P. chrysosporium* (Table 6.15). Addition of malt extract to fermentations with *P. sajo-caju* reduced digestibility below the value for controls. Sawdust supplemented with ammonium dihydogen phosphate fermented by *P. chrysosporium* had the lowest digestibility.

Spruce sawdust was fermented together with yeast extract, mycological peptone, or ammonium dihydrogen phosphate (Table 6.16) and assessed as a rumen substrate. Sawdust fermented with *C. versicolor* or *P. chrysosporium* evolved more carbon dioxide than control substrates. An exception was the fermentation with *P.chrysosporium* and supplemented by ammonium H_2 phosphate, when values for

TABLE 6.15 EFFECT OF NITROGEN SOURCES ON DELIGNIFICATION AND SAWDUST DIGESTIBILITY (%)

PARAMETER	ORGANISM(S)			
Malt extract(1%)	Control	*C.versicolor*	*P.sajo-caju*	*P.chrysosporium*
% Lignin loss	-	2.83	3.08	5.05
% NSP loss	-	1.51	2.19	2.52
DIG.	16.31 ± 0.5	18.3 ± 0.4	15.05 ± 1.3	21.24 ± 0.77
Yeast extract(1%)				
% lignin loss	-	36.27	8.98	16.06
% NSP loss	-	24.32	19.52	22.82
DIG.	5.68 ± 1.37	24.55 ± 2.4	8.98 ± 0.4	24.41 ± 3.04
Mycological peptone(1%)				
% lignin loss	-	16.50	nd	7.02
% NSP loss	-	14.67	nd	23.72
DIG.	14.23 ± 3.4	22.95 ± 2.5	nd	25.07 ± 2.45
Ammonium dihydrogen phosphate(1%)				
% lignin loss	-	-4.31	nd	-3.95
% NSP loss	-	11.87	nd	12.92
DIG.	12.16 ± 1.15	17.19 ± 2.84	nd	11.87 ± 1.55

All values are average of two determinations.

nd = not determined.

TABLE 6.16 *IN VITRO* RUMEN FERMENTATION CHARACTERISTICS OF SPRUCE SAWDUST SUPPLEMENTED WITH NITROGEN SOURCES.

PARAMETER	ORGANISM(S)			
Yeast extract(1%)	Control	*C.versicolor*	*P.sajo-caju*	*P.chrysosporium*
CO_2[a]	4.06	6.01	4.78	6.41
CH_4[a]	0.02	0.025	0.021	0.032
Mycological peptone(1%)[a]				
pH	6.95	6.85	nd	7.00
CO_2	7.84	8.76	nd	9.86
CH_4	0.79	0.89	nd	1.0
$NH_4H_2PO_4$(1%)[a]				
pH	7.24	7.20	nd	7.25
CO_2	5.94	6.20	nd	5.01
CH_4	0.46	0.40	nd	0.33

All values are means of two replications

[a]Analysed with rumen fluid collected from animal that was fed on "Mastertek" prior to sample collection.

a = rumen gases measured as $\mu mol\ ml^{-1}$

carbon dioxide and methane production were below those for controls, as was methane output with *C.versicolor* fermented wood with this nitrogen source.

6.3.13 Visual assessment of biomass production and sawdust discolouration

The effects of fungal attack on substrate texture and colour are summarised in Table 6.17. Fungal growth on spruce changed sawdust from pale white to a pinkish or brownish coloration (Plate 6.1) with an intensity dependent upon the nature of exogenous nutrients. This change in colour was likely to be due to enzymic oxidation of lignin or cleavage of lignin carbohydrate bonds yielding reactive radicals that generate chromophores (Plate 6.1).

With all three fungi, additions of minimal medium to sawdust promoted rapid colonisation and only traces or scanty hyphal growth was observed with distilled water. However, with *P. chrysosporium* addition of distilled water, followed by autoclaving, yielded maximal mycelial growth and substrate colouration. With the other two fungi greater colour change was observed with minimal medium.

With addition of 1% maltose, sucrose, lactose, or glucosamine, growth of *P. sajo-caju* and colouration was low. Similarly, 1% lactose or glucosamine repressed colouration by *P. chrysosporium* and glucosamine had a similar effect on *C. versicolor* fermentations.

TABLE 6.17 VISUAL OBSERVATION ON THE EFFECT OF SUGARS ON DISCOLOURATION OF SPRUCE SAWDUST DURING SOLID SUBSTRATE FERMENTATION.

SUBSTRATES(1%)	FUNGI		
	C.versicolor	*P.sajo-caju*	*P.chrysosporium*
glucose	+++++	++++	++++
xylose	++++	+++	++++
mannose	+++++	++	++++
galactose	+++	nd	++
arabinose	+++	nd	++
sucrose	+++++	++	++++
lactose	+++	-	+
D-glucosamine	++	++	+
maltose	+++++	++	+++++
malt extract	++++	++++	++++
yeast extract	++++	++++	+++
sterile water	++	++	++++
water	++	++	++++
minimal media	++++	++++	+++

nd= not determined

-= no decolourisation, + =traces, ++ =low, +++= average, ++++=high, +++++= very high.

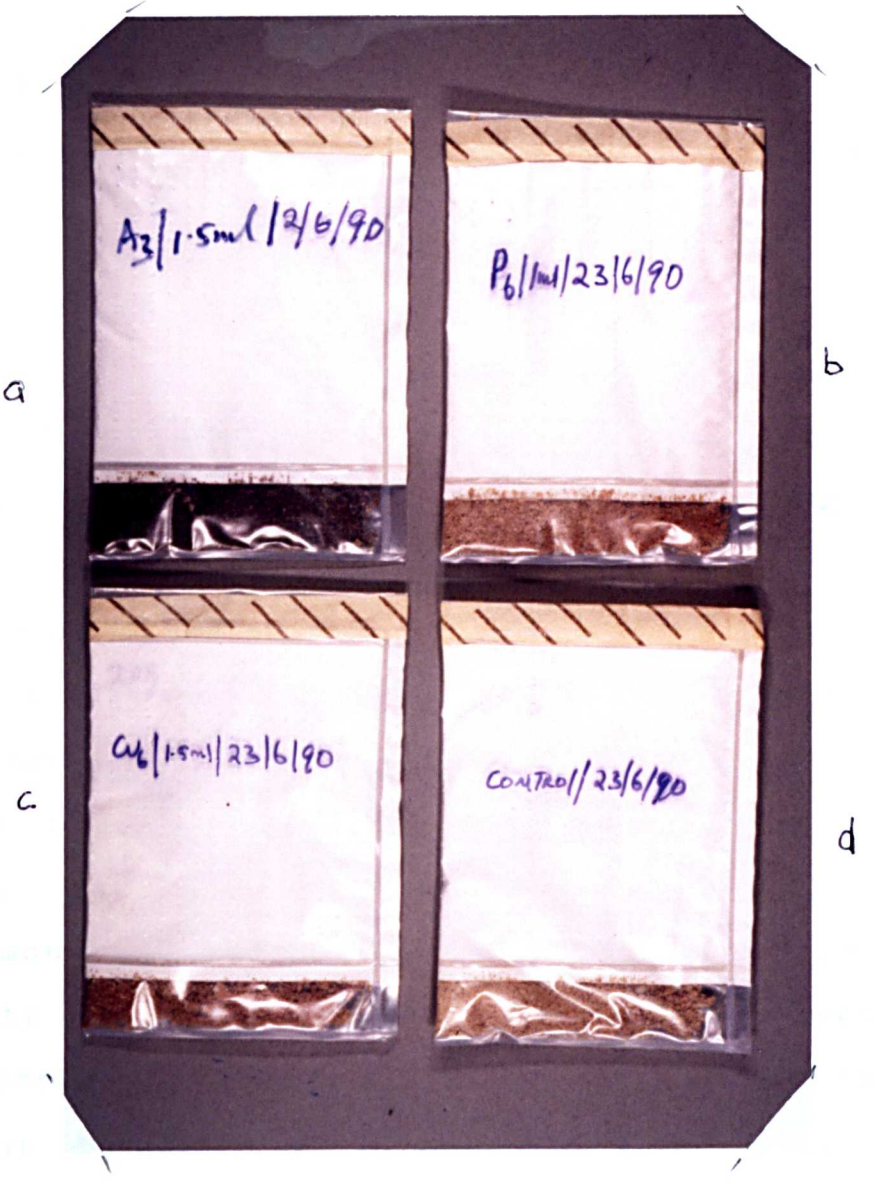


PLATE 6.1 Treated spruce wood showing colouration by (a) S. lignicola (b) P. chrysosporium (c) C. versicolor (d) Control

Addition of glucose, mannose, sucrose, maltose, or malt extract stimulated colouration by *C. versicolor* and *P. chrysosporium*. Rapid colonisation was observed when either 1% yeast extract or mycological peptone were added.

6.3.14 Synergy between strains

6.3.14.1 Effect of co-cultures on delignification and digestibility

With co-culture of fungi for 7 days, 3.5 - 10 % delignifications of substrates were achieved compared with 0 - 0.3% for monocultures (Table 6.18). Maximal reduction in lignin (16.2%) was recorded with a 3-week fermentation with all three fungi. Co-culture with *P. sajo-caju* had no effect on delignification by *P. chrysosporium*. Delignification was significantly higher with co-culture of *C. versicolor* and *P. sajo-caju* or *C. versicolor* and *P. chrysosporium* than with any monoculture.

With monocultures 180 - 250% increases in digestibility of sawdust were obtained after four weeks of incubation but mixed cultures achieved 250 - 370% increases in digestibility in less than three weeks of fermentation. However for both mono- and mixed cultures, cyclical fluctuations in dry matter digestibility were observed with fermented sawdusts (Table 6.18).

TABLE 6.18 RELATIONSHIP BETWEEN LIGNIN LOSS AND [a]DIGESTIBILITY INCREASES OF MONO AND MIXED CULTURE FERMENTED SAWDUST.

	PERIOD OF INCUBATION(WEEKS)					
	1	2	3	4	6	8
MONOCULTURES						
C.versicolor						
% lignin loss	-1.54	1.03	5.61	4.66	14.86	7.12
% DIG. increase	59.74	43.61	121.03	132.39	244.26	179.48
P.sajo-caju						
% lignin loss	0.26	-1.61	0.15	0.74	4.51	6.68
% DIG. increase	30.71	21.29	69.81	140.13	186.71	113.03
P.chrysosporium						
% lignin loss	0.04	5.12	8.24	2.67	8.28	9.35
% DIG. increase	163.33	151.73	161.48	113.83	182.10	120.98
MIXED CULTURES						
C.versicolor + P.sajo-caju						
% lignin loss	4.70	7.18	14.31	14.05	9.76	17.02
% DIG. increase	195.2	288.76	237.41	289.26	85.43	54.32
P.sajo-caju + P.chrysosporium						
% lignin loss	3.44	10.43	7.81	11.13	5.55	6.88
% DIG. increase	200.74	253.83	223.83	232.96	132.96	149.88
C.versicolor + P.chrysosporium						
% lignin loss	3.99	8.14	13.39	13.20	17.94	15.09
% DIG. increase	226.30	258.03	282.72	205.56	103.09	114.69
C.versicolor + P.sajo-caju + P.chrysosporium						
% lignin loss	9.80	10.06	16.24	14.39	9.95	13.54
% DIG. increase	156.05	258.52	364.93	314.81	115.06	179.75

$$a = \frac{\%\ \text{Digestibility of test sample} - \%\ \text{Dig. of control}}{\%\ \text{Digestibility of control(un-inoculated sawdust)}} \times 100$$

6.3.14.2 Depletion of cellulose and hemicelluloses with mixed cultures

Fermentations of 14 days resulted in depletions of cellulose of between 3.1% and 11.2% and of hemicellulose between 12.1% and 53.4% (Table 4.19). Cellulose losses were less in mixed cultures than in monocultures. With hemicellulose the opposite was true, with co-cultures of *P. sajo-caju* and *C. versicolor* and all three fungi. Co-culture of *P. sajo- caju* and *P. chrysosporium* gave a similar depletion to either monocultures. In contrast hemicellulose was broken down faster by monocultures of *C. versicolor* than co-cultures of *C. versicolor* and *P. chrysosporium*. With increasing incubation artefactual increases in cellulose and hemicellulose content of samples were observed.

Substrates fermented by mixed cultures evolved more rumen carbon dioxide than controls (Table 6.20), particularly when substrates were fermented for less than four weeks. More carbon dioxide was released from substrates fermented by monocultures for longer periods than that recovered during the early stages of fermentation. Maximal carbon dioxide release was observed with sawdust fermented with all three fungi and with co-cultures of *C. versicolor* and *P. chrysosporium*. In monocultures, fermentation with *P. chrysosporium* for 8 weeks gave the highest carbon dioxide yield. Only minimal or trace amounts of methane were detected with all fermented substrates.

TABLE 6.19 RELATIONSHIP BETWEEN DEGRADATION (%) OF SAWDUST POLYSACCHARIDE BY MONO AND MIXED CULTURES

	PERIOD OF INCUBATION(WEEKS)				
	Control	2	4	6	8
MONOCULTURES					
C.versicolor					
cellulose	43.5	42.17(3.06)[a]	42.76	37.86	40.22(7.5)
hemicellulose	18.4	10.49(43.0)	18.47	17.50	15.73(14.5)
P.sajo-caju					
cellulose	43.5	38.61(11.2)	40.58	41.01	40.28(7.4)
hemicellulose	18.4	16.17(12.12)	15.74	17.61	17.32(5.87)
P.chrysosporium					
cellulose	43.4	39.00(10.3)	40.22	39.79	40.02(8.0)
hemicellulose	18.4	13.66(25.8)	15.74	15.95	15.93(13.43)
MIXED CULTURES					
C.versicolor + P.sajo-caju					
cellulose	43.5	42.04(3.36)	41.01	42.76	41.77(3.98)
hemicellulose	18.4	8.99(51.14)	7.84	8.82	10.89(40.8)
P.sajo-caju + P.chrysosporium					
cellulose	43.5	41.62(4.32)	42.67	40.81	41.62(4.32)
hemicellulose	18.4	15.21(17.4)	9.99	12.36	12.04(34.57)
C.versicolor + P.chrysosporium					
cellulose	43.5	40.58(6.7)	40.16	40.71	40.28(7.4)
hemicellulose	18.5	14.21(23.19)	10.87	11.95	11.83(35.71)
C.versicolor + P.sajo-caju + P.chrysosporium					
cellulose	43.5	40.28(7.40)	41.94	40.71	41.34(4.97)
hemicellulose	18.4	8.57(53.42)	6.41	8.66	9.69(47.34)

All values are means of at least two determinations

[a]Values in parenthesis indicate percentage losses.

TABLE 6.19b TOTAL CELL WALL POLYSACCHARIDE(%) AFTER FERMENTATION WITH MONO- AND MIXED CULTURES

	PERIOD OF INCUBATION (WEEKS)				
	CONTROL	2	4	6	8
MONOCULTURES					
C. versicolor	61.9	52.66	61.23	55.36	55.95
P. sajo-caju	61.9	54.78	54.78	58.62	57.60
P. chrysosporium	61.9	52.66	55.96	55.74	55.95
MIXED CULTURES					
C. versicolor + P. sajo-caju	61.9	51.03	48.85	51.57	52.66
P. sajo-caju + P. chrysosporium	61.9	56.83	52.66	53.17	53.66
C. versicolor + P. chrysosporium	61.9	54.79	51.03	52.66	52.11
P. sajo-caju + C. versicolor + P. chrysosporium	61.9	48.85	48.35	49.37	51.03

All values are average of at least two replications.

TABLE 6.20 RELATIONSHIP BETWEEN INCUBATION TIME AND IN VITRO RUMEN PERFORMANCE OF MIXED AND MONOCULTURE FERMENTED SPRUCE SAWDUST.

	PERIOD OF INCUBATION(WEEKS)			
	Control	2	4	8
MONOCULTURES				
C.versicolor				
CO_2	5.51 ± 0.47	6.1 ± 0.15	6.30 ± 0.12	7.5 ± 0.25
CH_4	0.014	0.019	0.05	0.016
P.sajo-caju				
CO_2	5.51 ± 0.47	5.64 ± 0.22	4.9 ± 0.5	5.3 ± 0.15
CH_4	0.014	traces	traces	traces
P.chrysosporium				
CO_2	5.51 ± 0.47	6.01 ± 0.20	6.7 ± 0.5	8.1 ± 0.16
CH_4	0.014	0.016	0.08	0.027
MIXED CULTURES				
C.versicolor + P.sajo-caju				
CO_2	5.51 ± 0.47	6.22 ± 0.49	6.18 ± 0.53	6.52 ± 0.14
CH_4	0.014	0.010	traces	traces
P.sajo-caju + P.chrysosporium				
CO_2	5.51 ± 0.47	6.33 ± 0.73	6.55 ± 0.41	6.51 ± 0.86
CH_4	0.014	0.012	traces	0.016
C.versicolor + P.chrysosporium				
CO_2	5.51 ± 0.47	7.05 ± 0.78	6.93 ± 0.26	6.80 ± 0.15
CH_4	0.014	0.013	0.012	traces
C.versicolor + P.sajo-caju + P.chrysosporium				
CO_2	5.51 ± 0.47	7.87 ± 0.91	6.51 ± 0.46	6.4 ± 0.28
CH_4	0.014	traces	0.011	0.010

All values are average of two determinations.
Methane and carbon dioxide concentration reported as umol ml^{-1} gas space

6.3.14.3 Depletion of polysaccharides with mixed cultures

Polysaccharide depletion was more rapid with mixed cultures than with monocultures (Table 6.19b). Greatest loss of polysaccharide was observed in fermentations with all three fungal strains whereas in the monocultures, sawdust fermented with either *P. chrysosporium* or *C. versicolor* showed the greatest depletion. The increase in rate of depletion was not linear with time and with both mixed and monocultures polysaccharide depletion was reduced in the final four weeks of fermentations.

6.3.14.4 Changes in pH of fermented substrates

Mixed and monocultures produced similar changes in pH of fermented spruce sawdust (Figs. 6.1 & 6.5). With both mixed and monoculture fermentations with *P. chrysosporium*, decreases in the pH of fermented substrates were observed.

6.3.14.5 Changes in total solubles and reducing sugar

In all mixed cultures with the exception of co-culture of *C. versicolor* and *P. sajo-caju* a cyclical pattern of accumulation of total solubles and reducing sugar was observed (Table 6.21). There were 2-3 fold greater accumulations in mixed cultures than with monocultures, with the exception of *C. versicolor*. More reducing sugar accumulated in multiple cultures than in co- cultures and the maximum value was observed in multiple cultures after three weeks of fermentation. In

TABLE 6.21 [a]RATIO OF REDUCING SUGAR ACCUMULATION IN MONO AND MIXED CULTURE FERMENTED SAWDUST

	PERIOD OF INCUBATION(WEEKS)					
	1	2	3	4	6	8
C.versicolor + P.sajo-caju	1.84[a]	3.89	4.89	8.74	8.54	8.99
P.sajo-caju	1.21	1.33	1.36	0.94	1.38	0.94
C.versicolor	1.31	1.85	3.04	2.43	3.71	2.97
P.sajo-caju + P.chrysosporium	2.98	4.11	4.91	4.86	5.21	3.92
P.chrysosporium	1.59	2.87	3.63	3.04	3.43	3.14
Cversicolor + P.chrysosporium	3.48	5.68	6.41	9.65	9.49	7.52
P.sajo-caju + C.versicolor + P.chrysosporium	3.54	6.17	9.94	7.79	7.46	6.88

$$a = \frac{X \text{ mg ml}^{-1} \text{ sugar of test sample}}{y \text{ mg ml}^{-1} \text{ sugar of control sample(un-inoculated)}}$$

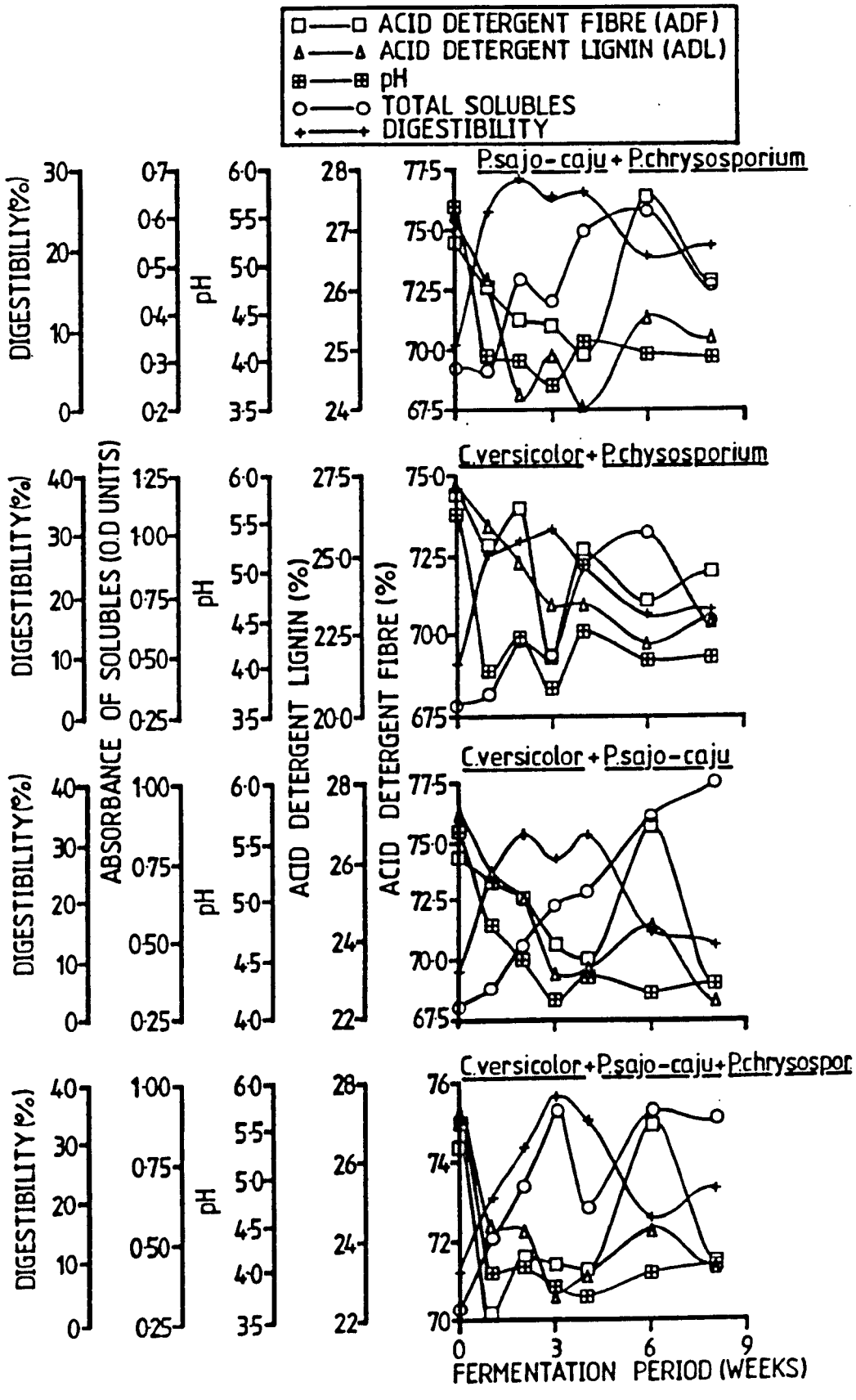


Fig. 6.5 Physiological changes during mixed culture fermentation of spruce sawdust.

co-cultures of *C. versicolor* and *P. sajo-caju* accumulation of reducing sugar was greatest at the end of 56 days.

6.3.14.6 The nature of mycelial interaction in surface culture

Photomicrographs of fungi growing on sawdust agar suggested intermingling of hyphal filaments in mixed cultures. Crossing of mycelial mats across hyphal boundaries and high concentration of hyphal filaments within interaction regions were observed in contrast to sparse hyphal cells within the domain of each fungus (Plates 6.2a - g). Co-culture of *C. versicolor* + *P. sajo-caju* on malt extract lignin cellulose agar suggested organisms were competing on an equal basis (Plate 6.4). With *P. chrysosporium* there was initially intermingling of hyphae on contact, but subsequently the faster growing *P. chrysosporium* appeared to overgrow the other organism (Plate 6.5 - 6.6). This may not, of course, be typical of behaviour on the native lignocelluloses because of the low nitrogen and soluble carbohydrate contents of agar media.

6.3.14.7 Interactions between ligninolytic and cellulolytic fungi

Synergy between ligninolytic and cellulolytic fungi was sought, to establish how fungi would behave in mixed culture fermentations under sterile and non-sterile conditions. Results obtained (Table 6.22) suggested that fungi with low moisture requirements such as *Trichoderma*

TABLE 6.22 INTERACTION BETWEEN LIGNOCELLULOSE DEGRADING FUNGI ON MALT EXTRACT CELLULOSE LIGNIN AGAR AT 0.98a_w AFTER 10 DAY INCUBATION PERIOD.

AT 25°C

	PSC	CV	P	CH	TR	C	R	TH
PSC	-	3*	3	3	4	4	4	4
CV	3	-	2	2	4	2	4	4
P	3	2	-	3	4	5	4	4
CH	3	2	3	-	4	2	4	4
TR	4	4	4	4	-	2	2	2
C	4	2	5	2	2	-	4	2
R	4	4	4	4	2	4	-	2
TH	4	4	4	4	2	2	2	-

AT 30°C

PSC	-	3	3	3	4	4	4	4
CV	3	-	2	3	4	4	4	4
P	3	2	-	nd	4	4	4	4
CH	3	3	nd	-	4	4	4	4
TR	4	4	4	4	-	2	2	2
C	4	4	4	4	2	-	4	4
R	4	4	4	4	2	4	-	2
TH	4	4	4	4	2	4	2	-

AT 40°C

PSC	NG	0	0	0	0	0	0	0
CV	0	NG	0	0	0	0	0	0
P	0	0	-	3	0	2	4	0
CH	0	0	3	-	0	2	2	0
TR	0	0	0	0	0	0	0	0
C	0	0	4	2	0	-	2	0
R	0	0	4	2	0	2	-	0
TH	0	0	0	0	0	0	0	0

PSC=*P. sajo-caju*, CV= *C. versicolor*, P= *P. chrysosporium*, CH=*C. cellulolyticum* TR= *T. reesei* , C= *Aspergillus terreus* , R = *A. corymbifera* , TH= *T. harzianum*

* See appendix 3 for description of numerical values.

nd = not determined

[1]PLATE 6.2a- g Microscopic observation of interaction between lignolytic fungi in sawdust agar media ;

(2a) Control culture

(2b) Mycelial structures observed within the domain of *C.versicolor* during interaction with *P. sajo-caju.*

[1] Note the presence of dense hyphal filaments within interation zones (Plates 6.2c, 6.2e & 6.2g) compared to scanty hyphal filaments at the domain of individual fungus (Plates 6.2b, 6.2d & 6.2f). **Mag. X 200**

PLATE 6.2a

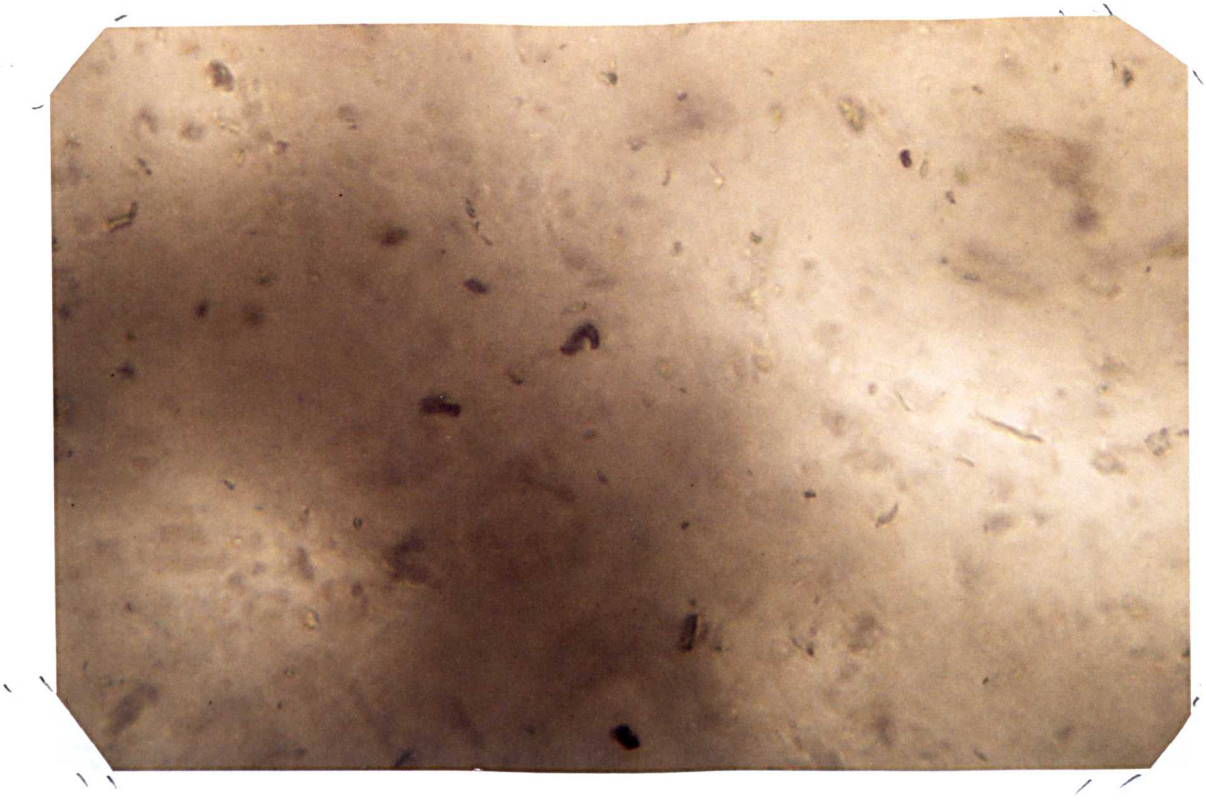
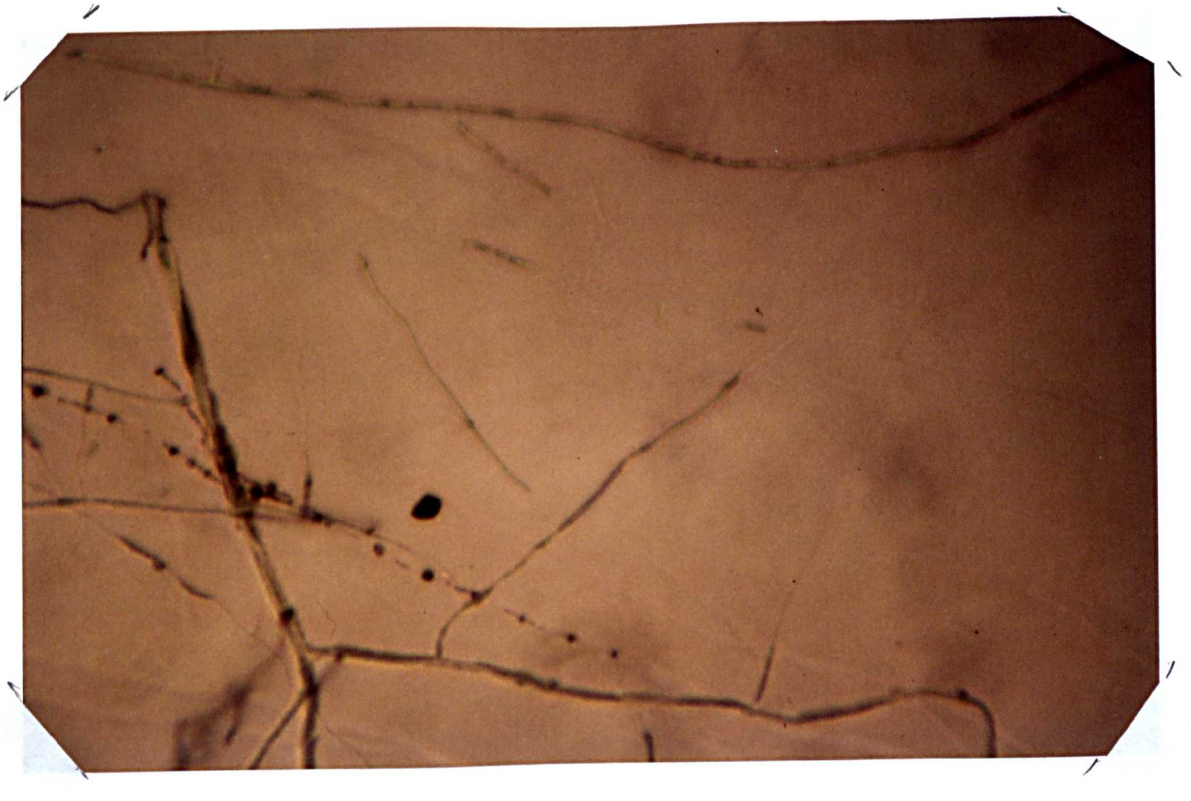


PLATE 6.2b



(2c) Mycelial structures observed within the interaction zone of *C.versicolor* and *P.sajo-caju.*

(2d) Mycelial structures observed within the domain of *P.sajo-caju*

PLATE 6.2c

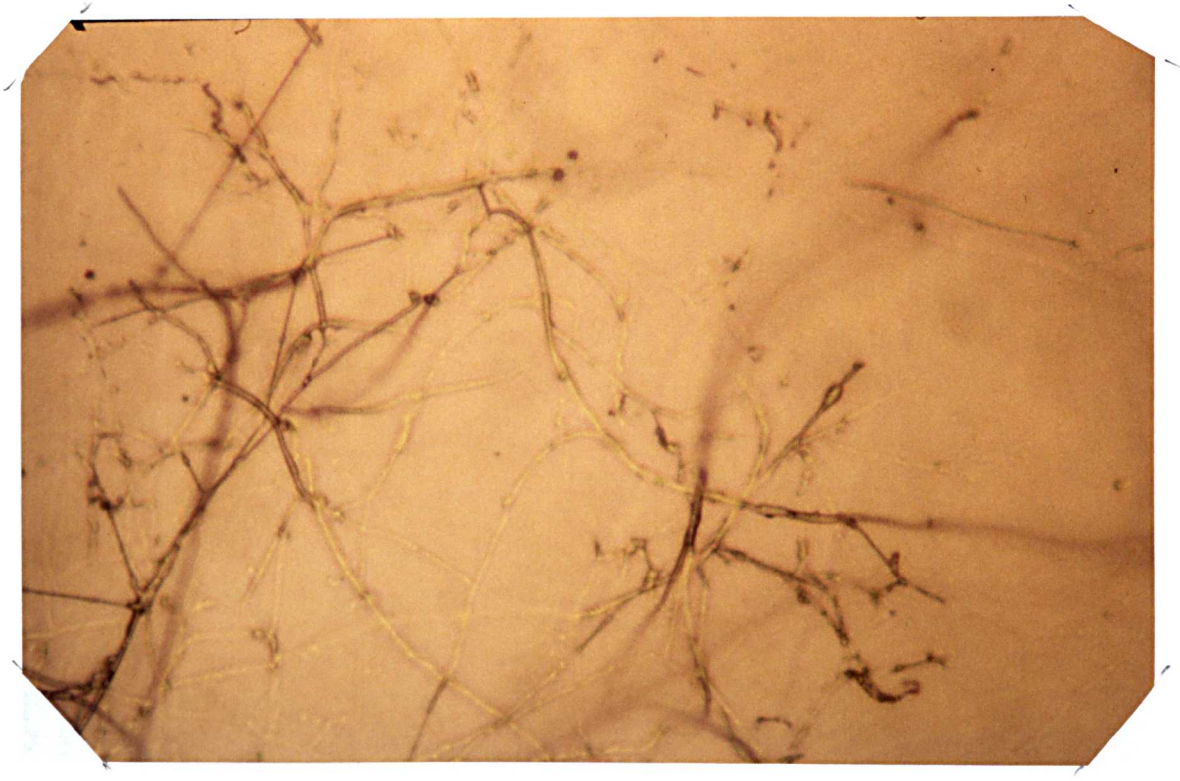
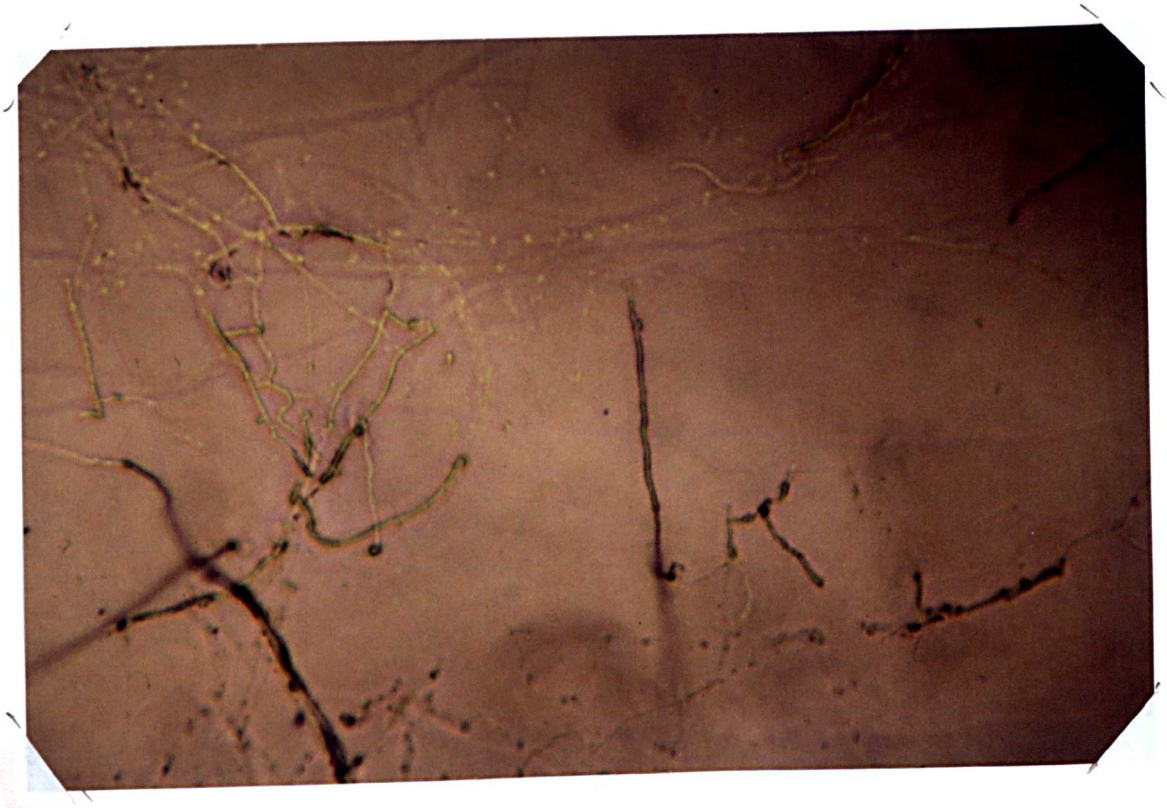


PLATE 6.2d



(2e) Mycelial structures observed within the interaction zone of *P.chrysosporium* and *P.sajo-caju*

(2f) Mycelial structures observed within the domain of *P.chrysosporium*

PLATE 6.2e

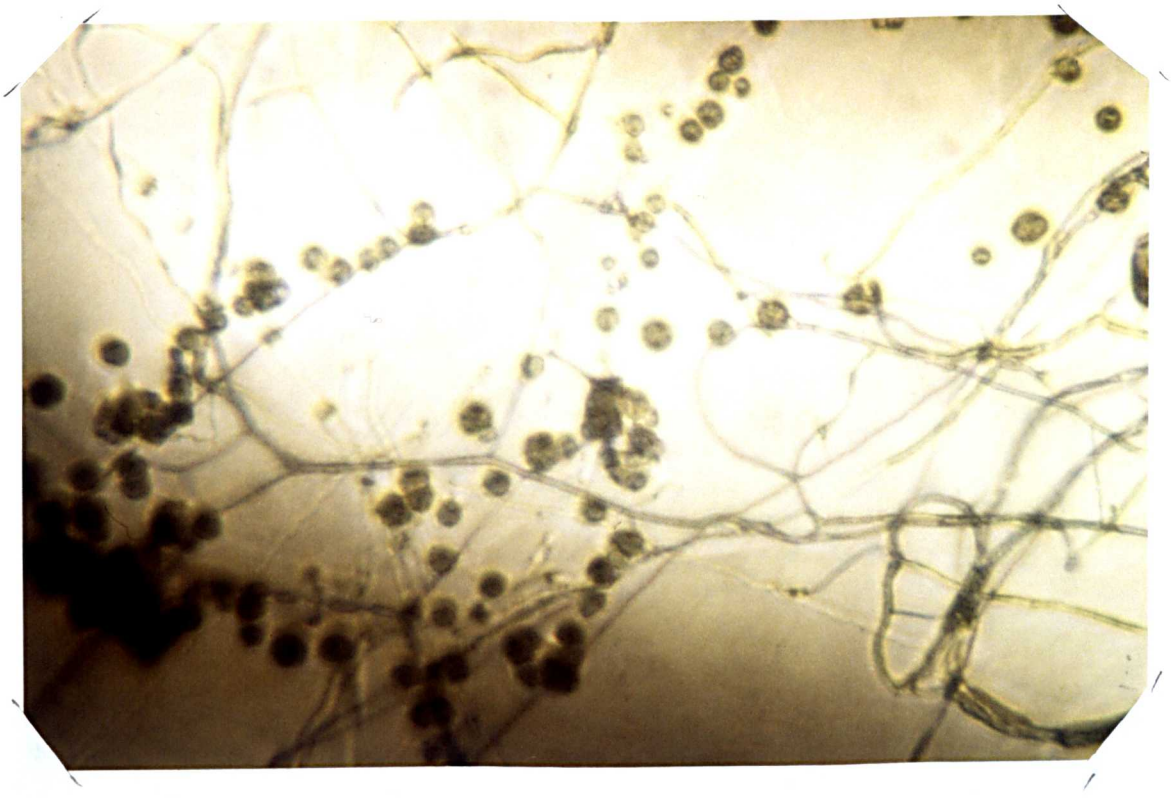
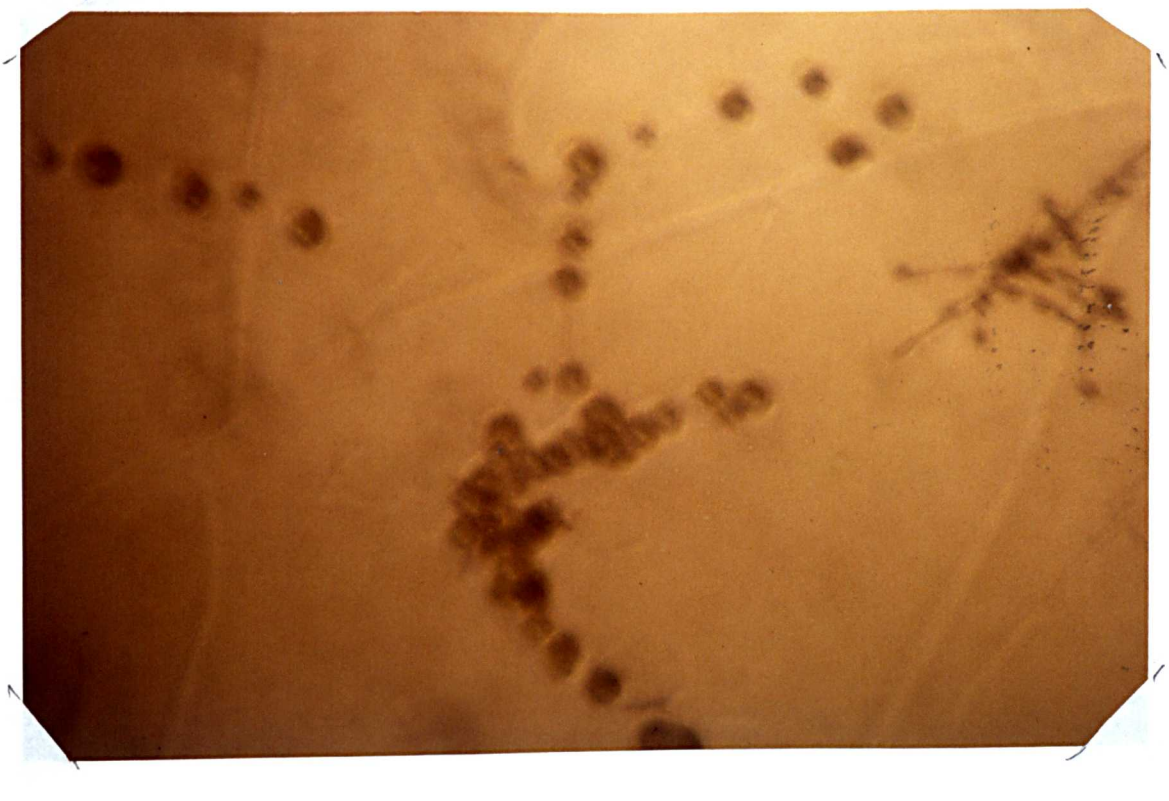
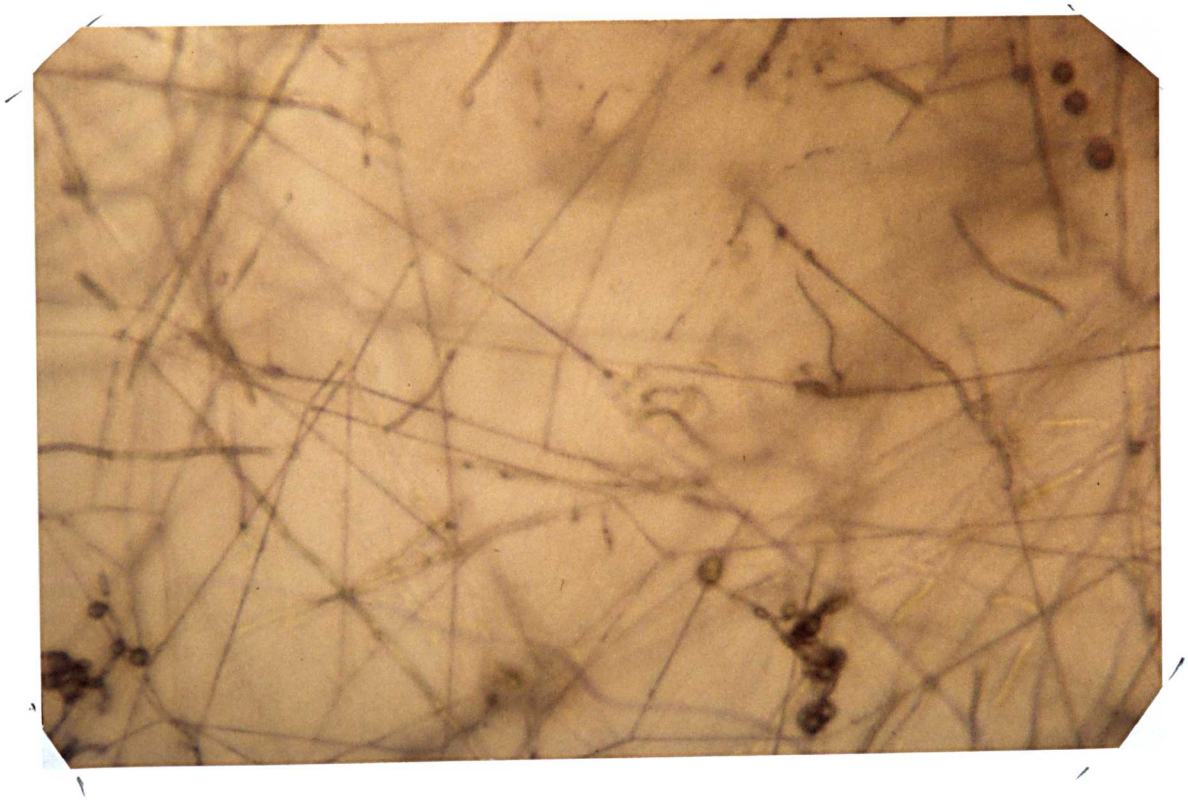


PLATE 6.2f





(2g) Mycelial structures observed within the interaction zone of P. chrysosporium and C. versicolor

PLATE 6.3 Interaction between *P.chrysosporium*(A) and *P.sajo- caju*(C), *C.versicolor* (B) on malt extract agar after 3 days at 28.5°C

PLATE 6.4 Interaction between *C.versicolor* (B) and *P.sajo- caju* (C) after 5 days on malt extract agar.

Plate 6.3

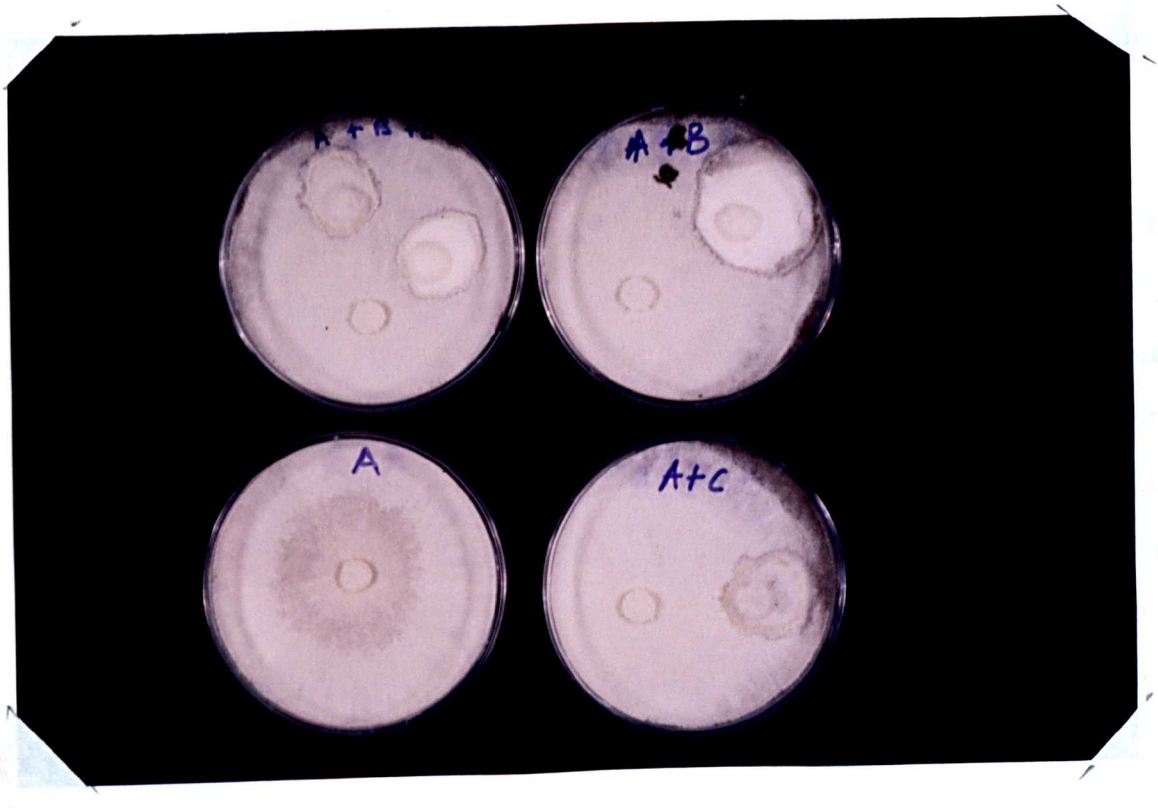
Plate 6.4

PLATE 6.5 Interaction between *P. chrysosporium* (A), *C.versicolor* (B) and *P.sajo-caju* (C) after 3 days on malt extract agar.

PLATE 6.6 Interaction between (i) all three lignolytic strains. (ii) *P.chrysosporium* (A) with either *C.versicolor* (B) or *P.sajo-caju* (C).

Plate 6.5

Plate 6.6



harzianum, *T. reesei*, *Aspergillus terreus*, and *Abysidia corymbifera*, were able to compete successfully under conditions that would inhibit competitors. In particular, slow-growing basidiomycete fungi such as *P. sajo-caju*, *C. versicolor* and the faster growing *P. chrysosporium* with higher moisture requirement were unable to compete (Plates 6.7 - 6.10). At 40°C growth of many fungi was inhibited but in co-culture of *P. chrysosporium* and *C. cellulolyticum*, and of *A. terreus* and *A. corymbifera* mutual antagonism was observed. However at 30°C, the primary ligninolytic fungi *C. versicolor*, *P. chrysosporium*, and *P. sajo-caju* appeared able to interact in co-culture given suitable levels of moisture(Table 6.23) but were not able to compete with other fungi. In contrast, the two most cellulolytic fungi (*T. harzianum* & *T. reesei*) were antagonistic towards each other and towards *A. corymbifera*(Table 6.22). However, under 0.98 a_w at 25 °C, *P. chrysosporium* was able to grow only in restricted form in co-culture with *C. versicolor*, *C. cellulolyticum* or *P. sajo-caju*.

Mixed cultures of ligninolytic fungi on malt extract cellulose lignin agar, without water activity adjustment, showed that *P. chrysosporium* intermingled with these fungi (Table 6.23) but *C. versicolor* and *P. sajo-caju* were mutually antagonistic. Under the microscope it could be seen that there was intermingling of hyphae from all three fungi below the surface of the agar, but dense mycelial mass was not formed on the surface of the

TABLE 6.23 INTERACTION BETWEEN THE THREE PHENOL OXIDASE SECRETING STRAINS WITHOUT WATER ACTIVITY ADJUSTMENT AT 28.5°C

	P. sajo-caju	*C.versicolor*	*P.chrysosporium*
In malt extract lignin cellulose agar[a]			
P.sajo-caju	-	1	4
C.versicolor	1	-	2
P. chrysosporium	4	2	-
In sawdust minimal media agar[b]			
P.sajo-caju	-	1	1
C.versicolor	1	-	1
P.chrysosporium	1	1	-

[a] by visual inspection after 3 - 5 days.

[b] by microscopic examination after 10 days.

See appendix 3 for description of numerical values.

PLATE 6.7 Interaction between ;

(i) *C. versicolor*(A) and *C. cellulolyticum* (B).

(ii) *C. versicolor*(A) and *P. chrysosporium* (C).

(iii) *C.cellulolyticum* (D) and *A. corymbifera*(E)

(iv) *C.versicolor*(A) and *A. terreus* (F).

PLATE 6.8 Interaction between ;

(i) *A.terreus* (A) and *P.chrysosporium* (B)

(ii) *T.harzianum* (C) and *P.chrysosporium* (B)

(iii) *A.fumigatus* (D) and *P.chrysosporium* (B)

(iv) *C. cellulolyticum*(E) and *P. chrysosporium* (B).

at 25^{o}C and 0.98 water activity.

Plate 6.7

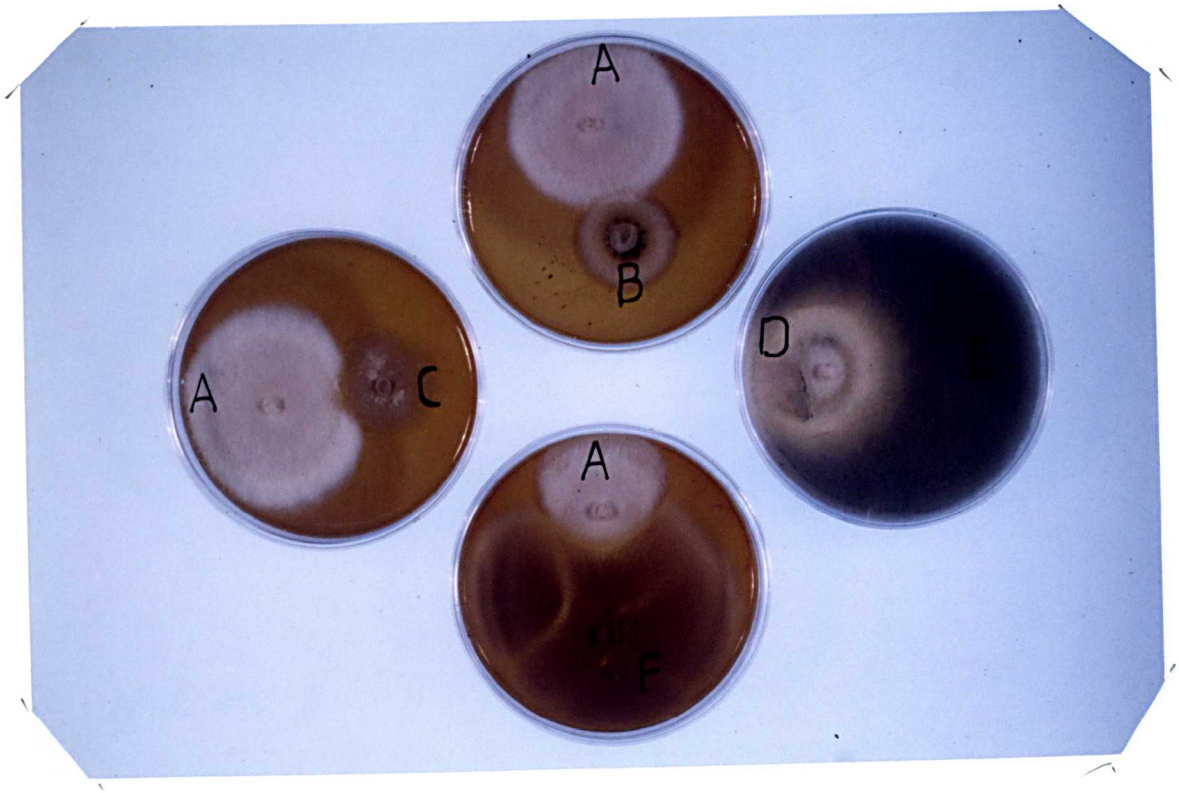


Plate 6.8

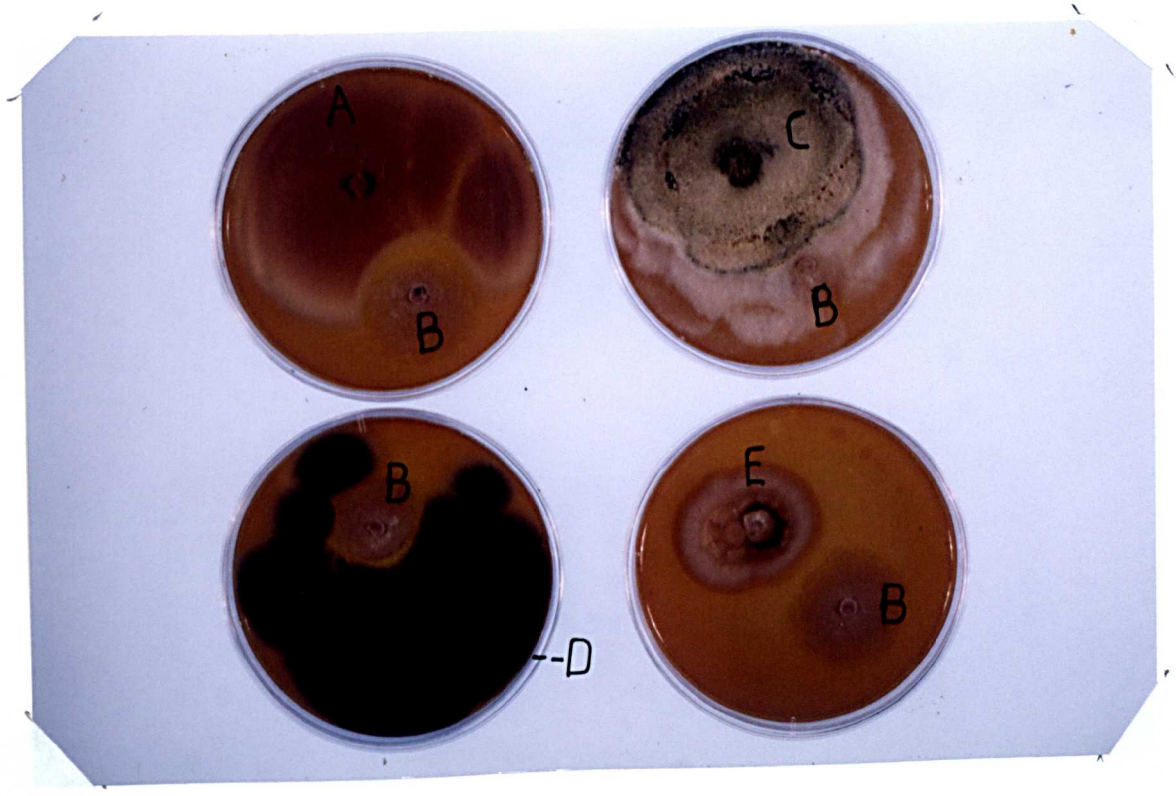


PLATE 6.9 Interaction between;

(i) *P. chrysosporium* (A) and *C.versicolor* (B)

(ii) *C.versicolor*(B) and *P. sajo-caju* (C)

(iii) *A. fumigatus* (D) and *C. versicolor* (B)

at 25°C and 0.98 water activity.

PLATE 6.10 Interaction between

(i) *A.terreus* (A) and *P.chrysosporium* (B)

(ii) *P.chrysosporium*(B) and *C.versicolor* (C)

(iii) *A.corymbifera* (D) and *P.chrysosporium*(B)

(iv) *P.chrysosporium*(B) and *T.reesei* (E)

at 30°C and 0.98 water activity.

Plate 6.9

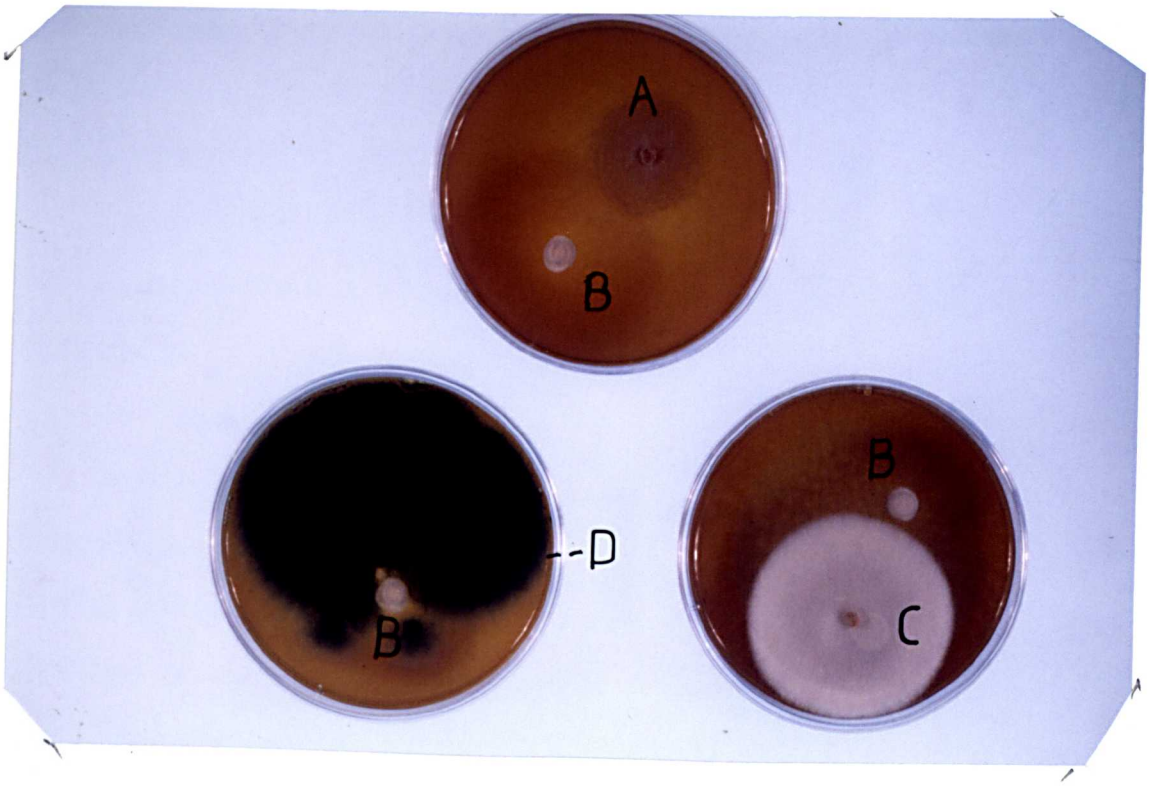
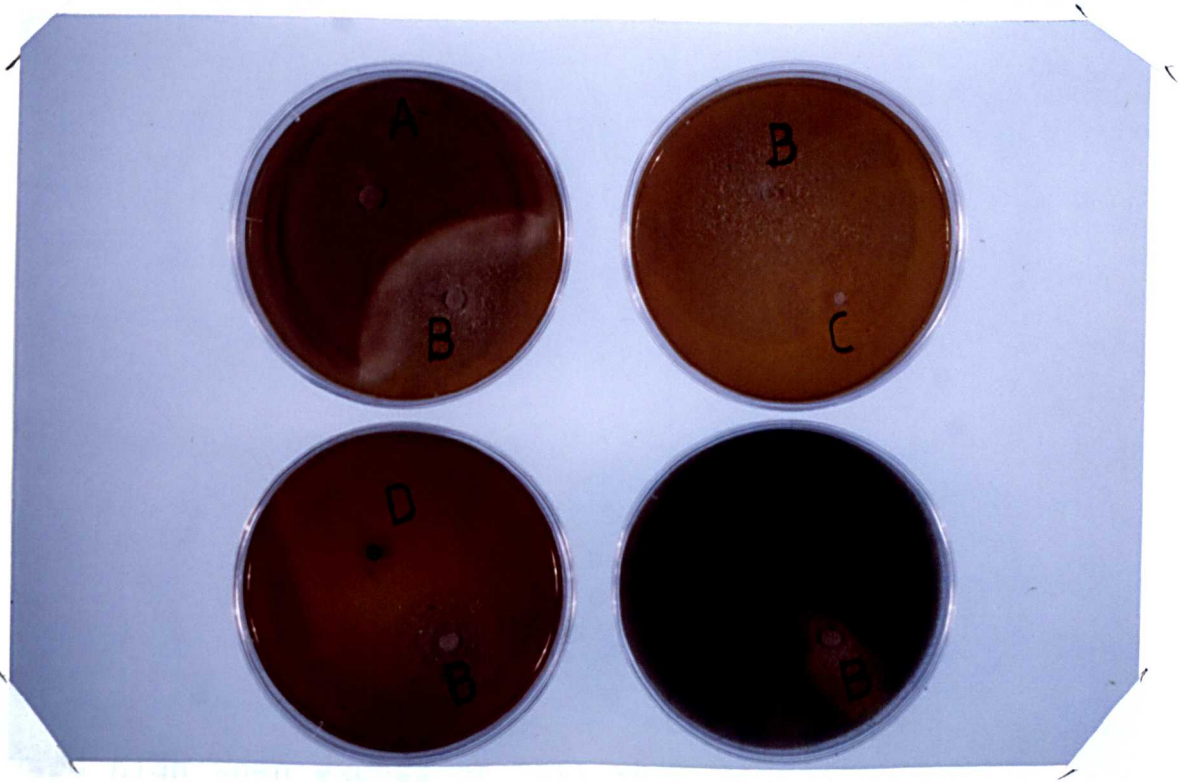


Plate 6.10



sawdust agar. This was observed with malt extract agar. Conidiospores of *P. chrysosporium* were scattered indiscriminately within sawdust agar media.

One major significant aspect of the present results is that culture pairs with reaction types 1 and 2 (Appendix 3) were considered suitable for use in mixed culture studies. Those with reaction type 4 and 5 were not deemed suitable pairs for solid state mixed culture fermentation under the conditions indicated. Culture pairs with reaction type 3 at the conditions stated may present a complex growth pattern especially when improved performance and yield is expected.

6.4 CELL FREE ENZYME EXTRACT

6.4.1 Preamble

In the last decade, the application of enzymes in feed preparation have received considerable attention. In the context of animal feed production, enzymes can have some of the following roles ;

(a) to enhance rate of digestion of fibrous feeds
(b) to improve the feed conversion ratio; and
(c) to utilise feeds with less desirable components.

Treatment of certain feeds with alpha galactosidase improves lactose utilisation by piglets whereas B-glucanases can reduce cereal B- glucan intoxication in poultry. Van der Meer(1989) noted that the risk of destruction of fibre structure and loss of carbohydrates are high when excess of cell wall degrading enzymes are

used. Moreover the application of such enzymes in ruminant feeds will be limited by their susceptibility to proteases secreted by rumen bacteria (Van der meer, 1990). However, Newbold and Wallace (1990)? have demonstrated a probiotic effect with certain fungal cultures (yeast and *Aspergillus oryzae*) in the rumen and it is possible that this is, in part, due to secretion of cell-wall hydrolysing enzymes.

6.4.2 RESULTS

Spruce sawdust was incubated in the presence of cell-free enzyme extracts of the 10-day cultures of ligninolytic fungi. Extracts of *P. chrysosporium*, used alone or blended with other fungal extracts, solubilised 1 - 2% of cellulose fractions and elicited slight reductions in lignin content (Table 6.24), whereas no cellulolytic activity was observed with other extracts either singly or in mixtures and increased ADL values were observed. It can be seen, in Table 6.25, that treated lignocellulosics gave slightly higher rates of rumen carbon dioxide evolution than control substrates. Once again, only *P. chrysosporium* had any noticeable effect. In addition, methane output did not significantly differ between treated and control samples. Increasing incubation time generally decreased carbon dioxide evolution (Table 6.26) except with *P. chrysosporium*. Evolution of methane was slightly higher with treated than with control substrates.

TABLE 6.24 EFFECT OF CELL FREE ENZYME EXTRACT(1:10) OF 10 DAY OLD FUNGI ON LIGNOCELLULOSE BREAKDOWN.

	ADL(%)	CELLULOSE(ADF - ADL)
MONOCULTURES		
Control	23.56	46.85
C.versicolor	23.96(-1.7)[a]	47.04(-0.4)
P.sajo-caju	23.94(-1.6)	47.02(-0.4)
P.chrysosporium	23.89(-1.4)	46.14(1.5)
MIXED CULTURES		
C.versicolor + P.sajo-caju	23.66(-0.4)	47.02(-0.4)
P.sajo-caju + P.chrysosporium	23.34(0.9)	46.19(1.4)
C.versicolor + P.chrysosporium	23.98(-1.8)	46.25(1.3)
C.versicolor + P.sajo-caju + P.chrysosporium	23.26(1.3)	46.11(1.6)

[a]Values in parenthesis refer to the extent of cellulose and lignin loss (%) after 48 h.

TABLE 6.25 EFFECT OF ENZYME TREATMENT (1:5) ON RUMINAL FERMENTATION CHARACTERISTICS OF SPRUCE SAWDUST.

	PERIOD OF INCUBATION(HOURS)	
	24HR	48HR
MONOCULTURES		
Control		
CO_2[a]	5.13 ± 0.32	5.13 ± 0.32
CH_4[a]	0.037	0.037
C.versicolor		
CO_2	5.58 ± 0.12	5.43 ± 0.04
CH_4	0.018	traces
P.sajo-caju		
CO_2	5.18 ± 0.07	5.23 ± 0.02
CH_4	0.020	0.040
P.chrysosporium		
CO_2	5.64 ± 0.03	6.44 ± 0.56
CH_4	0.013	0.020

All values are average of two determinations

a= rumen gases measured as μmol ml^{-1}

TABLE 6.26 RUMEN FERMENTATION CHARACTERISTICS OF SPRUCE SAWDUST EXPOSED CELL-FREE ENZYME EXTRACT(1:10)

MONOCULTURE	PERIOD OF INCUBATION(HOURS) 24	48
Control		
CO_2[a]	6.16 ± 0.029	6.16 ± 0.029
CH_4[a]	0.018	0.018
C.versicolor		
CO_2	5.90 ± 0.02	5.75 ± 0.06
CH_4	0.19	0.023
P.sajo-caju		
CO_2	6.21 ± 0.03	5.57 ± 0.06
CH_4	0.04	0.02
P.chrysosporium		
CO_2	5.57 ± 0.02	6.37 ± 0.06
CH_4	0.02	0.06
MIXED CULTURES		
C.versicolor + P.sajo-caju		
CO_2	4.77 ± 0.05	5.98 ± 0.01
CH_4	0.028	0.10
P.sajo-caju + P.chrysosporium		
CO_2	5.10 ± 0.02	5.65 ± 0.02
CH_4	0.01	0.074
C.versicolor + P.chrysosporium		
CO_2	5.27 ± 0.05	5.52 ± 0.07
CH_4	0.04	0.01
C.versicolor + P.sajo-caju + P.chrysosporium		
CO_2	5.88 ± 0.03	5.99 ± 0.03
CH_4	0.086	0.04

a = rumen gases measured as $\mu mol\ ml^{-1}$

6.5 : DISCUSSION

6.5.1 Substrates and organisms

Although biochemical pathways for the decomposition of lignocellulosic materials are not well understood it is believed that initially, soluble macromolecules are released from insoluble substrates. Subsequently compounds of lower molecular weight dominate (Giovannozi - Sermanni *et al.*, 1989). Thus, enhanced concentrations of accumulated soluble sugars and lignin degradation products can be taken as indices or evidence of degradation of cell wall polysaccharides and lignins, respectively.

Of the three classes of lignocellulosic studied, hay and barley straw were relatively rich in soluble carbohydrates(3.85 mg g^{-1} and 0.533 mg g^{-1}) and were considerably less lignified than spruce sawdust. Thus values for *in vitro* dry matter digestibility and output of rumen gases from native hay and barley straw were significantly higher than those obtained with spruce sawdust (Table 6.3). Fungal fermentations of hay enhanced apparent contents of cellulose and neutral detergent fibre (NDF). Such observations could arise from the amino sugars of fungal polysaccharides being deposited on substrates during microbial growth (Giovannozi-Sermanni *et al.*, 1989). Inoculation of barley straw with *T. harzianum*, *C. cellulolyticum* or *P. sajo-caju* appeared

to have no beneficial effect on delignification but promoted accumulation of soluble reducing sugars. However but fungal fermentations did not enhance digestibility possibly because the presence of fungal biomass reduced adhesion of rumen microbes, as proposed by Kamra and Zadrazil (1988).

With spruce sawdust there were however significant enhancements in production of rumen gases, soluble reducing sugars and slight reductions in lignin contents. In contrast, fermentation of hay (with the exception of *C. versicolor)* increased ADL values, and reduced *in vitro* dry matter digestibilities and, except with *P. sajo- caju,* depleted monosaccharides. It seems likely that structural and compositional differences in cell walls between the plant residues were the most important factors determining experimental results.

Growth of the three ligninolytic fungi - *C. versicolor, P. sajo-caju* and *P. chrysosporium* - on birch sawdust enhanced digestibility and rumen output of carbon dioxide, suggesting depolymerisation of cellulose and hemicelluloses and some degree of delignification. In contrast, fermented brans did not show improvements in rumen gas evolution and values for methane output were below those of control substrates except for bran treated with *C. versicolor.* This suggests metabolisable polysaccharides and soluble fractions had been utilised by the fungi for biomass production.

The results obtained suggest there is no advantage

in fermenting grass and cereal residues such as rice bran, hay, or barley straw, relatively rich in soluble carbohydrates, with low lignin contents and readily degraded by filamentous fungi. It would seem more effective to develop probiotic strategies such as addition of cell-free enzymes to enhance utilisation of carbohydrates in the rumen. With soft and hard woods, relatively low in soluble carbohydrates, highly lignified and recalcitrant to bacterial attack, fermentations with fungi will be more attractive. Thus this became the focus of this study.

6.5.2 Addition of ferulic acid

The effect of supplementing growth media with ferulic acid was determined in relation to delignification, accumulation of soluble sugars in media and enhancement of rumen fermentability. Although soluble sugars accumulated, rumen gas production remained low. This suggested that rumen microbial activities, including methanogenesis, were suppressed despite changes in ADL, NSP and NDF values for feeds. However varying rates for depolymerisation of cellulose, hemicelluloses and lignin were observed for the different fungi, as previously reported by Agosin *et al.* (1986). Where 0.05% ferulic acid stimulated delignification, as with *C. versicolor*, *P. sajo-caju*, and *P. chrysosporium*, it is possible that either the phenolic compound acted either as an inducer of lignin oxidases or as a reaction intermediary.

Giovannozi-Sermanni *et al.* (1982) reported that lignin oxidase production in the basidiomycete *Agaricus bisporus* was influenced by ferulic or chlorogenic acids and in other fungi ferulic acid has been shown to induce phenol oxidase production (Reihammer, 1984). However the relationship between phenol oxidase production and delignification in fungi is contradictory since Ander and Eriksson (1976) demonstrated a relationship that was later contradicted by Liwicki *et al.* (1985). Moreover it is also difficult to correlate extent of delignification with changes in rumen performance of feeds.

The transformation of spruce sawdust from pale white to pink or brownish coloration by *C. versicolor*, *P. sajo-caju* or *P. chrysosporium* may be attributed to enzymic oxidation of lignins or phenolics, linked to delignification, or possibly oxidation of carbohydrates to yield chromophores such as quinone structures. Zadrazil and Brunnert (1981) concluded that ideally maximal rate of delignification should be accompanied by minimal depolymerisation of carbohydrate . In this study it was observed that fungi which were unable to decolourise wood were preferentially attacking polysaccharide fractions of the lignocelluloses. Thus, it was concluded that although *T. harzianum* and *C. cellulolyticum* demonstrated good growth on hay and barley straw, no improvement in rumen performance was observed in these substrates after fermentation, and these strains were unlikely to prove suitable for enhancing the

nutritional value of such feeds

6.5.3 Effect of fermentation time on delignification

Weiland (1988) suggested that the length of fungal solid-substrate fermentations determined the extent of upgrading of lignocellulosics for ruminant feed production; incubation time was critical in solid state fermentation of lignocellulosic materials. Determination of the optimal fermentation period will be influenced by choice of fungal strain, nature of the substrate and rate of delignification, but may be influenced by the presence of other fermentation substrates, such as nitrogen, sulphur or trace metal sources. In this study six weeks was optimal for both enhancement of nutritional value and delignification in spruce sawdust. Agosin and Odier (1985) reported that periods varying from three to eight weeks were required, depending on substrate, for appreciable delignification and enhancement of digestibility. Zadrazil (1984) concluded *in vitro* digestibility and lignin content were inversely related and Giovannozi-Sermanni *et al.* (1989) reported that mineralization of lignin rather than solubilization of cellulose and hemicellulose were correlated with improvements in the nutritional value of high fibre plant residues.

The results obtained in this study suggested that during initial growth on lignocellulosics, fungi metabolised readily solubilised feed components,

resulting in a decrease in digestibility. Further fermentation led to significant depolymerisation of carbohydrates with release of low-molecular weight sugars and other components. During this phase delignifying fungi would produce increases in dry matter digestibility, whereas other fungi would metabolise carbohydrates accessible to rumen microbes yielding an apparent reduction in digestibility (Zadrazil & Brunnert, 1980).

6.5.4 Effect of particle size

In this study it was observed that lignocellulose which had not been ball milled, with a particle sizes of <0.5 mm, was optimal for delignification and enhancement of digestibility. Reid (1989b) also concluded that there was an optimal relationship between surface to volume ratio and ability for oxygen and hyphae to penetrate the substrate mass. However Zadrazil and Brunnert (1981), found that varying particle sizes in the range of 1mm to 8mm had no adverse effect in fermentation of relatively digestible wheat straw by *P. chrysosporium* or *Dichomitus* sp. It is certainly clear that ball milling materials leads to decreases in inter particle spaces which may adversely effect gas transfer. This may be reflected in exaggerated production of aerial mycelium. Reid (1989) noted that particle size reduction was energy intensive and thus expensive. Moreover use of larger particles ensures adequate gas exchange. In this study

particle sizes in the range between 0.5 mm and 1.5 mm were optimal, although delignification was not maximal. In fermentation studies, it has been observed that rumen microbes and particulate matter adhere to feeds blocking attack on substrate polysaccharides and lowering values for digestibility. Reid (1989) observed that hammer milled aspen wood was delignified by *Phlebia tremellosa* as rapidly as in finer particles but significantly faster than in shavings. Thus it can be concluded that the effect of particle size on delignification varies between substrates.

6.5.5 Effect of temperature on delignification

Both delignification and enhancement of digestibility have been reported to be influenced by fermentation temperature (Zadrazil and Brunnert, 1981). In this study rate of lignin loss was higher at a low temperature (30^{o}C) for *P. chrysosporium*, and greater at a temperature of 28.5^{o}C for *C. versicolor* and *P. sajo-caju*. Zadrazil and Brunnert (1981) showed that the elevated temperature of 30^{o}C had an adverse effect on delignification of wheat straw by either *P. serotinus* or *Ganoderma applanatum* and that both increasing temperature and fermentation period shifted degradation towards total organic matter breakdown by *P. sajo-caju*, *G. applanatum* and *P. ostreatus*. In this series of experiments it is possible that the lower temperature (25^{o}C) supported greater biomass production by *C. versicolor* and *P. sajo-*

caju than 28.5 ^{o}C which adversely affected detergent extractions yielding artefactually high ADL values. However there was little difference in dry matter digestibilities between substrates incubated at different temperatures, except with fermentations by *C. versicolor* where digestibility was optimal at 28.5 ^{o}C. It is possible that with *C. versicolor* mycelial biomass may reduce lignocellulose digestibility. Reid (1985) observed similar values for digestibility of aspen wood fermented between 25 and 30 ^{o}C for 8 weeks by *Merulius tremellosus*. It has been suggested that elevated temperatures result in accumulation of metabolic heat, generated by biomass, that leads to fermentation temperatures that are inhibitory (Lonsane *et al*, 1985: Reid, 1989). Other authors have suggested that forced air or water circulation or covering fermenters with wet burlap can stabilise temperatures in solid-substrate fermentations (Silman *et al.*, 1979). However, the small scale of fermentations in this project obviated the necessity for such procedures.

6.5.6 Effect of water activity on delignification

Water activities will influence delignification in solid state fermentation of lignocellulosics and Zadrazil and Brunnert (1981) concluded that a water content of 3 ml/g substrate was optimal for fermentations of straw and recorded increased production of aerial mycelium at higher water contents of 125 - 150ml/25g of substrate. In

contrast, Arora and Sandhu (1986) found moisture levels of 5 ml g^{-1} optimal for degradation of angiosperm wood by *P. versicolor*. Yadav (1988) observed an optimum of 65% for wheat straw fermentations with *Coprinus* sp for enhancement of digestibility.

In this study, the effect of water content on delignification and substrate digestibility varied . Although with *P. chrysosporium* optimal delignification was observed at 80% moisture content digestibility was not enhanced. At 70% moisture content, rates of lignin degradation and enhancement of digestibility were optimal with sawdust fermented with *P. sajo-caju* or *C. versicolor*. It is possible that low moisture contents repress mycelial growth and reduce depolymerisation through the lower degree of swelling or rapid drying of the cellulosic substrate, and high water tension (Zadrazil & Kamra, 1989). High moisture levels could reduce substrate porosity, and gas exchange, leading to the observed enhanced formation of aerial mycelium. Moreover, in the absence of humidity control, the moisture content of substrates may decrease during fermentations through evaporation promoted by heat arising from fungal metabolism. However, in this series of experiments, moisture loss was minimised by continous infusion of humid air into the bioreactor, maintaining the high relative humidity of 98% (Cuero *et al.*, 1985).

6.5.7 Changes in pH and lignin content of fermented sawdust

Following fermentations, spruce sawdust showed losses in total organic matter and increases in crude protein contents, as well as changes in pH, soluble sugar content, ADF and ADL values.

Although the influence of pH on delignification was not assessed, studies on surface culture suggested that growth of the three fungi was optimal at pH 4.5 - 5.5 and minimal at pH 3.5. Reid (1985) noted that although pH 4 - 6 was optimal for growth of *M. tremellosus*, during delignification the pH of wood was reduced to pH 3.5, although it was not clear whether this was through excretion of organic acids, uptake of basic molecules or generation of acidic carbohydrate and lignin degradation products. Reversal of acidification, by such strategies as addition of $NaHCO_3$, did not enhance delignification. Reid and Seifert (1982) had earlier showed that with growth of *P. chrysosporium* on aspen wood, pH dropped to 3.8 after three weeks of fermentation and with sawdust, pH dropped from 5.6 to 4.0 - 4.5 in the same period. Although such significant decreases in pH during solid substrate fermentations may reduce metabolic activity in ligninolytic fungi it may not inhibit enzymic oxidation of lignin. Palmer *et al.* (1987) showed that the lignin peroxidase enzyme of *P. chrysosporium* retained optimal enzymic activity at pH 2.5. Such a hypothesis is supported by the findings of Agosin *et al.* (1989) that

during growth of fungi on lignocellulosic materials, acidification of substrates to pH levels which inhibit other microbes takes place does not alter fungal metabolism .

Strategies for stabilising pH during solid substrate fermentations have not proven as successful as those in submerged batch fermentations. The addition of calcium carbonate or ammonium salts and urea (Raimbault & Alzard, 1980) or dimethyl succinate buffer (Fenn & Kirk, 1979) have been reported.

6.5.8 Accumulation of total solubles and reducing sugars

Accumulation of total solubles and reducing sugar is observed at the later stages of fermentation when higher rates of lignin depolymerisation are observed (Fig. 6.2) , suggesting rapid polysaccharide breakdown. However, the observed cyclical pattern in soluble sugar accumulation may suggest complex morphogenetic regulation.

6.5.9 Changes in cellulose, hemicellulose and ADL content

In this study it was observed that the relatively amorphous hemicellulose was the initial substrate to be depolymerised followed by the more crystalline cellulose. A similar observation was made by Blanchette and Abad (1988) who reported that the hemicellulose of the wood middle lamella was removed before the secondary wall is attacked. Ruel (1990) also showed, in cytological

studies, that *P. chrysosporium* initially attacked hemicellulose and lignin and only subsequently cellulose. It is clear that oxidative ligninases may be able to diffuse across hyphal walls but are unable to penetrate woody cell walls. Suggestions of selective attack were not supported by the conclusions of Buswell and Odier (1987), amongst others, who suggested basidiomycetes are able to deplete the three main polymers at similar rates. However, Martinez *et al.* (1990) noted preferential degradation of lignin and pentosans by *Ganoderma australe* and the conservation of glucans at different stages of huempe formation. Such differences may reflect differences in analytical procedures utilised by the different authors. With isotopic labelling of cellulose, lignin or hemicellulose comparisons of rates of mineralization can be made (Crawford & Crawford, 1978) which will not be influenced by the presence of microbial biomass. However production of such substrates was beyond the scope of this series of experiments although used by other authors in analyses of animal feedstuffs (Van soest, 1963; Reid & Seifert, 1982 ; Giovannozi-Sermanni *et al.*, 1989).

It was interesting to note that at later stages of fermentations increases in residual cellulose and hemicelluloses were observed in woods. It was predicted that contents of such carbohydrates would decrease with time. However, other authors have reported similar findings (Al-Ani & Smith, 1986; Moyson *et al.*, 1991).

Blanchette (1984) showed extensive degradation of wood hemicellulose and lignin by a wide range of white rot fungi where chemical analyses of delignified materials indicated that the residues were composed primarily of cellulose. In degraded woods higher contents of glucose and lower of xylose or mannose were observed than in sound wood. For example, 80.6% glucose from birch wood degraded by *Ganoderma applanatum* compared to 44.1% glucose in controls and 9.3% xylose and 0.85% mannose in degraded and 22.66% and 2.70%, respectively, in controls. Giovannozi - Sermanni *et al* (1989) considered that fungal polysaccharides are deposited on complex substrates during growth and Rosenberg,(1976) reported that the polysaccharide content of certain fungi, especially basidiomycetes contain S-glucan and heteropolymers which may yield glucose, mannose or galactose following acid or enzymic hydrolyses. Such artefacts may exaggerate recoveries of glucose which may be quantified as cellulose or hemicellulose. Attempts to minimise such errors by detergent extractions prior to analyses did not reduce apparent yields, although it is possible that such treatments are advantageous in the early stages of the fermentation.

6.5.10 Enhancement of protein contents of feeds

Crude protein contents of substrates increased in parallel with fermentation. This may be through accumulation of amino sugars in fungal biomass with the

slight decrease at the end of fermentations arising through cell autolysis. Such increases in nitrogen in fermented substrates may indirectly serve as a nitrogen source to rumen microbes which is often limiting in the rumen and only present in trace amounts in control lignocelluloses. High ratios of fungal biomass relative to digestible carbohydrate in fermented substrate may, however, not be beneficial to the ruminant as observed with fermented hay and rice bran.

6.5.11 Other factors in solid substrate fermentations

With *P. chrysosporium*, use of distilled water, as opposed to minimal salts medium, was found to enhance delignification but the converse was observed with *P. sajo-caju* and *C. versicolor*. Ammonium salts in minimal media may suppress delignification (Glenn & Gold, 1983 ; Buswell *et al.* , 1984) although this has not been reported for either *P.sajo-caju* and *C.versicolor* (Leatham & Kirk, 1983). Moreover, autoclaving in the presence of distilled water enhances delignification by *P.sajo-caju* and *C.versicolor* over non-sterile controls. Reid (1989) reported that autoclaving inhibited germination, colonisation and growth of microflora indigenous to substrates on wetting. Moreover fast-growing and antagonistic fungi, such as cellulolytic ascomycetes and hyphomycetes, which often suppress wood decay by white rot fungi are eliminated in sterilised or autoclaved substrates. On the other hand, comparison of ADL values

suggests delignification may have been enhanced in non sterile cultures and similar observations were made for total solubles and output of rumen gases which were higher than in sterile cultures. However, ADL values of non-sterile wood, and other parameters suggest the presence of contaminating fungi on natural substrates. This hypothesis is supported by the observation that during incubations control sawdusts were decolourised. Such contaminants have been noted by other authors to inhibit delignification and deplete soluble sugars in solid state fermentation (Reid, 1989). The contaminants observed in this study may secrete phenol oxidases since uninoculated control sawdusts are decolourised. A further relevant observation is that of Mudget and Paradis (1985) who concluded that autoclaving birch sawdust partially inhibited lignin degradation and enhanced degradation of polysaccharides by *P. chrysosporium*.

6.5.12 The effect of level of atmospheric oxygen

A number of authors have concluded that fungal delignification is influenced by the ratio of oxygen to carbon dioxide present in the gas phase (Leisola *et al.*, 1983; Reid & Seifert, 1982). Reid and Seifert (1982) reported that in an atmosphere of 100% oxygen *P. chrysosporium* and *Gloeoporous dichrous* mineralised aspen wood more rapidly than in air. In this series of experiments it was found that oxygen the utilised was directly proportional to the output of carbon dioxide by delignifying fungi. This may partly explain the increased

rates of degradation of organic matter and lignin in the presence of oxygen rather than air observed by other authors (Reid & Seifert, 1982 ; Zadrazil & Kamra, 1989). From the results obtained (Table 6.10 & Fig. 6.4), it appears that *C. versicolor* and *P. chrysosporium* have comparable and *P. sajo-caju* least requirement for oxygen. Zadrazil and Kamra (1989) had earlier observed that *Pleurotus eryngii* degraded lignin more rapidly in air than *P. sajo-caju*. Moreover, Zadrazil *et al.* , (1990) reported that during solid state fermentation of poplar wood in air cultures, carbon dioxide content increased for the first 12hr and then slowly decreased. The reduced rate of carbon dioxide evolution in cultures flushed once during the entire sampling period compared to substrates flushed weekly may be attributed to accumulation of high concentrations of gaseous metabolites which could affect fungal metabolism (Mudgett & Paradis, 1985 ; Zadrazil and Kamra, 1989). It is probable that the low oxygen content of atmospheric air may be the limiting factor in most lignin degradation systems. Although constant flow of oxygen to enhance organic matter decomposition may seem attractive, the economics of such a process may restrict it to laboratory scale operation

The influence of addition of exogenous carbohydrates sources on delignification has been reported to be strain-specific (Ander & Eriksson, 1975; Bone & Levonen-Munoz, 1984). The highest rates of lignin degradation by

C. versicolor and *P. sajo-caju* were evoked by the presence of glucose and mannose respectively. From Tables 6.8 & 6.11 , it can be seen that in these organisms mannose, glucose and rhamnose were preferentially attacked in complex substrates. With *P. chrysosporium* the greatest delignification was observed in supplementations with glucose, also preferentially metabolised by this organism (Table 6.8). Other sugars such as xylose or galactose appeared to reduce the rate of delignification. In contrast, Bone and Levonen-Munoz (1984) reported that addition of xylose to oat straw accelerated lignin degradation by either *Polyporous* sp. or *Irpex lacteus* and other authors have emphasised the importance of supplementary carbon sources especially glucose in lignin degradation (Wallace *et al.*, 1984).

Leisola *et al.* (1982) concluded that lignin depolymerisation in *P. chrysosporium* was evoked after exhaustion of available nitrogen and was partially inhibited by high glucose concentrations, possibly due to the physical barrier provided by extracellular glucan synthesised by this organism at high carbon/nitrogen ratios in media (Liwicki *et al.* , 1985). Eriksson *et al.* (1983) noted that exogenous glucose increased weight loss during solid state fermentation of pine and spruce wood by a cellulase-less mutant of *P. chrysosporium*. Results obtained with exogenous disaccharides are more difficult to interpret. It was found that the behaviour of these fungi in the presence of exogenous

carbohydrates in surface culture assays paralleled those obtained in solid substrate fermentations. For instance, ligninase production in agar surface culture was found to be either completely repressed in the presence of glucosamine (*P. chrysosporium*) or considerably reduced (*C. versicolor* and *P. sajo-caju*). This correlated well with observed delignification in solid state fermentations in the presence of this sugar.

Minimal loss of polysaccharide was observed with glucose supplementation in each of the three fungi whereas the greatest depletion with *P. chrysosporium*, *C. versicolor* and *P. sajo-caju* was with supplementations of sucrose or mannose (Table 6.11). This suggests that in each organism cellulase activity was suppressed by the presence of glucose. Supplementation of sawdust with sugars prior to fungal fermentation decreased production of methane in the simulated rumen suggesting that either lignin or its oxidation products may repress methanogenesis.

6.5.13 Supplementation of fermentations with complex carbohydrates

Enhancing delignification by supplementation with complex substrates such as rice bran or cow concentrate does not appear to have been widely studied. Addition of 30% rice bran to spruce sawdust enhanced delignification by *C. versicolor* whereas for *P. chrysosporium* and *P. sajo-caju* less than 10% bran was optimal. Yadav (1987)

reported that addition of molasses or whey to solid substrate fermentations of wheat straw decreased the extent of delignification by *Coprinus* species. Addition of birch sawdust did not enhance delignification of spruce by *P. sajo-caju* and *P. chrysosporium*. Supplementations, such as rice bran or cow concentrate, provide a readily accessible growth substrate in terms of susceptibility of carbohydrates and proteins to hydrolysis. However ready access to protein nitrogen led, as predicted, to reductions in the rate of delignification by *P. chrysosporium*(Table 6.15).

6.5.14 Effect of addition of nitrogen sources to fermentations

Addition of yeast extract enhanced the rate of delignifications more than alternate nitrogen sources, as reported previously by Reid (1989a). It has also been shown that simple nitrogen sources such as ammonium nitrate and amino acids repress delignification more than complex sources such as malt extract, albumin and yeast extract (Al-Ani & Smith, 1986; Reid, 1983). However in a number of fungi nutrient nitrogen has no effect on delignification (Leatham & Kirk, 1983). Ander and Eriksson (1977) also noted rapid delignification of pine wood blocks by *Pycnopora cinnabarinus* with supplements of either malt extract, asparagine or ammonium dihydrogen phosphate. However, a number of authors have reported the converse with *P. chrysosporium*, *Phlebia tremellosa*, *Irpex*

lacteus and *Pleurotus ostreatus* (Commandy & Macy, 1985: Al -Ani & Smith, 1986). McCarthy *et al.* (1984) using mineralization of ^{14}C-labelled lignin as an indicator of lignin breakdown showed that with *C. versicolor*, *P. chrysosporium* or *S. pulverulentum* reduction of nutrient nitrogen stimulated mineralisation. Ulmer *et al.* (1983) suggested that *P.chrysosporium* could mobilise sufficient biomass nitrogen to allow cycles of new growth at 10 - 15 day intervals, enabling growth in woods of low nitrogen content. Moreover these authors considered that fungal growth could be measured most accurately as formation of novel carbohydrate and such cyclical growth would be reflected as fluctuations in mycelial dry weight and carbohydrate content. However formation of extracellular glucan may reduce the rate of oxygen transfer to the site of delignification. In contrast, Zadrazil and Brunnert (1980) observed no correlation between lignin degradation and nitrogen supplementation with *Ganoderma applanatum* and *Pleurotus* sp. *florida* although ammonium nitrate stimulated delignification by *Agrocybe aegerita*.

6.5.15 Delignification by mixed fungal cultures

The possibility of synergy in delignification of spruce sawdust by growth of mixed fungal cultures was evaluated by assessing changes in dry matter digestibility(Fig. 6.5) and gas production (Table 6.20) in the simulated rumen. It was observed that substrates fermented by consortia of fungi showed increased levels

of accumulated solubles, accelerated delignification and enhancements of rumen gas evolution when compared to substrates fermented by monocultures.

Studies of consortia in surface agar culture, utilising photomicroscopy, revealed that *P. chrysosporium*, *C. versicolor* and *P. sajo-caju* were able to grow with crossing of hyphae, showing no signs of antagonism. Since the lignolytic fungi differed in their physiology it seemed likely that synergy could be observed in delignification of sawdust. Since both *C. versicolor* and *P. sajo-caju* secrete laccases, increased levels of this enzyme could be achieved that could give elevated levels of delignification. Growth of *C. versicolor* and *P. chrysosporium* would ensure both elevated levels of lignin oxidases and the presence of laccase (Evans, 1985) and novel combinations of lignin-attacking enzymes could be achieved. The presence of oxidative cellulases would also ensure that an appropriate balance between hydrogen peroxide production and consumption could be maintained.

Synergy in attack on insoluble cell wall polysaccharide by cellulases and hemicellulases in mixed cultures has been demonstrated by Wood (1980). Such studies may clarify why such traditional fermented lignocellulosic feedstuffs as the Chilean palo podrido have greater enhancements of digestibility than substrates fermented by laboratory cultures. With palo podrido the fermentation is effected by a sequential

mixed culture rather than monocultures (Gonzalez *et al.*, 1989) as in mushroom composting. Gonzalez and his co-workers (1989) characterised the natural rain forest fermentation and identified 68 different strains of yeast and a succession of filamentous fungi, including *Ganoderma applanatum* and *Rhodotorula* sp.

6.5.16 Interaction of lignolytic fungi with other cellulolytic fungi

A number of workers have analysed interactions between organisms and enzymes to elucidate the basis of biological control and the dynamics of fungal consortia in decaying wood (Carruthers & Rayner, 1979; Magan & Lacey, 1984a & b). A series of experiments were set up to examine the effect of such strategies on fungal delignifications. Although growth of mixed cultures on semisolid agar culture has been studied (Magan & Lacey, 1984 ; Faraj, 1990) such experiments have been criticised because of differences in concentration, availability and distribution of nutrients when compared with natural substrates and solid substrate fermentations (Dowding, 1978). However, Magan and Lacey (1984b) suggested such strategies were the best available for analysing interactions between fungi. Although Dowding (1978) expressed doubts about extrapolating results obtained on surface culture to natural situations, Carruthers and Rayner (1979) concluded that behaviour of basidiomycetes on 3% malt agar was very similar to that on wood. Magan

and Lacey (1984a & b) suggested that dominance among interacting fungi would vary in relation to water activity, temperature and substrate.

The three lignolytic fungi studied in this project appeared to have similar abilities to degrade lignocelluloses and could thus cohabit successfully.

It was clear that growth of the lignin-degrading white rot basidiomycetes was slower than other fungi on malt extract lignin cellulose agar at 30 ^{o}C. Carruthers and Rayner (1979) reported that *Phlebia merismoides*, a lignolytic isolate, occurred more frequently and dominated other species in similar studies with wood media, suggesting carbohydrates in malt extract may stimulate growth of certain fungi over their competitors.

Results in co-culture of *C. versicolor* and *P. sajo-caju* were particularly promising and culture with *P. chrysosporium* also of interest. In surface sawdust cultures, *P. chrysosporium* could establish itself by both spores and mycelium and spread quickly to occupy the remaining agar being a fast growing strain.

Results in this study showed clearly that both temperature and substrate water content can play a major role in influencing the pattern of fungal interaction patterns as reported by Magan and Lacey (1984b) for storage fungi. It must be concluded that organisms with similar nutritional requirements are likely to be able to coexist on a substrate at a particular time. Moreover

it seemed that the white-rot basidiomycetes would be the most suitable fungi to effect delignification in mixed cultures.

6.5.17 The effect of cell-free enzyme extracts on substrates

Although cell-free extracts of enzymes from 10-day cultures of ligninolytic fungi produced slight improvements in rumen gas production from treated spruce sawdust, analysis of values for ADL and cellulose content revealed no marked differences between treated and control lignocelluloses. However other authors have considered that treatment of forages with hydrolytic cellulases should increase availability of soluble carbohydrates (Van der Meer & Van Es, 1987; Van der Meer & Keetelaar, 1990). However, Van Vuuren and Spoelstra (1987) reported that cellulase treatments had no effect on the less-degradable fibrous fraction of organic matter and cell wall constituents.

Van der Meer *et al.* (1988) made similar observations subsequent to the incubation of maize silage and maize gluten feed with hemicellulases (including xylanase) and cellulase preparation. Yu *et al* (1975) investigated the effect of commercial cellulase enzyme on native oat straw, recording slight increases in values for ADL and hemicellulose and slight reductions in cellulose contents.

Van der Meer and Keetelaar (1990) recently concluded

that nutrient availability in enzymically treated feedstuffs could not be estimated accurately by traditional chemical methods such as detergent extractions, which do not assess degree of structural changes in cell wall polymers and changes in the degradation characteristics of the structures. Thus in this project the simulated rumen was employed to determine whether such changes had been effected. Unfortunately the results of such experiments were inconclusive. Van der Meer and Keetelaar (1990) evaluated enzyme treatments for improving silage digestibility and reported that grass silage treated with cellulase and hemicellulases achieved significantly higher contents of acetic acid and sugar than control silages.

Although the cell free enzyme extracts utilised in this study were rich in cell-wall degrading activities conditions cannot be considered optimal for delignifications and were perhaps more suited to solubilization of natural celluloses. It has been reported that certain actinomycete enzymes are able to hydrolyse the bonds between hemicelluloses and graminaceous lignins and some pectinolytic enzymes (polygalacturonases and pectin lyases) have been reported to cleave pectin lignin bonds (Van der Meer, 1990). Such disruptions of the components of cell walls should have been reflected in this study as enhancements of delignification. However such depolymerisation reactions may be more relevant to forage feeds containing less than

10% lignin since the structure of the cell wall may be more limiting to enzyme attack in these substrates than in the more highly lignified woods.

SECTION 7

STUDY OF*IN VITRO* DIGESTIBILITY

7.1 Preamble

Reid (1989), in a review, concluded that the feed quality of wood (Kirk & Moore, 1972) and cereal straws (Zadrazil, 1984) could be enhanced by solid state fermentation with white rot fungi. In most published reports, *in vitro* digestibility has been quantified utilising crude preparations of cellulase enzymes. This does not permit the acquisition of data on ruminal performance when unconventional feeds are used. Krishna *et al.* (1986) suggested that both *in vitro* and *in vivo* assays should be evaluated for suitability for enhancing the value of agro-industrial by-products as animal feeds.

A major objective of the present study was to analyse fungal-fermented lignocellulosic materials using compositional analyses and a technique for simulating the rumen (RUSITEC). From available literature reports, this latter approach would seem to be novel.

7.2 EXPERIMENTAL - *in vitro* rumen digestibility

7.2.1 Rumen simulation technique(RUSITEC)

7.2.1 Description of the RUSITEC system (Czerkawski & Breckenridge, 1977).

The apparatus (Fig. 7.1a) consisted of four vessels. Each vessel had a capacity of 1000 ml and was secured to the base of a perspex water tank with a "Bayonet" fitting. The temperature of the water tank was maintained at 39 ^{o}C.

Each reaction vessel (R) consisted of a Perspex cylinder (254 x 76 mm) with saliva inlet at the base (I), sealed by means of a flat Perspex cover provided with a screw flange. This Perspex cover had two holes ; one for a sampling tube provided with a three way tap (V) (Pharmaseal, Liege, Belgium) and the other for an overflow tube (O) fitted through a rubber stopper into a 1 l collection flask (E). The rubber stopper also had an outlet connected to a 5 l gas bag(M) (Jencons, Hemel Hempstead, Herts), provided with a glass tap.

The food container (C) inside the reaction vessel was made of a polyethylene reagent bottle with a screw cap of similar material with an outer diameter which gave a sliding fit inside the reaction vessel. The food was put into nylon bags (125 mm X 60 mm) with 50 µm diameter perforations (Boots Co. Ltd, Nottingham). The bags (N) were closed by means of a plastic binder or cable ties

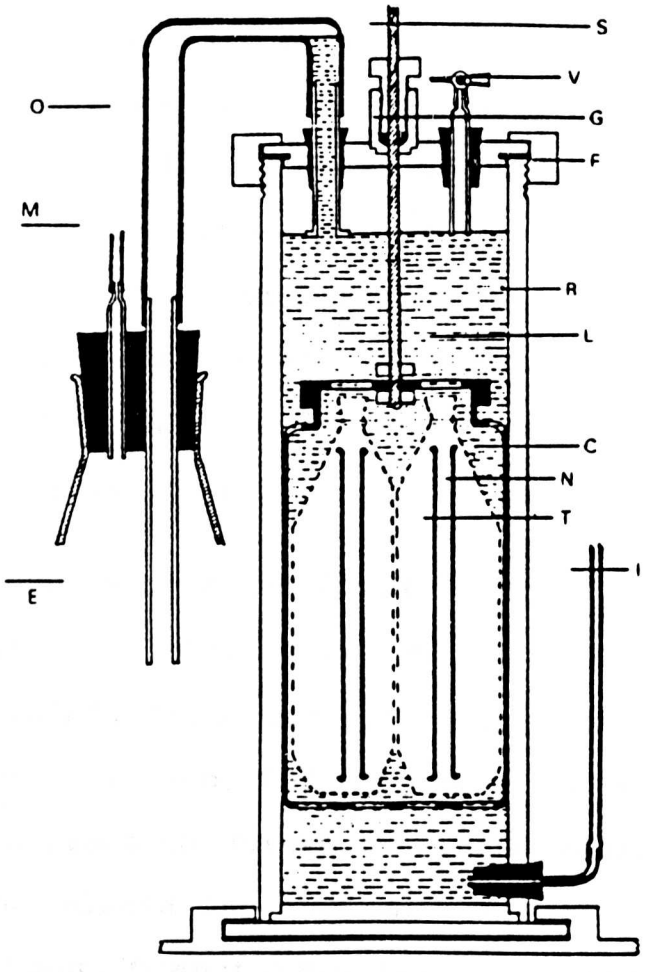


Fig. 7.1a Schematic diagram of one unit of the four-vessel long-term artificial rumen. (□), Made of perspex, (■), made of rubber or polyethylene. The driving shaft (S) was made of stainless steel. V, Sampling valve; G, gland (gas-tight); F, flange; R, main reaction vessel; L, rumen fluid; C, perforated food container; N, nylon gauze bag; T, rigid tube; I, inlet of artificial saliva; O, outlet through overflow; M, line to gas-collection bag; E, vessel for collection of effluent.

(RS Components Ltd, Belgium ; RS 543 -428) and placed into the polyethylene container. The container was moved up and down by means of a stainless steel rod passing through a gland (G), and connected to the screw cap of a food container and a crank actuated by a motor (B). The liquid could pass through the container by holes punched in the bottom and on the shoulder. The motor speed was 20 rev min^{-1} and this produced a vertical stroke of 50 - 80 mm at 1 cycle per min.

7.2.2 Source of rumen fluid

Rumen microbial inocula used throughout this study were obtained from fistulated cows (British Friesian cow 370 ; Plate 7.1a-b) fed only on a hay diet 24 h prior to sample collection unless otherwise indicated . The rumen fluid was placed in a 500 ml conical flask sealed with a rubber bung, transferred into a plastic disposable bag and tied with cable ties. Thereafter the rumen contents were placed in anaerobic jars (BTL Ltd, England), which was made anaerobic with an Oxoid gas generating kit (Code no. BR 38, Oxoid Ltd, Hampshire). The jar together with rumen fluid was transferred into an insulated box containing warm water (39 $\pm$ 1^{o}C). Sample were taken from the sample collection station (Hannah Research Institute, Ayr, Scotland) to Strathclyde University by road taking approximately 50 min.

PLATE 71a Fistulated fresian cow.

PLATE 7.1b Collection of rumen fluid from fistulated cow.

7.2.3 Artificial saliva (McDOUGAL, 1948)

	gl^{-1}
Na_2HPO_4	3.71
$NaHCO_3$	9.8
NaCl	0.47
KCl	0.57
$CaCl_2$	0.04
$MgCl_2$	0.06

Chlorides, carbonates and phosphates were dissolved separately before mixing. The pH was adjusted to 7.1 ± 0.1 with 1M H_2SO_4.

7.2.4 General incubation procedure for rumen simulation technique (Plate 7.2)

(A) Pairs of food or test samples (3.5 - 4.5 g) were weighed into nylon bags containing plastic rods or glass marbles as a stiffener and closed with a plastic tie. The bags were marked with plastic discs of different colours (serially numbered) attached with the plastic tie.

(B) Initiating the experiment; the apparatus was set up containing

300 ml rumen fluid ;

600 ml artificial saliva ;

50 g solid rumen food placed in nylon bags;

+ 2 food bags.

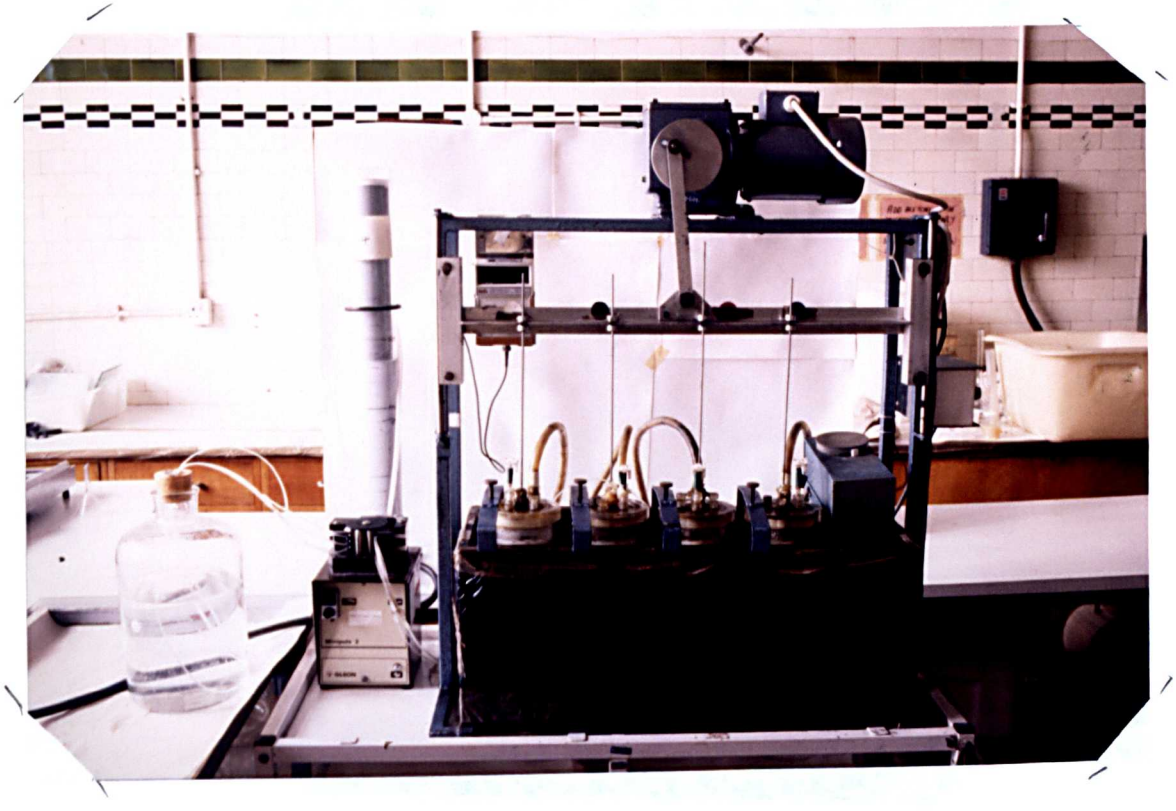


PLATE 7.2a The "RUSITEC" system (Front view).

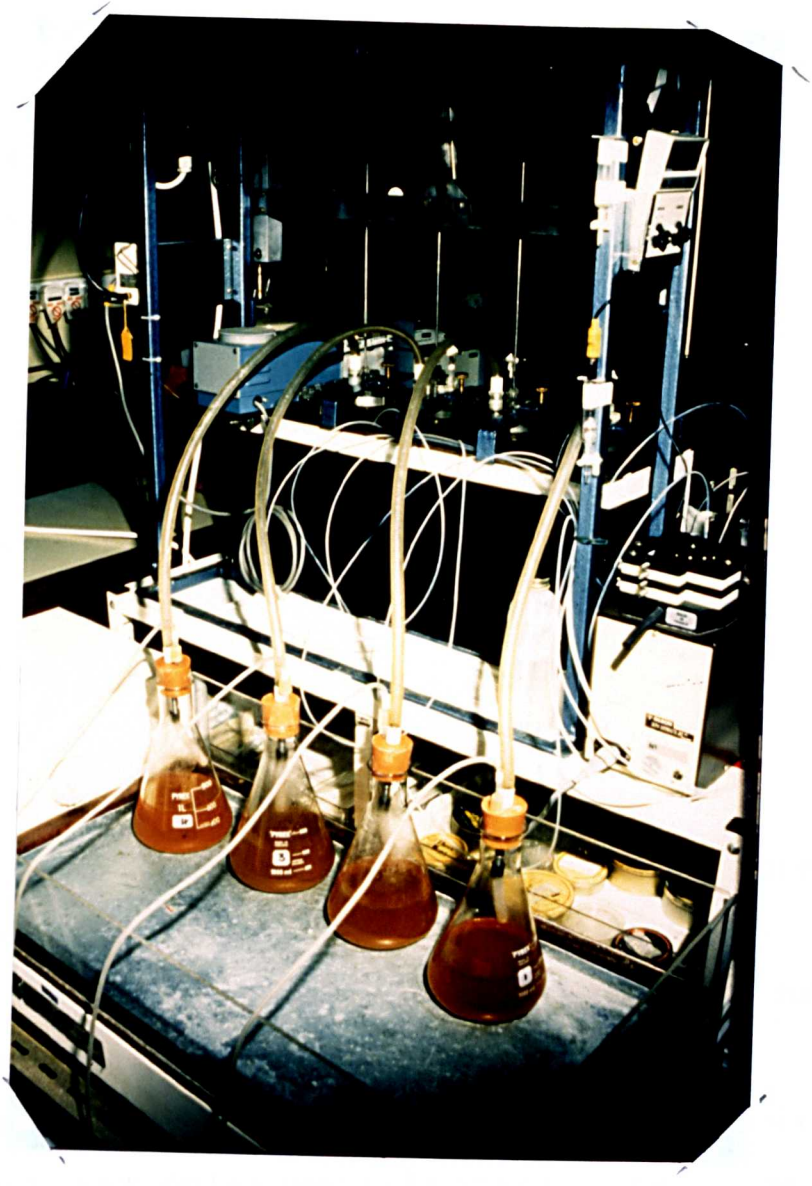


PLATE 7.2b The "RUSITEC" system (Back view)

1. The bag of solid rumen content and 2 bags of food were put in the plastic cage and placed in the reaction vessel containing rumen liquor and artificial saliva.

2. The rim of the reaction vessel was wiped to remove residues of food particles or dirt, greased with vaseline and lid replaced.

3. The vessel was placed in the water bath at 39 ± 1°C and

4. The effluent flask was connected

5. The system was then flushed with 1l 5% carbon dioxide / 95% nitrogen.

6. The empty gas bags were connected and locks opened.

7. The infusion pump(Minipuls 2, Gilson, France) and the motor was started .

(C) ; Day 2 of experiment

8. After 24 h the infusion pump and motor were stopped

9. The system was flushed with 1 l of gas (5% CO_2 / 95% N_2) the tap on the gas bag closed and the bag disconnected from the system.

10. pH was measured with the aid of Extech digital pH meter model 607 (Clandon Scientific, UK). and the effluent flask was disconnected and emptied.

11. The reaction vessel was removed from the water bath, opened and the bag of solid rumen content removed. The bag was placed in a glass beaker washed with 40 - 50 ml aliquots of artificial saliva and the washings were returned to the vessel.

12. A new pair of food bags were placed in the cage (beside the one already there). The cage was then returned back in the reaction vessel.

13. Steps 2 - 7 of the initiation were repeated.

14. The volume of effluent was measured and aliquots of fluid (10 ml) from the vessels collected, centrifuged and used for volatile acid analyses.

15. Gas samples were analysed and total gas volume quantified using a wet meter (Alexander Wright Ltd , UK) and a vacuum pump (Plate 7.3).

16. The procedures were repeated at the end of every 24 h incubation period. Food samples which had been incubated for 48 h were washed, put in a foil tray, dried overnight at 105 ^{o}C and weighed again.

17. Digestibility was calculated as % solubility of dry matter.

7.2.5 Total population of rumen bacteria

Colony counts of viable cells present in dilutions of rumen fluid were produced using modified anaerobic culture media as described by Varel and Jung (1986). The composition of the medium (per 1) was as follows; Clarified rumen fluid (150 ml), yeast extract (1.0 g), cellulose powder (2.0 g), Na_2CO_3 (4.0 g), cysteine hydrochloride (0.030 g), sodium sulphide (0.030 g) and purified agar (10.0 g). Plates were incubated in anaerobic jars for 48 h.

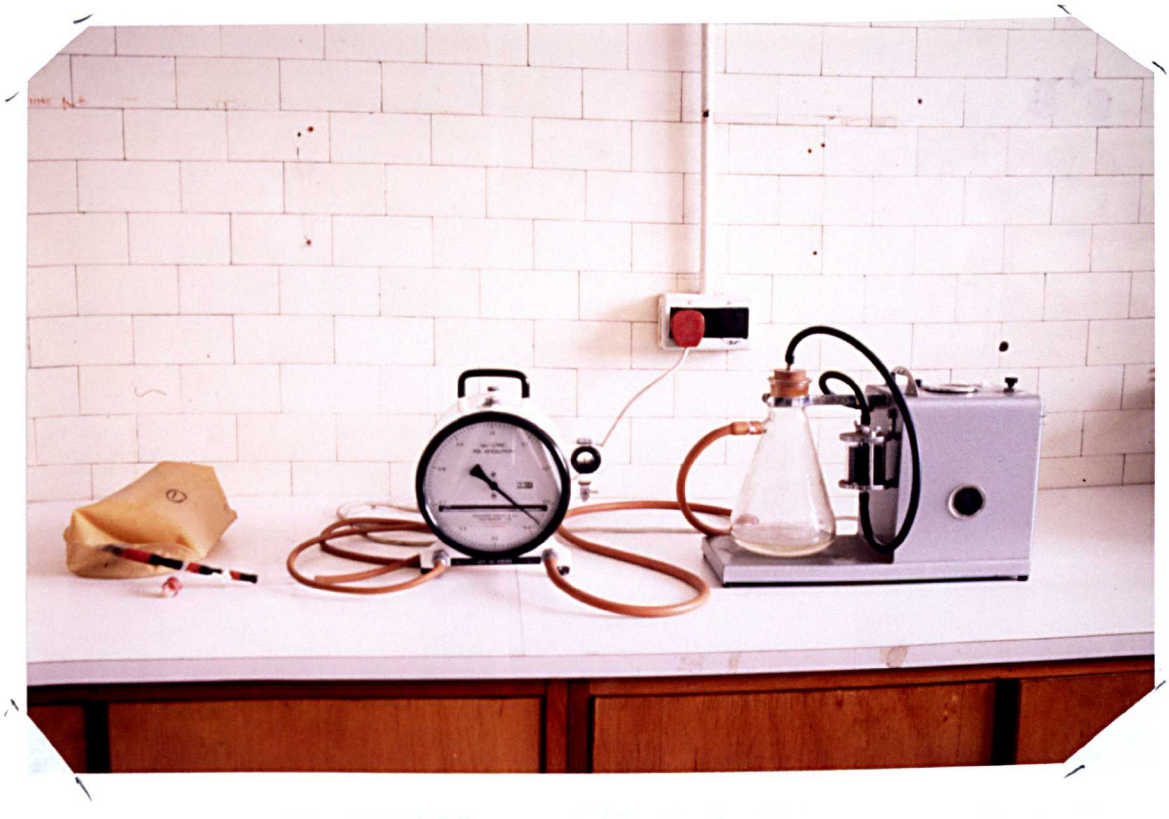


PLATE 7.3 Wet meter for measuring gas volume.

7.2.6 Experimental variables for RUSITEC

In all the experiments, the mean rate of outflow ranged from 700 - 800 ml day^{-1} . Thus the average dilution rate was 0.75 day^{-1} as the volume of the reaction vessel was 1 l. The same artificial saliva (pH 7.1 $\pm$ 0.1) was used in all vessels.

7.2.6.1 Experiment 1 ; Investigation of rumen fermentation characteristics of native spruce sawdust

In this experiment, three replicate vessels were used. The daily rations for the first 3 days of the experiment was hay (10 g day^{-1} : 2 X 5 g bags). The daily ration for subsequent days was spruce sawdust (10 g day^{-1} : 2 X 5 g bags).

7.2.6.2 Experiment 2 - Investigation of rumen fermentation features of fermented lignocelluloses

In the second experiment, four vessels were used. The system was supplied hay diet (10 g per day) within the first 3 days of the experiment. Thereafter, vessel 1 received substrates (7- 8 g day^{-1}) fermented by *Coriolus versicolor* while vessels 2 & 3 were supplied with substrates (7 - 8 g day^{-1}) fermented by *Pleurotus sajo-caju* and *Phanaerochaete chrysosporium*, respectively. Vessel 4 was supplied with untreated substrates (7 - 8 g day^{-1}). The treatment period lasted for 2 days for each fermented sample.

7.2.6.3 Experiment 3 - Investigation of the effect of different levels of soluble sugars on the digestibility of spruce sawdust

As described above, four vessels were used. With the exception of vessel 4 which received a basal diet of hay (8 g day^{-1}) throughout the experiment, the other three vessels were supplied with hay for the initial 3 days. Thereafter they were supplied with untreated spruce sawdust (8 g day^{-1} : 2 X 4 g bags). The basal diets for vessels 1 & 2 were supplemented with soluble sugars while the basal diet in vessel 3, acting as control was unsupplemented. Each treatment period for vessels 1 & 2 was divided into two periods of 4 days. In vessel 1, 0.025 % xylose in artificial saliva was infused from day 4 - 7 and 0.05% xylose infused with artificial saliva from day 8 - 11. For vessel 2 , 0.025% glucose and 0.05% glucose was infused for similar periods . The experiment was repeated using fructose.

7.2.6.4 - Experiment 4 ; To investigate the effect of complex carbon sources on digestibility of spruce sawdust

Four vessels were used in this experiment, all vessels being kept on a hay diet for the first 3 days of the simulated fermentation. Thereafter sawdust was supplied to all four vessels for days 4 - 6 . During days

7 - 20 , the rations in all vessels were different. In vessel 1 , the ration (9 g d^{-1} : 2 x 4.5 g bag) was a mixture of untreated sawdust and rice bran in the following proportions 95 : 5 ; 90 : 10 ; and 70 : 30% respectively (w/w). These were introduced into the vessel as mixtures in nylon bags. For vessel 2, the ration (9 g) was a mixture of untreated sawdust and cow feed concentrate in the ratio described above. Similarly for vessel 3 the daily ration (9 g) was a mixture of untreated sawdust and chopped hay , in the above proportions above. The treatment period for vessels 1 - 3 was divided into 3 periods of 4 d per set of ration. For vessel 4, the control, untreated spruce sawdust was supplied throughout the experiment.

7.2.6.5 Experiment 5: To investigate the effect of chitin and D glucosamine on ruminal fermentation

Three vessels were employed. As before each vessel received hay ration (9 g day^{-1}) for days 1 - 3 followed by a ration of untreated spruce sawdust for days 4 - 6. Throughout the remaining period of the experiment, the basal ration of spruce sawdust in vessel 1 was supplemented with differing concentrations of D-glucosamine(0.5%, 1% & 2% (w/v). Each was infused as a solution in artificial saliva. Each concentration had a treatment period of 3 days. For vessel 2, the basal ration was a mixture of spruce sawdust and chitin. The two substrates were mixed in the proportions described in

section 7.2.6.4. Similarly, treatment period for each set of rations lasted for 4 d. After the third day of the experimental period, the basal ration (spruce sawdust) for vessel 3 was not supplemented.

7.2.6.6 Experiment 6: Optimised condition for the investigation of rumen fermentation of fungal fermented lignocellulosics

In vitro digestibility of fungal fermented spruce sawdust was investigated using the optimised condition except where indicated otherwise. For the entire period of this experiment ,the basal ration was supplemented with 0.05 % glucose (w/v) and infused in artificial saliva. Four vessels were used. Each vessel was supplied with a hay diet during the initial 3 days of the experiment. All four vessels also received a basal ration of untreated spruce sawdust from days 4 to 6. Fermented rations were supplied to the vessels as from day 7 . The maximum treatment period was 12 days.

7.3 RESULTS

7.3.1 Hay and spruce wood as RUSITEC substrates

Performance as a ruminant feed was evaluated as gases (carbon dioxide and methane) and volatile fatty acids produced. Both gas produced and *in vitro* digestibility were significantly lower with sawdust than

TABLE 7.1 RUMEN FERMENTATION CHARACTERISTICS OF HAY AND SPRUCE SAWDUST IN RUSITEC.

DAYS OF EXPERIMENT	pH	Digestibility (%)	Total gas ($L\ g^{-1}\ d^{-1}$)	$^{a}CO_2$ ($\mu mol\ g^{-1}\ ml^{-1}$)
DAY 4 - 6				
HAY	6.63 ± 0.06	47.50 ± 3.70	0.169 ± 0.01	0.851 ± 0.18
SPRUCE	7.08 ± 0.15	8.93 ± 1.23	0.127 ± 0.002	0.337 ± 0.12
DAY 7-10				
HAY	6.71 ± 0.02	35.40 ± 1.30	0.145 ± 0.01	0.520 ± 0.05
SPRUCE	7.25 ± 0.05	4.84 ± 1.22	0.118 ± 0.004	0.159 ± 0.04
DAY 11 - 14				
HAY	6.70 ± 0.05	35.86 ± 1.02	0.148 ± 0.005	0.572 ± 0.14
SPRUCE	7.30 ± 0.05	4.58 ± 0.48	0.114 ± 004	0.153 ± 0.03

All values are means of at least 3 daily determinations

[a] CO_2 measured as $\mu mole\ g^{-1}\ ml^{-1}$ gas space.

with hay (Table 7.1). Spruce sawdust produced 0.114 - 0.127 l g^{-1} d^{-1} whereas with hay 0.148 - 0.169 l g^{-1} d^{-1} was obtained. In addition, spruce wood had an adverse effect on rumen activity (Fig. 7.1b) such that addition of sawdust at day 4 of the RUSITEC experiment resulted in a sharp decrease in concentration of fermentation end-products, notably volatile fatty acids (Table 7.2). This reduction in fermentation activity was accompanied by marked loss of the characteristic greenish colour and odour of rumen fluid until days 7-10 when a plateau in reduction of digestibility was reached. With hay, rumen pH was maintained between 6.25 - 6.8 whereas with spruce sawdust a more alkaline fermentation (7.3 $\pm$ 0.1) was observed.

The effect of spruce sawdust addition (Table 7.3) to rumen was stabilisation of pH at a higher pH, decline in the population of microbes, and observed catabolic enzyme activity. With spruce sawdust evolution of carbon dioxide was markedly lower than with hay (Table 7.3). However differences in methane production were less. Volatile acid production was considerably higher with hay than with spruce sawdust(Table 7.3) although values for iso-butyrate, iso-valerate and valerate were comparable. Output of acetic, propionic and butyric acid were markedly greater with hay than with spruce sawdust (Table 7.3). Total soluble sugars released in batch culture rumen fermentation with hay (0.220 mg ml^{-1}) were much higher than with spruce sawdust (0.113 mg ml^{-1}) which may

TABLE 7.2 CHANGES IN VOLATILE FATTY ACID (VFA) PRODUCTION DURING RUSTIEC STUDIES ON SPRUCE WOOD

		DAYS OF EXPERIMENT		
VFA(mmol d^{-1})	Fresh rumen fluid	4 - 6	7 - 10	11 - 14
Acetic acid	68.96 ± 3.16	11.69 ± 4.86	4.68 ± 0.95	4.01 ± 0.06
Propionic acid	24.70 ± 0.32	2.27 ± 1.10	0.79 ± 0.19	0.47 ± 0.03
Isobutyrate	0.78 ± 0.25	0.35 ± 0.09	0.16 ± 0.04	0.11 ± 0.02
Butyrate	9.65 ± 0.56	1.46 ± 0.38	0.66 ± 0.19	0.55 ± 0.05
Isovalerate	0.98 ± 0.19	0.23 ± 0.04	0.13 ± 0.03	0.11 ± 0.04
Valerate	1.71 ± 0.67	0.49 ± 0.05	0.46 ± 0.08	0.34 ± 0.01
Hexanoic acid	0.647 ± 0.12	traces	traces	traces

All values are average of at least 3 daily determinations except fresh rumen fluid

TABLE 7.3 RUMEN FERMENTATION CHARACTERISTICS OF HAY AND SPRUCE SAWDUST IN BATCH CULTURES

PARAMETERS	RUMEN FLUID alone	+ HAY	+ SPRUCE WOOD
pH	7.30	5.75	7.40
cultureable bacteria[b]	1.0×10^5	1.3×10^6	2.0×10^4
ENZYME ACTIVITIES[a]			
CMc-ase activity(mm)	16	21	16
Total cellulase (mm)	24	15	16
Soluble sugar(mg ml^{-1})	0.100	0.220	0.113
GASES(µmol ml^{-1} gas space)			
CO_2	3.53	21.29	3.94
CH_4	0.04	0.01	0.026
O_2	traces	traces	traces
VFA(mMol)			
Acetate	12.66	34.57	13.16
Propionate	3.90	47.20	4.39
Isobutyrate	0.24	0.35	0.47
Butyrate	1.66	8.86	2.11
Isovalerate	0.50	0.77	0.38
Valerate	0.41	0.61	0.45
Hexanoic acid	0.25	traces	0.10

All values are means of at least two replications.

[a] Enzyme activities measured as diameter of clearance of polysaccharide (mm).

[b] Bacterial population per ml

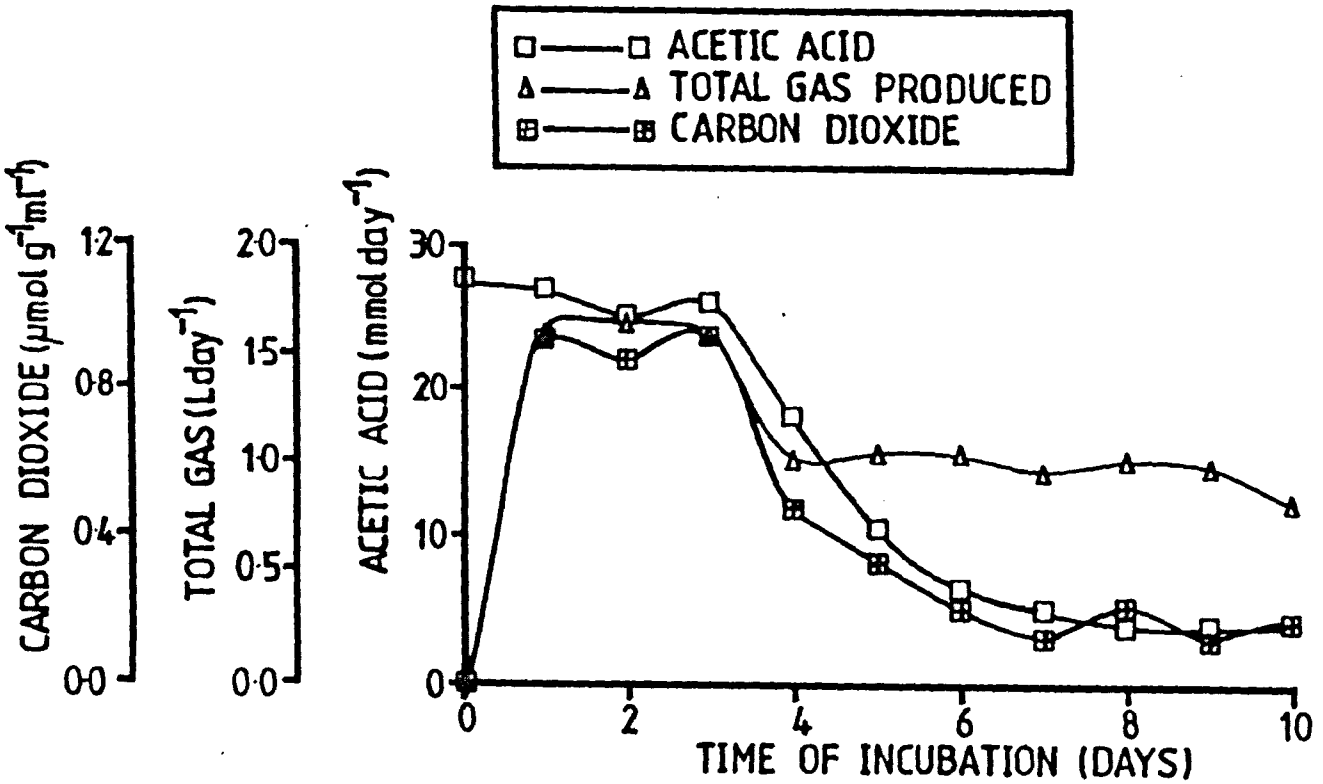


Fig. 7.1b TYPICAL OUTPUT FROM "RUSITEC" UTILISING SPRUCE SAWDUST AS SUBSTRATE
FIBRE DIET FOR DAY 1 TO DAY 3 WAS HAY.
FIBRE DIET FROM DAY 4 TO DAY 10 WAS SPRUCE SAWDUST.

be through either enhanced cell wall hydrolysis activity or greater access to hydrolysable substrates with the former substrate.

7.3.2 Fermented spruce sawdust as a RUSITEC feed

Fermented sawdusts yielded significantly higher outputs of rumen gases, notably carbon dioxide, than control untreated sawdusts as observed in batch and culture and the simulated rumen (Figure 7.2). As the incubation in the simulated rumen proceeded there was a reduction in concentration of volatile fatty acids (Table 7.2) which was not reversed by addition of fermented sawdust (data not shown).

7.3.3 Digestion of spruce sawdust in the presence of glucosamine and chitin

Infusion of the simulated rumen with the soluble amino sugar D-glucosamine) led to substantial increases in volatile acid production especially for acetate and propionate. Only trace amounts of iso-butyrate were detected when concentration of D-glucosamine was increased (Table 7.4) and at 2% (w/v) rumen pH was reduced to 6.05, with parallel increases in the output of gases. *In vitro* dry matter digestibility was also slightly improved at 10.59 + 0.25. However increases in D-glucosamine beyond 1% showed no further enhancement in digestibility. The presence of chitin slightly stimulated both total gas and volatile acid output. Polymeric chitin appeared to have been less utilised than glucosamine by

TABLE 7.4 EFFECT OF D(+) -GLUCOSAMINE AND CHITIN ON RUMINAL FERMENTATION CHARACTERISTICS OF SPRUCE SAWDUST

	pH	DIG(%)	[a]Total gas	AA[b]	PA	IBA	BA	IVA	VA
SAWDUST[1]	7.25	4.84	0.98	4.68	0.79	0.16	0.66	0.13	0.46
+ 0.5% glucosamine(w/v)	6.73	10.31	1.12	19.71	0.84	0.04	1.63	0.10	0.48
+ 5% chitin(w/w)	7.03	13.8	1.01	4.99	0.52	0.01	0.93	0.11	0.33
SAWDUST[2]	7.30	4.58	0.85	4.01	0.47	0.11	0.55	0.11	0.34
+ 1% glucosamine	6.55	10.59	1.71	34.11	1.74	traces	1.51	0.15	0.74
+ 10% chitin	7.00	15.23	1.00	5.38	0.56	traces	1.18	0.11	0.43
SAWDUST[3]	7.30	4.62	0.86	3.85	0.29	traces	0.34	traces	0.27
+ 2% glucosamine	6.05	10.52	2.29	44.24	2.56	traces	1.37	0.16	0.69
+ 30% chitin	6.92	15.57	1.01	5.50	0.63	traces	1.59	0.13	0.55

All values are average of at least 3 daily determinations

[a]Total gas obtained as L day^{-1}

[b]Volatile acid concentration recorded as mmol day^{-1}

AA= acetate, PA = propionate, BA= butyrate , IBA = isobutyrate, VA = valerate

IVA = isovalerate.

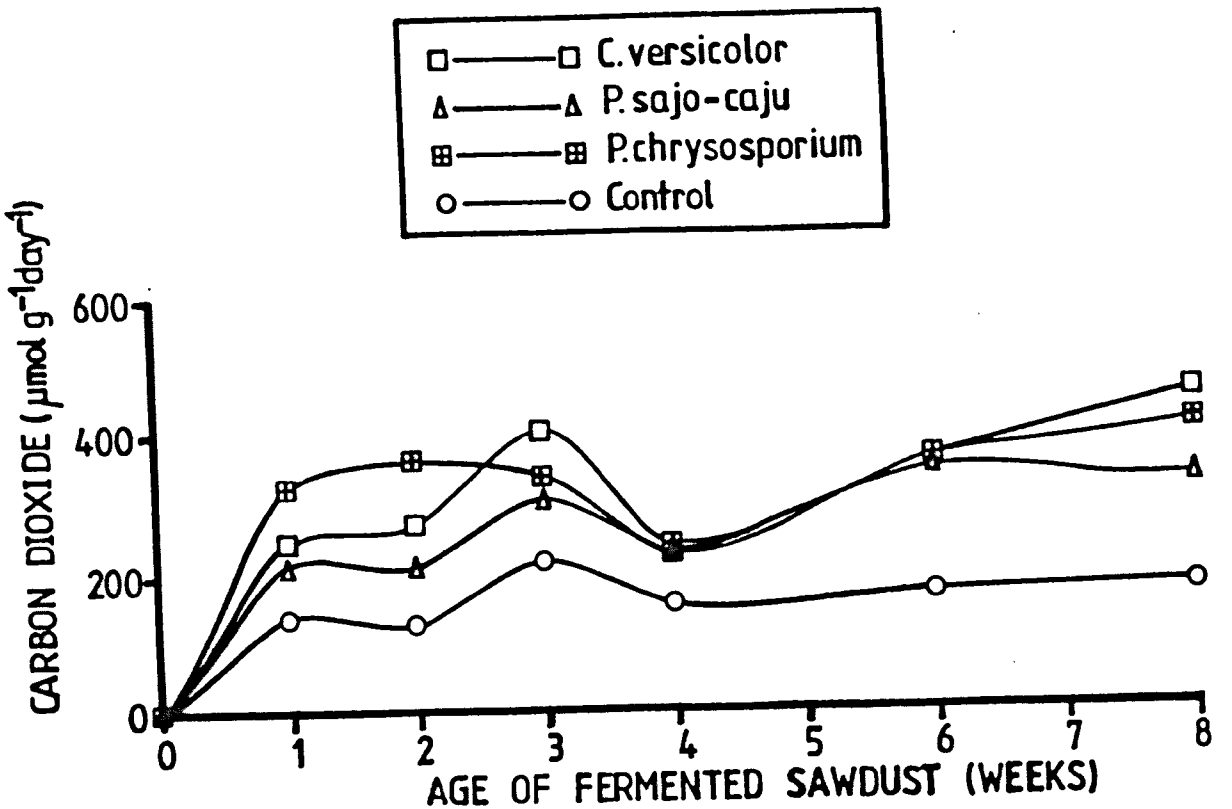


Fig. 7.2 CARBON DIOXIDE PRODUCTION DURING IN VITRO DIGESTIBILITY OF FERMENTED SPRUCE SAWDUST IN " RUSITEC".

rumen microbes as determined by yield of acetate and reduction in rumen pH when compared to D- glucosamine.

7.3.4 Effect of mixing of spruce sawdust with rice bran, hay or cow concentrate

The inclusion of either hay, cow concentrate or rice bran appeared to partially reverse the negative effects of spruce sawdust on the simulated rumen metabolism. In each case enhancement of gas output, increases in VFA production and dry matter digestibility were observed.

Higher concentrations of volatile acids were produced with rice bran or cow concentrate than with hay. Rumen pH fluctuated within a range that supported active microbial growth and activity. Values for dry matter digestibility and end products of rumen fermentation increased in parallel with addition of rice bran, cow concentrate or hay (Table 7.5).

7.3.5 Infusion of the simulated rumen with soluble sugars

Infusion of the simulated rumen with soluble carbohydrates in artificial saliva was evaluated for stabilisation of rumen pH, microbial population and enhancement of wood digestibility. The results obtained (Table 7.6) showed that with spruce sawdust as sole energy source, rumen pH drifted away from neutrality (0.25 pH units). Addition of 0.025% of xylose, glucose or fructose did not have marked effects on rumen activity, except for a slight increase in dry matter digestibility.

TABLE 7.5 INFLUENCE OF COMPLEX CARBOHYDRATE SOURCES ON RUMINAL FERMENTATION CHARACTERISTICS OF SPRUCE SAWDUST

	pH	DIG(%)	[a]Total gas	AA[b]	PA	IBA	BA	IVA	VA
+ 5% HAY[1]	6.92[c]	10.89	1.01	4.75	0.63	0.18	0.56	0.21	0.38
+ 5% COW CONCENTRATE	6.90	11.63	1.02	5.66	0.67	0.20	0.78	0.16	0.58
+ 5% RICE BRAN	6.90	10.34	1.03	5.20	0.98	0.17	0.80	1.15	0.51
+ 10% HAY[2]	6.88	12.56	1.01	5.62	0.74	0.24	0.82	0.22	0.47
+ 10% COW CONCENTRATE	6.88	13.54	1.04	6.16	0.83	0.27	1.42	0.27	0.65
+ 10% RICE BRAN	6.87	14.13	1.06	6.01	1.93	0.26	1.17	0.12	0.56
+ 30% HAY[3]	6.80	14.73	1.03	6.12	1.15	0.29	1.72	0.22	0.63
+ 30% COW CONCENTRATE	6.78	20.73	1.09	6.62	1.47	0.30	2.38	0.30	0.76
+ 30% RICE BRAN	6.80	24.98	1.09	7.25	3.07	0.34	1.95	0.13	0.55

All values are average of at least 3 daily determinations.

AA= acetate, PA = propionate, BA= butyrate , IBA = isobutyrate, VA = valerate IVA = isovalerate.

[a] Total gas measured as L day^{-1}

[b] VFA quantified as mmol day^{-1}

[1,2,3] The corresponding control values are shown in Table 7.4.

TABLE 7.6 EFFECT OF SOLUBLE SUGARS(XYLOSE, GLUCOSE , FRUCTOSE) ON RUMINAL FERMENTATION OF SPRUCE SAWDUST IN RUSITEC.

	pH	DIG(%)	[a]TOTAL GAS	[b]CO_2	[b]CH_4
Spruce wood(alone)	7.25	4.84 + 1.22	0.118	0.159	6.18×10^{-4}
XYLOSE(W/V)					
+ 0.025 %	7.30	6.24 + 0.45	0.126	0.177	4.10×10^{-4}
+ 0.05 %	7.10	8.79 + 0.52	0.138	0.199	3.08×10^{-4}
Glucose(W/V)					
+ 0.025 %	7.20	5.82 + 0.51	0.119	0.160	1.68×10^{-3}
+ 0.05 %	7.05	8.48 + 0.64	0.132	0.183	5.68×10^{-3}
FRUCTOSE(W/V)					
+ 0.025 %	7.15	5.52 + 0.98	0.126	nd	nd
+ 0.05 %	7.05	7.46 + 0.96	0.127	nd	nd

All values are average of at least 4 daily determinations

[a] Total gas quantified as $l\ g^{-1}\ day^{-1}$

[b] CO_2 and CH_4 measured as $\mu mol\ g^{-1}\ ml^{-1}$ gas space

Supply of any of these sugars at 0.05% stabilised rumen pH (7.05 ± 0.05) and also enhanced substrate digestibility.

Total gas released, and carbon dioxide production, were slightly higher than with controls at low concentration of the sugars. Methane production was low throughout experiments but slightly higher when glucose was infused (Table 7.6). In addition, infusion of sugars at 0.05% increased turbidity of rumen fluid suggesting increases in microbial biomass.

7.4 DISCUSSION

Incubations in simulated rumen and batch cultures were performed under controlled conditions facilitating collection of quantitative information on the rumen fermentation of highly lignified spruce sawdust before and after fungal delignification. Despite the high polysaccharide content of spruce sawdust, it had a low nutritional value (Tables 3.1 & 7.3). Analyses of this substrate showed low contents of fermentable carbohydrates and relatively high contents of polymeric lignin as well as possibly less condensed polyphenols and tannins. This polyphenol content may have reduced values for digestibility and methane production even following fungal delignification. Krishna *et al.* (1986), in a study with spent hops, noted that this feed reduced methane and acetate production by rumen microbes. It was concluded that the high lignin content and presence of

inhibitory compounds such as terpenes, methylsulphides, flavanol glycosides and bitter isohumulones in hops was responsible for this reduction in rumen activity.

Fungal delignification of spruce sawdust(Table 6.7 & Fig. 7.2) yielded substantial improvement in dry matter digestibility and increased production of gases, especially carbon dioxide, by rumen microbes. Such increases could either be attributed to depolymerisations and solubilization of cellulose or hemicellulose, increasing concentrations of sugars fermentable by rumen microbes. Enhancement of rumen gas output may also be through depolymerisation or modification of lignin structure that enhances access of rumen enzymes to substrate polysaccharides. Furthermore delignification may generate breakdown products, monomeric or dimeric units, that can yield methane and carbon dioxide from rumen bacterial metabolism (Chen *et al.*, 1985). However the observed lower production of volatile acids with spruce wood than with hay (Table 7.2) could be explained either by reaction between phenolics in the wood with proteins or other fermentable components to produce complexes that are resistant to microbial attack (Kumar & Singh, 1984). An alternative would be that tannins and lignin degradation products are inhibitory to rumen microbes or their catabolic activities. Tamir and Alumot (1969) reported that tannin compounds extracted from carob pods inhibited trypsin, amylase and lipase production. It seems likely that if concentrations of

fermentable nutrients were low, accumulation of volatile acids and gases, such as carbon dioxide and methane, evolved from volatile acid degradation would also be reduced. At extreme nutrient shortage, VFA could be metabolised by other acid utilising bacteria to form hydrogen and carbon dioxide. Boran and Czerkawski (1983), showed that the end-products of carbohydrate breakdown in the rumen are volatile acids and that 80% of dietary hexose was converted to higher volatile acids and, in turn, to acetate. This may explain the preferential accumulation of acetate over other volatile acids. Thus VFA's production will depend largely on the diet of the animal and spruce will give only limited yields of volatile fatty acids.

Addition at 5 - 30% of either rice bran, cow concentrate or hay to spruce sawdust stimulated production of volatile acids and enhanced rumen fermentations. This may be attributed to the addition of fermentable carbohydrates. However, spruce sawdust appeared to reduce the nutritional quality of feeds. Although feed additives enhanced substrate digestibility, it is possible this was through attack on the additive rather than the sawdust.

The infusion of glucosamine resulted in enhancement of spruce sawdust digestibility and output of rumen fermentation end products which can be attributed to increased rumen microbial activity stimulated by the presence of the readily utilised amino sugar. It is

however difficult to explain why with glucosamine high levels of acetate and relatively low concentrations of other VFA's were produced. In contrast, Boran and Czerkawski (1983), noted that increasing glucose concentration led to production of less acetate and enhanced output of propionate and butyrate. The amino sugar, based on glucose, was chosen as it represented a precursor of fungal polysaccharide synthesis, and from results obtained it would seem that the presence of autolysed fungal cells in fermented feeds may prove beneficial to rumen fermentations.

The addition of monosaccharides at 0.05% stabilised rumen pH, and enhanced dry matter digestibility and evolution of gases. In the experiments described, the first two of these factors appeared to be central to adaptation of rumen microbes to new feeds or substrates. Such factors are thus important in evaluation of nutritional value of fungal fermented sawdust. Consequently in subsequent experiments, 0.05% glucose was infused in to rumen simulations in assessments of digestibility to ensure maintenance of high, stable rumen microbial populations since pH stability reflected optimal microbial activity.

These experiments demonstrated that feed substrates vary in rumen fermentation characteristics. Whereas certain agricultural residues, such as rice bran, have nutritive values comparable to those of forage feeds, like hay, others such as spruce sawdust have less

nutritional value and a negative effect on rumen metabolism. However such highly lignified substrates forming high fibre dietary components may be of value in the manipulation of rumen fermentations.

7.5 THE LIMITATIONS OF RUSITEC AS A RUMEN SIMULATION SYSTEM

Although RUSITEC is a simple simulation with low-level technology coupled with easy of operation, it is in practice often difficult to maintain absolutely anaerobic conditions. Although minimal amounts of oxygen have been detected in fistulated animal rumen, most investigators share the view that it would be better if the incubations in simulations could be performed without the need to open the vessels (Czerkawski & Breckenridge, 1979).

SECTION 8

8 THE EFFECT OF LIGNIN MODEL COMPOUNDS AND FUNGAL METABOLITES

8.1 Preamble

The simulated rumen performance of high - fibre plant residues is influenced not only by feed composition but also by the presence of microbial metabolites in the feedstuff. Delignification of plant material has been reported to enhance exposure of cellulose fibres to attack by rumen microbes and enzymes (Chen *et al.*, 1985). However, the products of oxidative attack and solubilization of lignin complexes by white rot fungi will undoubtedly lead to accumulation of low molecular weight phenolic compounds in feeds. The effects of such compounds on polymer breakdown and overall rumen fermentation have not been fully studied. Many authors have reported anaerobic degradation of phenolic monomers such as benzoate and vanillin in enrichment cultures (Chen *et al*, 1985; Mountfort & Bryant, 1983) and Colberg and Young (1985) observed that lignin derived oligomers were metabolised anaerobically into monoaromatic compounds or mineralised.

On the other hand, a probiotic effect of fungal cultures on ruminal fermentation has recently been reported by Newbold and Wallace (1990) who observed that

addition of live cultures of *Aspergillus oryzae* or *Saccharomyces cerevisiae* to feeds led to enhanced dairy milk production, increased fibre digestibility and stabilisation of the rumen environment. Addition of *S. cerevisiae* appeared to result in increases in total rumen bacterial population.

A possible problem with such additions is the effect of metabolites from toxigenic strains of fungi on rumen fermentations. Sinha and Arora (1982) reported that the presence of aflatoxin (0.1 - 1.25 ppm) led to decreases in cellulase digestibility and microbial protein synthesis.

The effects of both classes of fungal metabolite, phenolic breakdown products and mycotoxins on fibre digestibility and output of rumen fermentation end products was studied.

8.2 EXPERIMENTAL -Effects of lignin model compounds and fungal metabolites on ruminal fermentation

8.2.1 Mycotoxin production - organism & substrate

Aspergillus flavus (IMI 102566) was maintained at 30 ^{o}C on malt extract agar slants. Maize grains were obtained from United States Department of Agriculture, America by courtesy of Dr. Raul Cuero.

Culture conditions; Previously cracked maize grains (100 g) were moistened with minimal medium (1 part grain : 2 parts medium). Moistened grains were equilibrated at

4°C for 24 h and aliquots (50 g) were dispensed into microporous bags. The bags were sterilised and inoculated with 1 ml samples of spore suspension of *A.flavus* (4.5 x 10^6) . Bags were incubated at 30 °C for 10 d in a Fisons Environmental Cabinet at 98 % relative humidity.

8.2.2 Treatment of *A. flavus* fermented maize grain

(a) Fungal fermented maize grain (50 g) containing *A. flavus* spores, mycelial cells and toxins was blended with 500 ml distilled water in a metal blender for 1 min at high speed. Thereafter aliquots of the blended culture were used either (i) directly as fresh culture or (ii) as protein denatured cells after autoclaving at 121 °C for 15 min or (iii) as wholly denatured cultures after vigorous boiling for 1 h.

(b) Unpurified toxin : Toxin (1 ml) in 30% methanol extracted after step 2 of aflatoxin extraction procedure (8.2.5) was used in the rumen fermentation study.

(c) Purified toxin : 1 ml of laboratory extracted aflatoxin was similarly used for the study.

8.2.3 Batch culture *In vitro* rumen fermentation of crystalline cellulose

Whatman crystalline cellulose powder (1.5 g) was dispensed into a 150 ml bottle with varying concentrations (0%, 0.05%, 0.1%, 0.5%) of lignin model compounds (ferulic, cinnamic, and p-hydroxybenzoic acid, vanillin, guaiacol, indulin lignin AT and Klason lignin).

Alternatively bottles were supplemented with varying concentrations (1 ml, 5 ml, 10 ml) of live or killed cultures of A.flavus in fermented maize or aliquots (1 ml) of purified (10.5ppm) or unpurified toxin. Freshly buffered rumen fluid (50 ml) was added to each bottle, which was stoppered, made anaerobic and incubated as detailed previously (Section 2.3.8).

8.2.4 Preparation of samples for analyses

Replicate samples (15 ml) from pre-shaked bottles were collected, centrifuged at 5000 X g at 4 ^{o}C for 30 min. Aliquots were used for enzyme, volatile acid and soluble sugar analyses. Methane and carbon dioxide in the gas space were analysed prior to removal of liquid samples.

Enzyme assay;

Endoglucanase activity was determined using the plate screening assay of Teather and Wood (1982). Agar plates containing 1.0% CMC (low viscosity) as substrate in sodium citrate buffer(pH 5.5, 0.1 M) were used as described in 4.2.3 using 100 ul of samples and incubation at 39^{o}C for 24 h. Total cellulase activity was quantified similarly except that dye solution(1 mg ml^{-1}) was incorporated into the crystalline cellulose(0.1% w/v) - citrate buffer media.

8.2.5 Extraction and quantification of aflatoxin

The aflatest affinity column method (Rhone Poulenc, Diagnostics Ltd, Glasgow) described by Faraj (1990) was used (Appendix 4).

8.3 RESULTS

8.3.1 Production of the mycotoxin Aflatoxin

Aflatoxin was produced by growing a toxigenic strain of *Aspergillus flavus* on previously moistened cracked maize grain for 10 d (Table 8.1). Using semi-quantitative ultraviolet detection, 22.5 ppm aflatoxin were determined; using a fluorometric assay the value was 10.5 ppm.

8.3.2 The effect on rumen pH of fungal metabolites and lignin model compounds

A simulated rumen microbial population was grown on 3% cellulose powder in presence of varied concentrations of ferulic , p-hydroxybenzoic and p- cinnamic acids, vanillin, or guaiacol and the industrial - lignin Indulin AT. Addition of each of these compounds at levels between 0.05 and 0.1% generally reduced rumen pH to values compareable to control cultures (6.75). However with 0.5% Indulin lignin, Klason lignin (also at 0.1%), vanillin or guaiacol pH increased to 7.25 - 7.45 (Table 8.2).

TABLE 8.1 ESTIMATION OF AFLATOXIN USED FOR RUMEN FERMENTATION STUDIES.

	REPLICATES 1	2	Average
Qualitative*	20 µg/Kg	25 µg/kg	22.5 µg/kg = 22.5ppm ± 2.5
Quantitative	11.0 ppm	10.00 ppm	10.5 ppm ± 0.5

* 1 µg/kg = 10^{-9} = 1 ppb aflatoxin = 0.001 ppm.

Therefore total aflatoxin = concentration(ppb) multiplied by dilution factor (1000 X)

Moreover, anaerobic saccharification of crystalline cellulose in presence of a fresh extract of aflatoxin-contaminated maize reduced the observed rumen pH to 6.3 whereas with controls or 1 ml aliquots of boiled or autoclaved fermented maize, pH was maintained near neutrality. Addition of 5ml autoclaved extract, however, induced a reduction in pH to 6.7 (Table 8.2). Although no marked change in pH could be demonstrated in the presence of pure aflatoxin, over controls, cultures containing crude mycotoxin extracts had a slightly reduced pH (6.75) (Table 8.2).

8.3.3 The effect on rumen bacterial population

Table 8.3 shows the effect on rumen bacterial populations of growth in the presence of phenolics or mycotoxins. It can be seen that control cultures, containing cellulose with or without addition of aliquots (10 ml) of maize extract maintained high populations of rumen bacteria. Addition of 0.5% Indulin AT, ferulic or t-cinnamic acids, or vanillin led to significant decreases in rumen population. Vanillin had a more adverse effect than either ferulic acid or industrial lignin. Addition of fresh mycotoxin-contaminated maize extracts had no negative effect on bacterial multiplication whereas addition of purified *A.flavus* toxin slightly reduced bacteria numbers as compared to controls.

TABLE 8.2 CHANGES IN IN VITRO RUMEN pH IN PRESENCE OF DIFFERING CONCENTRATIONS OF EITHER CELL WALL PHENOLIC OR FUNGAL METABOLITES

	pH VALUES			
	(a) Lignin model compounds			
	0.0%	0.05%	0.1%	0.5%
Control	6.75	-	-	-
+ Indulin lignin	-	nd	6.85	7.35
+ Klason lignin	-	nd	7.25	7.25
+ Ferulic acid	-	6.60	6.65	6.55
+ p-Hydroxybenzoic acid	-	6.70	6.70	6.70[a]
+ Vanillin	-	6.70	6.65	7.25
+ t-cinnamic acid	-	6.75	6.65	6.75
+ guaiacol	-	6.60	6.80	7.45
	(b) *A. flavus* fermented maize extracts			
		1ml	5ml	10ml
Control		-	-	6.65
Fresh culture		6.55	6.25	5.80[a]
Boiled culture		7.05	nd	nd
Autoclaved culture		7.15	6.70	nd
	(c) Toxin			
		1ml		
Control		6.85		
Pure aflatoxin (10.5 ppm)		6.85		
Crude mycotoxin		6.75		

[a]Incubated for 24hrs.

nd = not determined

TABLE 8.3 EFFECT OF CELL WALL PHENOLIC OR FUNGAL METABOLITES ON RUMEN BACTERIAL POPULATION[1]

	CULTUREABLE BACTERIA POPULATION / ml
Rumen fluid (diluted)	1.0×10^5
+ cellulose (3%)	7.8×10^6
+ cellulose + indulin lignin (0.1%)	2.6×10^6
+ cellulose + indulin lignin (0.5%)	6.5×10^4
+ cellulose + ferulic acid (0.05%)	1.0×10^5
+ cellulose + ferulic acid (0.5%)	4.0×10^4
+ cellulose + t-cinnamic acid (0.05%)	5.0×10^5
+ cellulose + t-cinnamic acid (0.5%)	2.5×10^5
+ cellulose + vanillin (0.5%)	1.0×10^3
+ cellulose + 10ml untreated maize liquor	3.5×10^7
+ cellulose + 1ml fresh A.flavus treated maize	5.1×10^6
+ cellulose + 5ml fresh A.flavus treated maize	3.6×10^6
+ cellulose + 1ml methanol	2.2×10^6
+ cellulose + 1ml aflatoxin(10.5 ppm)in methanol	1.5×10^6
+ cellulose + 1ml mycotoxin in 60% methanol	4.2×10^6

All values are average of two replications.

[1]Samples were incubated anaerobically for 48 h.

8.3.4 Effect on production of soluble sugars

Figure 8.1 demonstrates a typical enrichment experiment to monitor the solubilization of crystalline cellulose in the presence of either phenolics or fungal metabolites. After 42 - 44 h of incubation, maximal accumulation of soluble sugars in culture media was observed on addition of 0.5% ferulic acid (0.297 mg ml^{-1}) or 0.5% guaiacol (0.240 mg ml^{-1}). Least accumulation of sugars was observed with 0.05% of phenolics (Figure 8.1) and with 0.5% of either Klason lignin or vanillin.

Furthermore, cellulose saccharification did not appear to be influenced by addition of mycotoxin extracts and there was no marked difference in rates of sugar accumulation with fresh *A. flavus* fermented maize and the killed fungal biomass (Fig. 8.1). Comparable soluble sugar levels were obtained in the presence or absence of aflatoxin or crude mycotoxin (Table 8.4).

8.3.5 Effect on rumen cellulase production

The influence of phenolics and fungal metabolites on simulated rumen performance was evaluated by observing the effect on rumen cellulase production (Fig. 8.2). Initial cellulose saccharification or total cellulase production was inhibited by presence of either 0.5% ferulic acid, 0.1 - 0.5% t-cinnamic acid, 0.05% guaiacol, 0.1 - 0.5% p-hydroxybenzoic acid, 0.1% vanillin or 0.1 - 0.5% Klason lignin (Fig. 8.2). Figure 8.2 presents the results of assays of rumen endocellulase production in

TABLE 8.4 INFLUENCE OF A. flavus TOXIN ON IN VITRO RUMEN SACCHARIFICATION OF CRYSTALLINE CELLULOSE.

	Rumen fluid/ cellulose	+ 1ml methanol	+ 1ml aflatoxin	+ 1ml crude toxin
Soluble sugar(mg/ml)	0.126	0.110	0.110	0.120
Total cellulase activity (mm)	23.5	24.5	26.0	13.0
CMc-ase activity(mm)	16.0	23.0	23.5	16.0
CO_2(µmol ml^{-1} gas space)	9.65	8.48	8.04	11.88
CH_4(µmol ml^{-1} gas space)	0.55	0.32	0.27	0.78
Acetate (mMol)	24.87	25.43	21.15	35.05
Propionate	20.68	13.75	14.92	25.40
Isobutyrate	0.93	0.26	0.47	0.39
Butyrate	3.57	3.49	2.82	4.75
Isovalerate	0.88	0.71	0.54	0.93
Valerate	0.75	0.34	0.41	0.60

All values are average of at two replications

KEY TO THE FIGURES

Lignin model compounds

Q1	Ferulic acid
Q2	p-hydroxybenzoic acid
Q3	Vanillin
Q4	t-cinnamic acid
Q5	Guaiacol
Q6	Indulin lignin
Q7	Klason lignin

A. flavus metabolites

H1	Fresh metabolites
H2	Boiled metabolites
H3	Autoclaved metabolites

*Values for 0.5% levels of p-hydroxybenzoic acid were based on 24 h incubation.

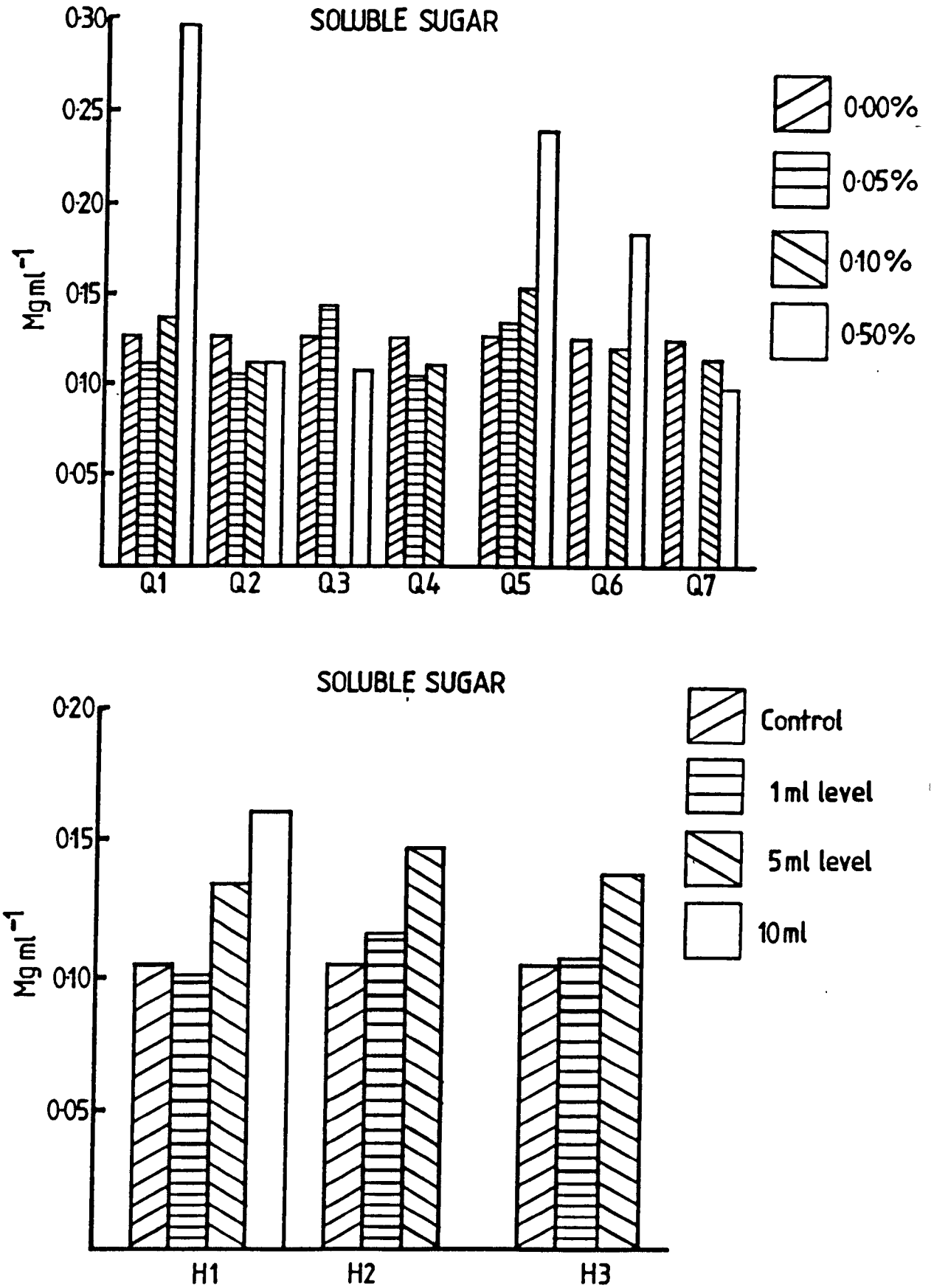


Fig. 8.1 Effect of fungal metabolite (*A. flavus*) or lignin model compounds on rumen soluble sugar accumulation during cellulose fermentation (0.05% levels of Q6, Q7 & 10 ml levels of H2 & H3 were not assessed).

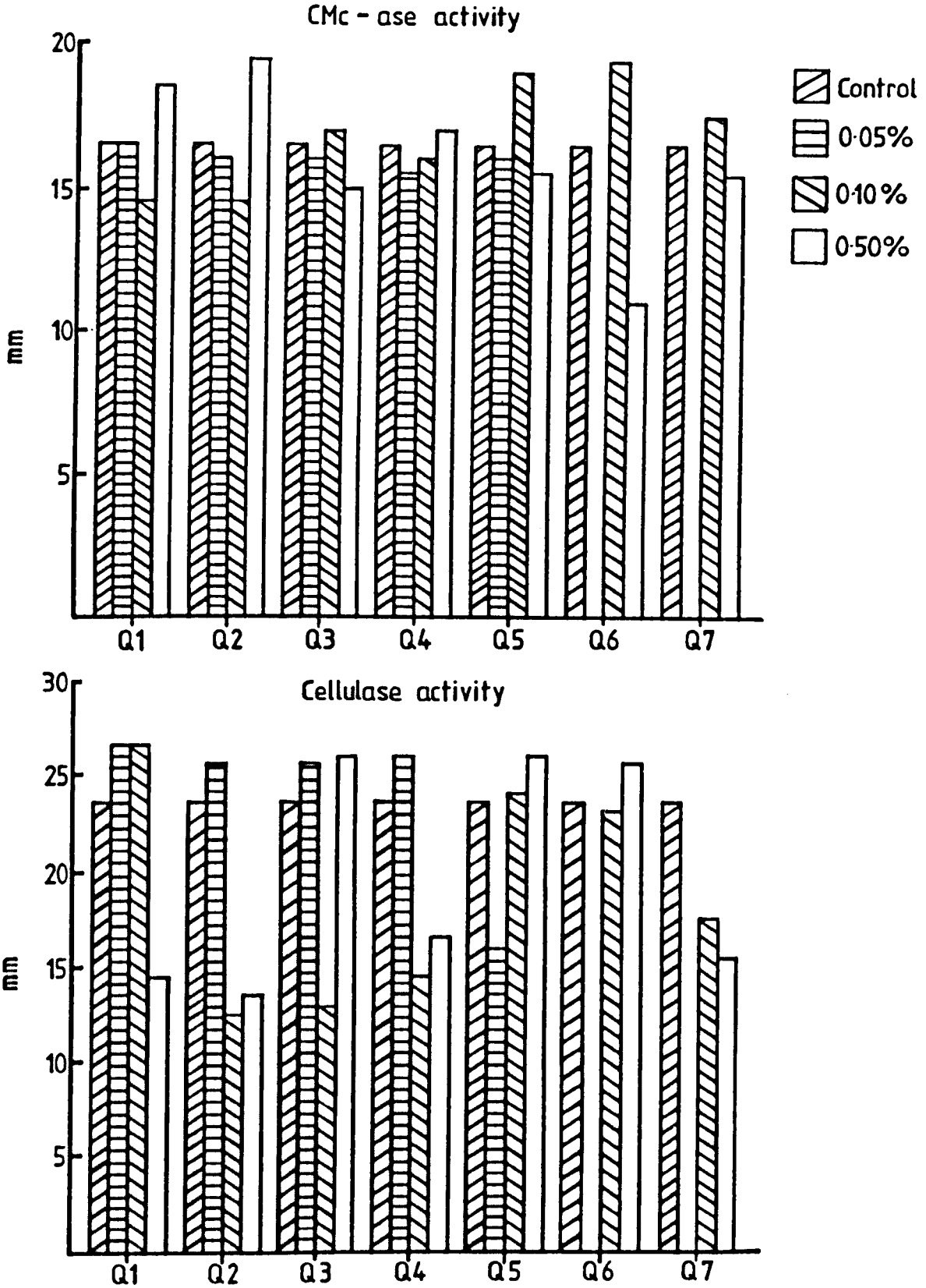


Fig. 8.2 Effect of lignin model compounds on rumen cellulolytic enzyme formation (0.05% of Q6 & Q7 levels were not determined).

presence of the phenolic. It was clear that rumen cultures containing 0.5% ferulic, p-hydroxybenzoic, or t-cinnamic acid or Klason lignin and 0.1% guaiacol, vanillin, indulin or Klason lignin had enhanced the release of endocellulases (Cmcase) whereas at other concentrations these compounds either repressed or had no marked effect on this enzyme.

Addition of 1 ml aliquots of mycotoxin-contaminated maize slightly reduced cellulase production over that observed with boiled or autoclaved controls although marked differences in either cellulose saccharification or endocellulase activities were not observed (Fig. 8.3; Fig. 8.3). Neither was there any marked differences in total cellulase and endocellulase production in rumen cultures supplemented with pure aflatoxin over control. Cultures containing crude mycotoxin extract had reduced total cellulase but slightly higher endocellulase activities (Table 8.4)

8.3.6 Evolution of gases in the simulated rumen

8.3.6.1 Methane

Rumen methane production (Figure 8.4) was significantly suppressed on addition of 0.5% ferulic or p-cinnamic acids, guaiacol, Indulin or Klason lignin . However, addition of 0.05% ferulic or p-cinnamic acid, guaiacol, or vanillin enhanced methane output (Fig. 8.4). Moreover 0.1% ferulic or t-cinnamic acid, Indulin or Klason lignin showed modest inhibitions of methane

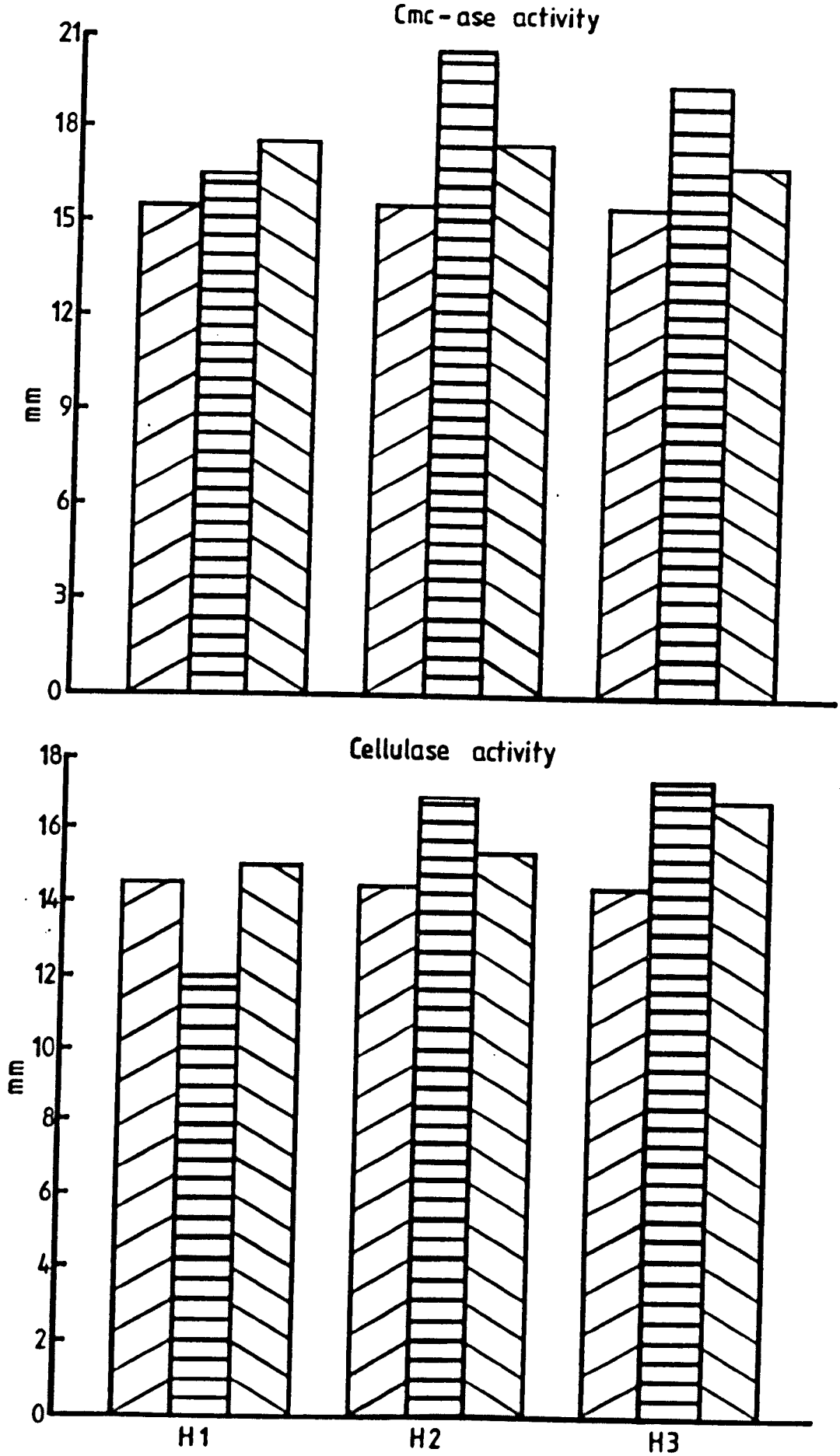


Fig. 8.3 The effects of fungal metabolite (*A. flavus*) on rumen endocellulase and total cellulase activity.

production.

Evolution of methane was stimulated with increasing addition of fresh *A. flavus* cultures and, unexpectedly, less with either boiled or autoclaved fungal cultures. A 10 - 20 fold stimulation was observed on supplementation with fresh *A. flavus* metabolites (Table 8.4). Methane production was slightly lower with cultures containing pure aflatoxin than with controls containing cellulose with or without methanol. With cultures containing crude mycotoxin extract, methane production was particularly high (Table 8.4).

8.3.6.2 Carbon dioxide

Addition of ferulic acid to batch fermenters stimulated carbon dioxide evolution whereas suppression was observed with high concentrations (0.5%) of guaiacol, t-cinnamic acid, vanillin, Indulin or Klason lignin (Fig 8.4). Stimulation of carbon dioxide production was recorded with 0.05% or 0.1% vanillin or t-cinnamic acid. Similarly carbon dioxide production was higher with 0.05% guaiacol than with elevated guaiacol concentrations. Carbon dioxide evolution was stimulated in parallel with increasing concentration of p-hydroxybenzoic acid and at 0.5% the lid of the experimental bottle was forced open by gas pressure. A similar observation was recorded with addition of 10 ml aliquots of extracts of *A. flavus* fermented maize. Thus results recorded for each of these were were based on 24 h incubations. Furthermore, carbon dioxide evolution was

KEY TO THE FIGURES

Lignin model compounds

Q1	Ferulic acid
Q2	p-hydroxybenzoic acid
Q3	Vanillin
Q4	t-cinnamic acid
Q5	Guaiacol
Q6	Indulin lignin
Q7	Klason lignin

A. flavus metabolites

H1	Fresh metabolites
H2	Boiled metabolites
H3	Autoclaved metabolites

*Values for 0.5% levels of p-hydroxybenzoic acid were based on 24 h incubation.

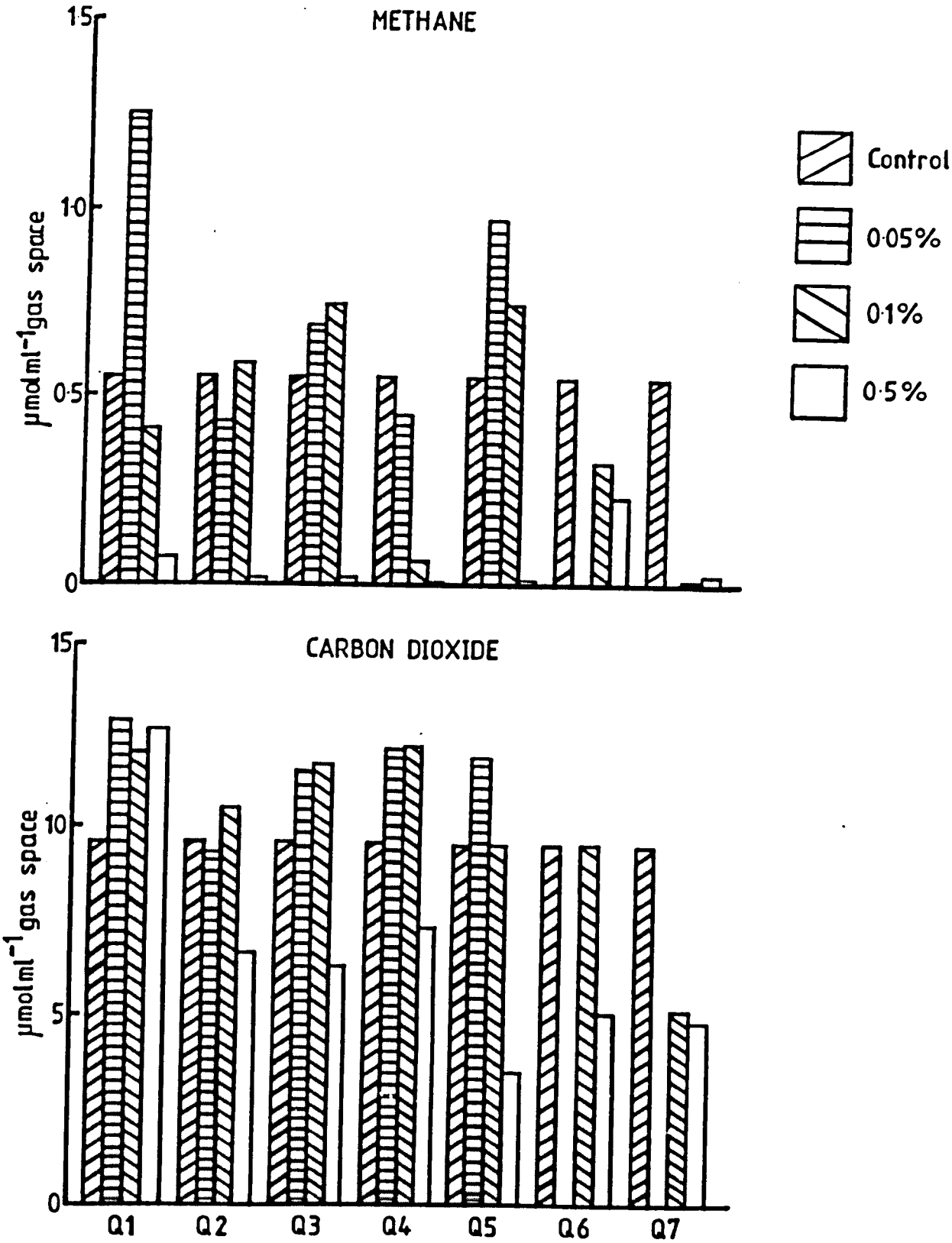


Fig. 8.4 Effect of lignin related phenolic compounds on the output of rumen gases (methane and carbon dioxide). (0.05% levels of indulin-Q6 & klason-Q7 lignin were not determined).

1.5 - 2-fold higher in vessels supplemented with 5 ml aliquots of either fresh or autoclaved *A. flavus* cells than in controls and at 1 ml addition slightly higher values were recorded than in controls of untreated maize liquor (Table 8.5). However, in cultures containing aflatoxin, carbon dioxide production was considerably lower than in controls with cellulose alone, but slightly less than those containing methanol. Carbon dioxide formation was exceptionally high with cultures supplemented with crude mycotoxin extract when compared to controls with or without added methanol (Table 8.4).

8.3.7 Production of volatile fatty acids

8.3.7.1. Acetate

The effect of phenolic compounds and fungal metabolites on volatile fatty acid production in the rumen simulation is shown in Fig. 8.5. Acetate production was slightly lower with addition of 0.05% p-hydroxy benzoic acid , vanillin or t- cinnamic as compared to control values. With 0.5% p- hydroxybenzoic acid (data for 24 h incubation shown), 0.05% ferulic acid and 0.05% guaiacol, results comparable with values obtained in controls were recorded. Marked reductions in acetate output were observed with 0.5% vanillin, guaiacol, Indulin, or Klason lignin. In general, it was observed that acetate production in the presence of phenolic compounds was either comparable or slightly less than obtained with control vessels (Fig 8.5).

TABLE 8.5 EFFECT OF FRESH OR BOILED OR AUTOCLAVED *A. flavus* CULTURES ON RUMEN GAS PRODUCTION DURING CELLULOSE SACCHARIFICATION

	VOLUME OF CULTURE EXTRACTS ADDED			
	Control	1ml	5ml	10ml
Fresh *A. flavus* cells				
CO_2[b]	10.81	12.31	20.76	13.33[a]
CH_4[b]	5.23×10^{-3}	1.52	4.31	0.023[a]
Boiled *A.flavus* cells				
CO_2	10.81	7.33	nd	nd
CH_4	5.23×10^{-3}	0.018	nd	nd
Autoclaved *A.flavus* cells				
CO_2	10.81	5.63	11.46	nd
CH_4	5.23×10^{-3}	0.021	0.016	nd

[a]Incubated for 24 hr.

nd = not determined

[b]methane and carbon dioxide measured as micromoles per ml gas space

Acetate production was stimulated with addition of 1 ml or 5 ml aliquots of extracts of *A. flavus* fermented maize whether or not such extracts had been boiled although acetate accumulation was 2-3 fold greater with fresh than with heated extracts (Fig. 8.5). However, acetate formation was slightly lower in cultures containing pure aflatoxin than with controls whereas the opposite was observed with crude mycotoxin extracts (Table 8.4).

8.3.7.2. Propionate

The results shown in Figure 8.6 revealed that propionate production is markedly reduced with increasing concentrations of ferulic or p-hydroxybenzoic acids, guaiacol, vanillin, Indulin, or Klason lignin. It was observed that 0.5% guaiacol had a particularly severe effect on production. However phenolic compounds had a very limited influence at the lowest 0.05% concentrations.

Extracts of fresh *A. flavus* cultures stimulated propionate production more than boiled or autoclaved cells, with boiled extracts stimulating more than autoclaved extracts (Fig. 8.6). Pure aflatoxin reduced propionate production whereas no significant effect was observed on addition of crude mycotoxin extract (Table 8.4).

KEY TO THE FIGURES

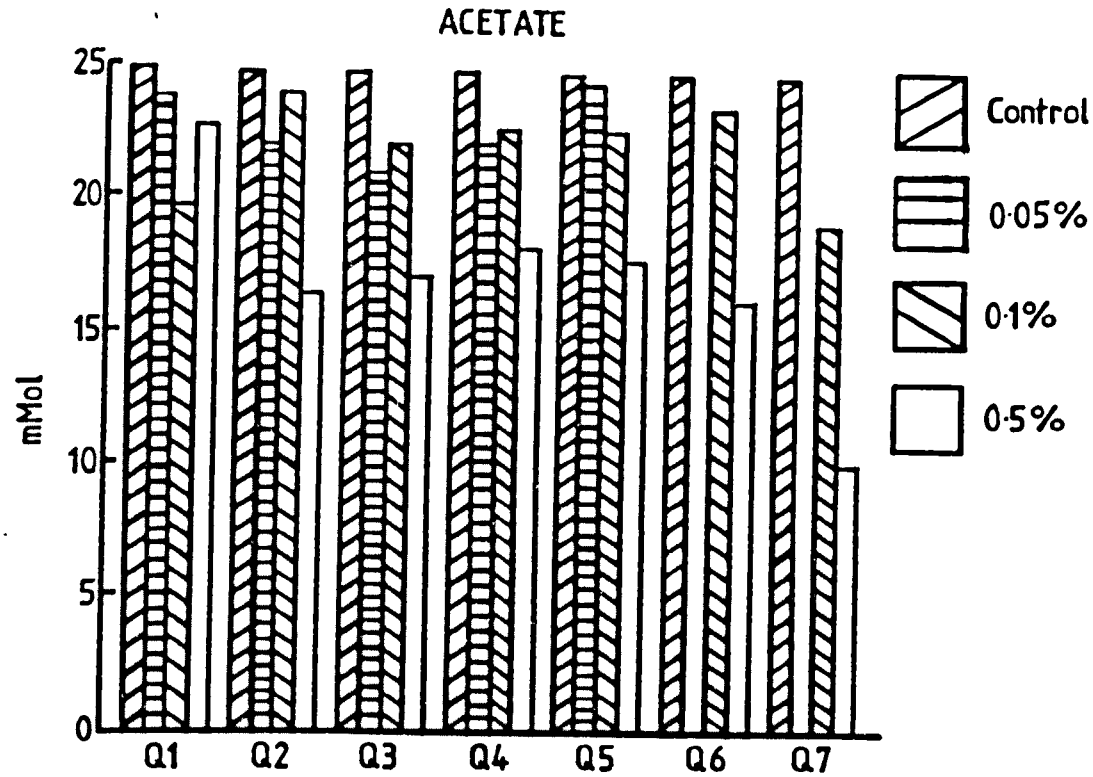
Lignin model compounds

Q1	Ferulic acid
Q2	p-hydroxybenzoic acid
Q3	Vanillin
Q4	t-cinnamic acid
Q5	Guaiacol
Q6	Indulin lignin
Q7	Klason lignin

A. flavus metabolites

H1	Fresh metabolites
H2	Boiled metabolites
H3	Autoclaved metabolites

*Values for 0.5% levels of p-hydroxybenzoic acid were based on 24 h incubation.



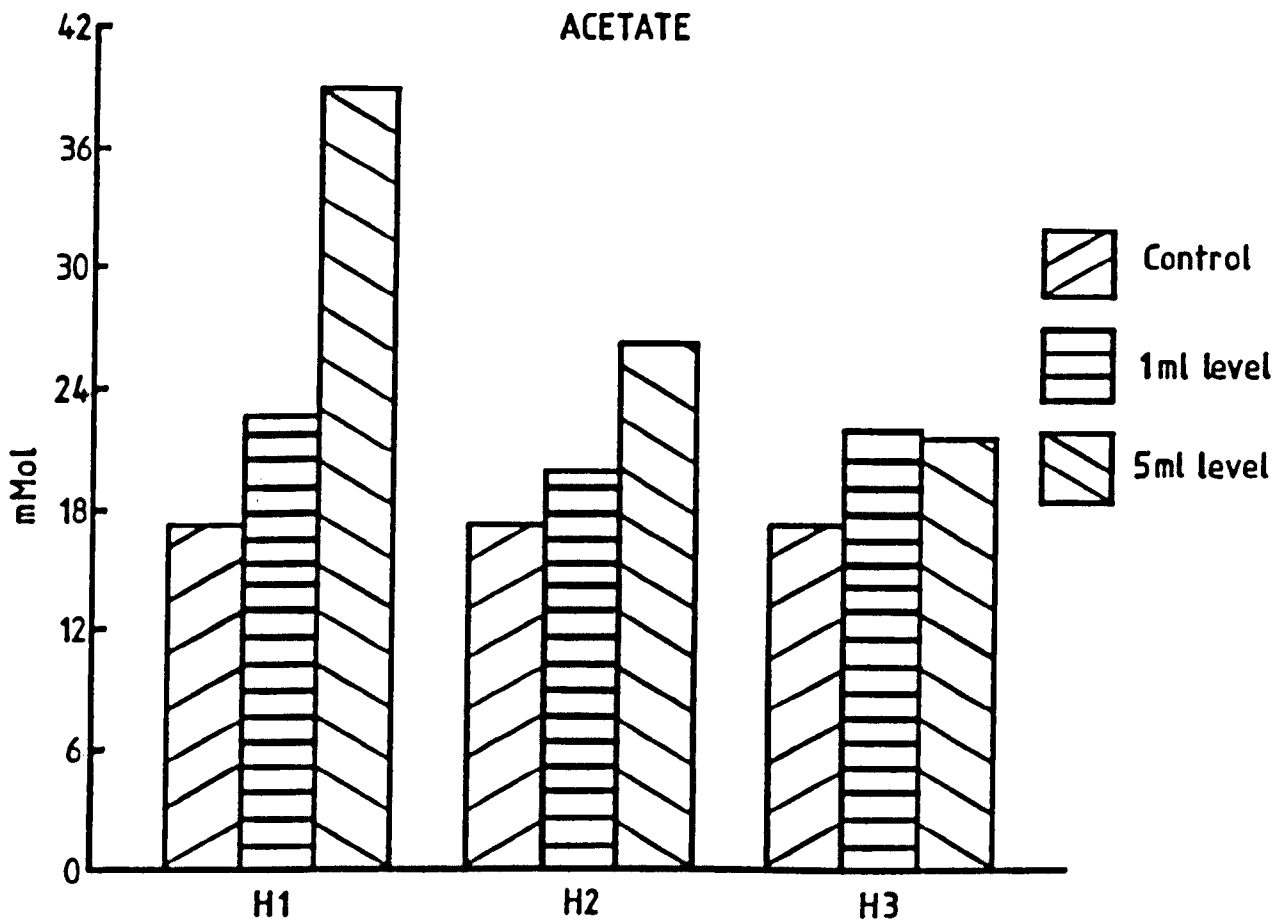


Fig. 8.5 Effect of fungal metabolite (*A. flavus*) or lignin model compounds on rumen acetate production (0.05% levels of Q6 & Q7 were not determined).

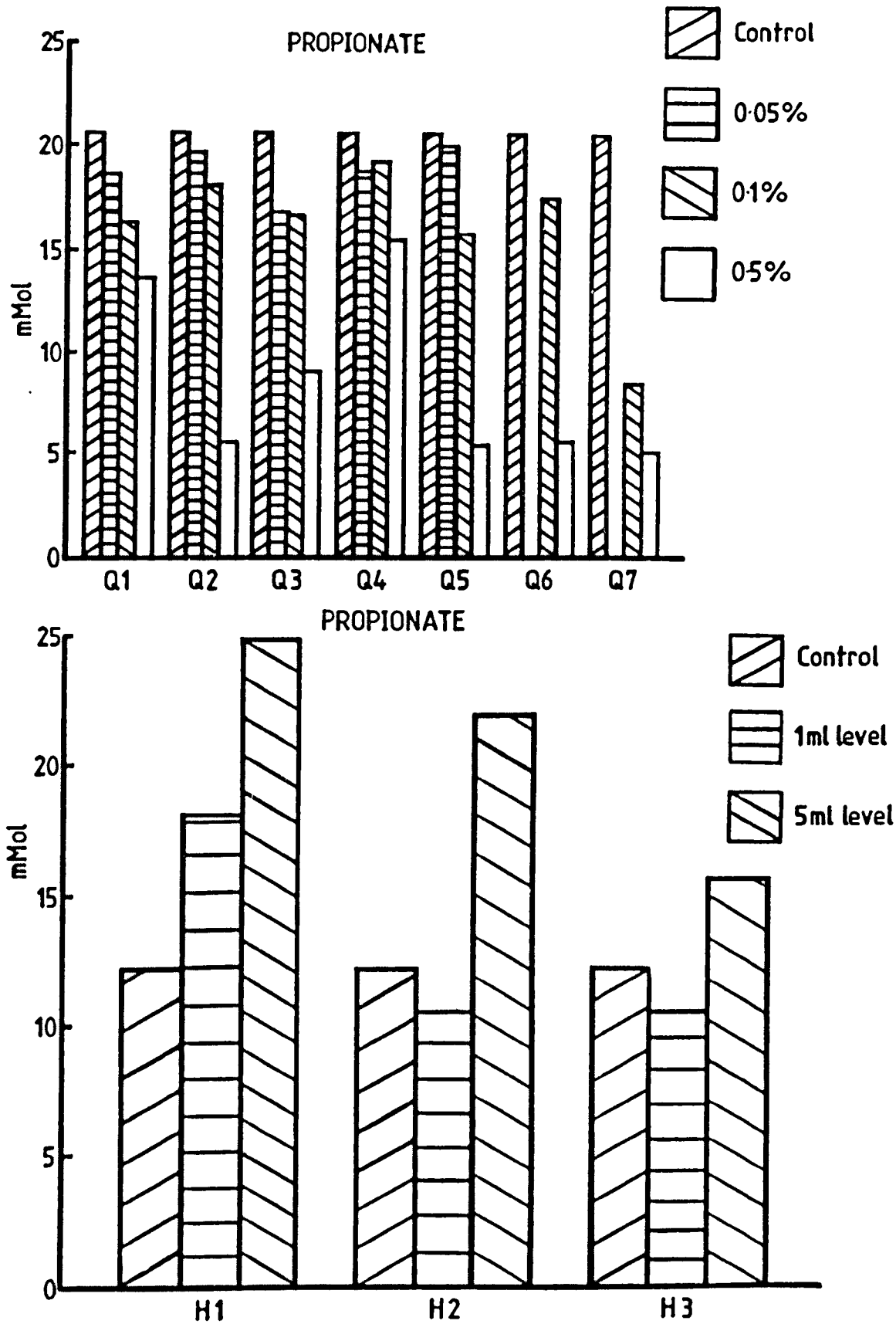


Fig. 8.6 Effect of fungal metabolite (*A. flavus*) or lignin model compounds on rumen propionate production (0.05% levels of Q6 & Q7 were not determined).

8.3.7.3. Butyrate, Isobutyrate, Valerate and Isovalerate production

Isobutyrate production was inhibited by phenolic compounds with the exception of 0.05% p-hydroxybenzoic acid, 0.05 - 0.1% vanillin or 0.1% guaiacol (Fig. 8.7a). Production of butyrate was slightly enhanced by 0.05% p-hydroxybenzoic acid, 0.5% vanillin, 0.1 - 0.5% Klason lignin and 0.1% guaiacol but was low in the presence of other concentrations of the phenolic compounds (Fig. 8.7a). Comparable results were recorded for isovalerate and valerate (Fig. 8.7c). Extracts of *A.flavus* fermented maize, whether fresh or heated, stimulated production of the four volatile acids in proportion to concentration (Fig. 8.7b & d). Purified aflatoxin reduced concentrations of butyrate and isovalerate below levels recorded for control samples whereas supplementation with crude mycotoxin appeared to stimulate production of butyrate and isovalerate and reduce concentrations of isobutyrate and valerate as compared to controls with cellulose alone (Table 8.4).

8.4 DISCUSSION

The results obtained indicated that both lignin-related phenolic compounds and fungal metabolites had an influence on the volatile fatty acids produced in simulated rumen fermentations.

KEY TO THE FIGURES

Lignin model compounds

Q1	Ferulic acid
Q2	p-hydroxybenzoic acid
Q3	Vanillin
Q4	t-cinnamic acid
Q5	Guaiacol
Q6	Indulin lignin
Q7	Klason lignin

A. flavus metabolites

H1	Fresh metabolites
H2	Boiled metabolites
H3	Autoclaved metabolites

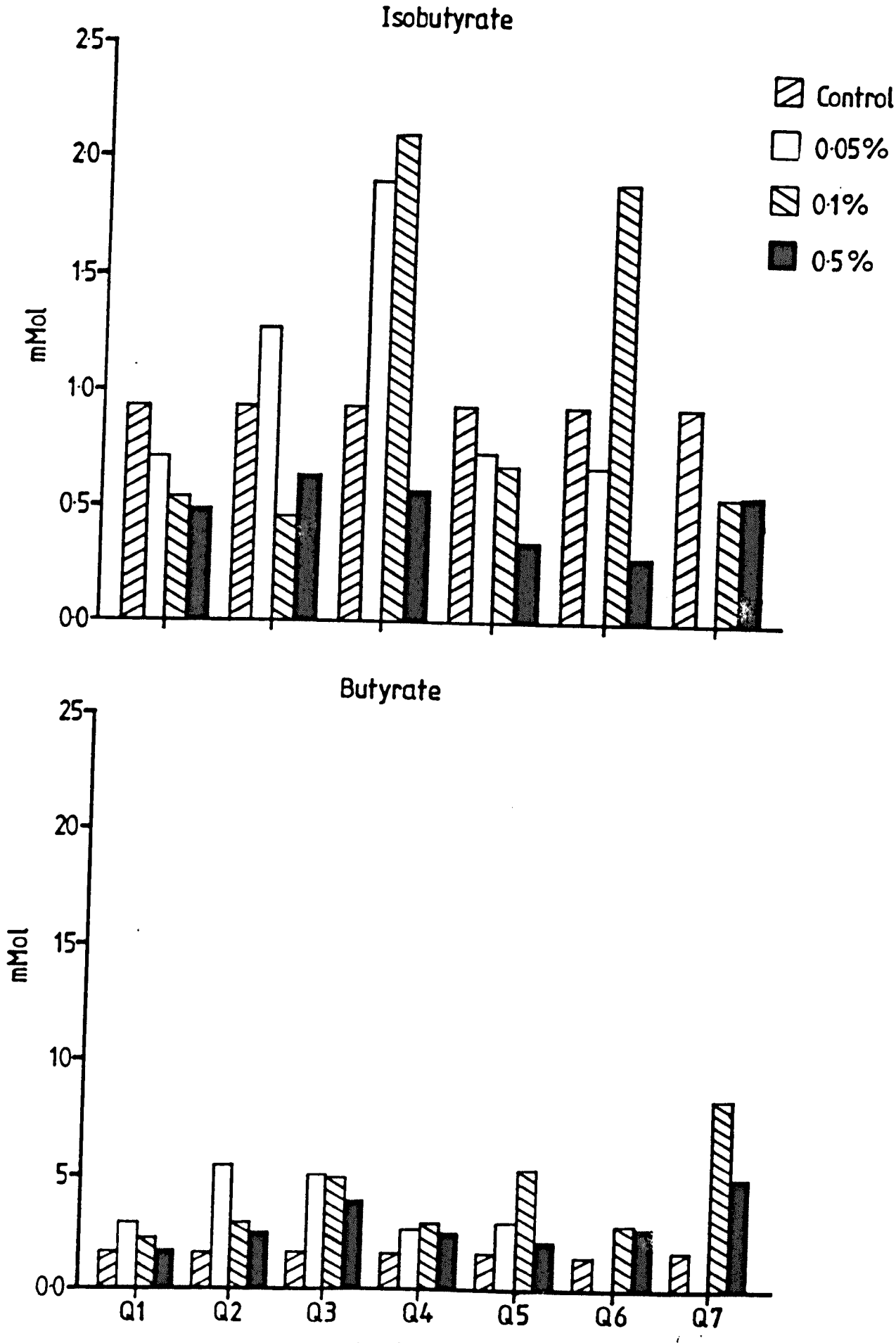


Fig. 8.7a Effect of lignin related compounds on rumen isobutyrate and butyrate production (0.05% of Q6-indulin & Q7-klason lignin was not assessed).

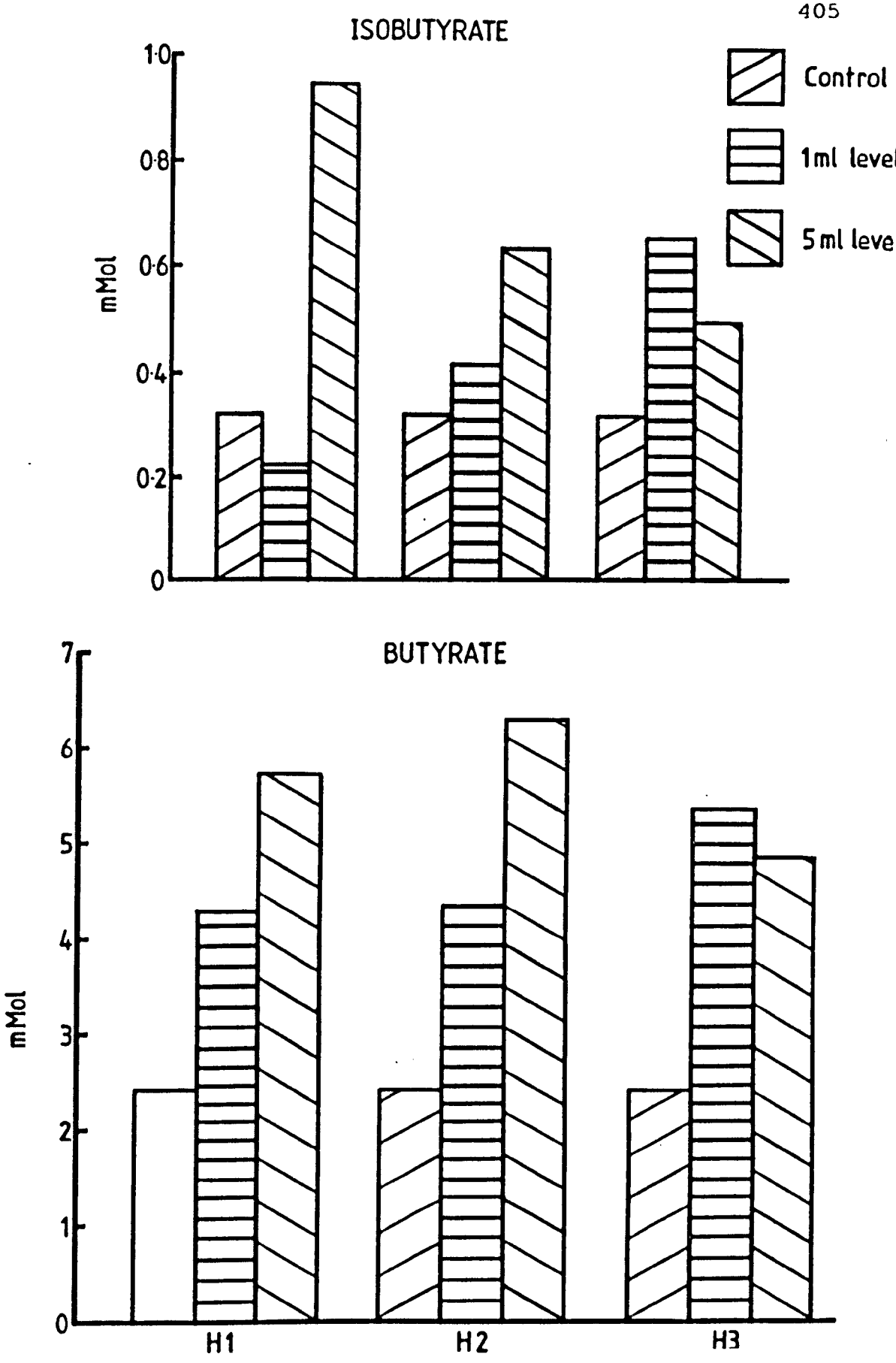


Fig.8.7b The effect of *A.flavus* metabolite on the output of isobutyrate and butyrate during rumen saccharification of crystalline cellulose.

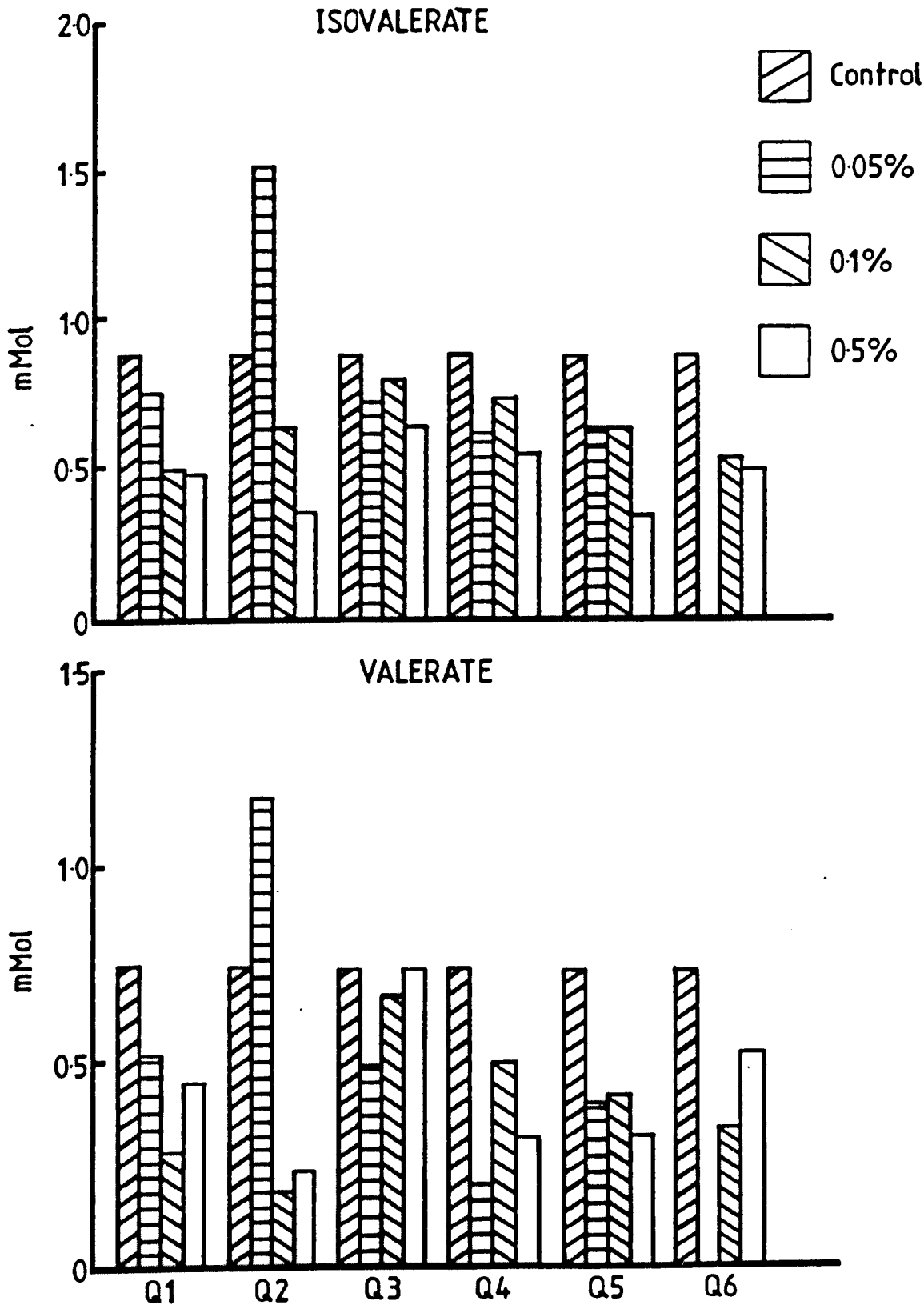


Fig. 8.7c Effect of lignin model compounds on valerate and isovalerate production during fermentation of crystalline cellulose (0.05% of Q6 was not determined).

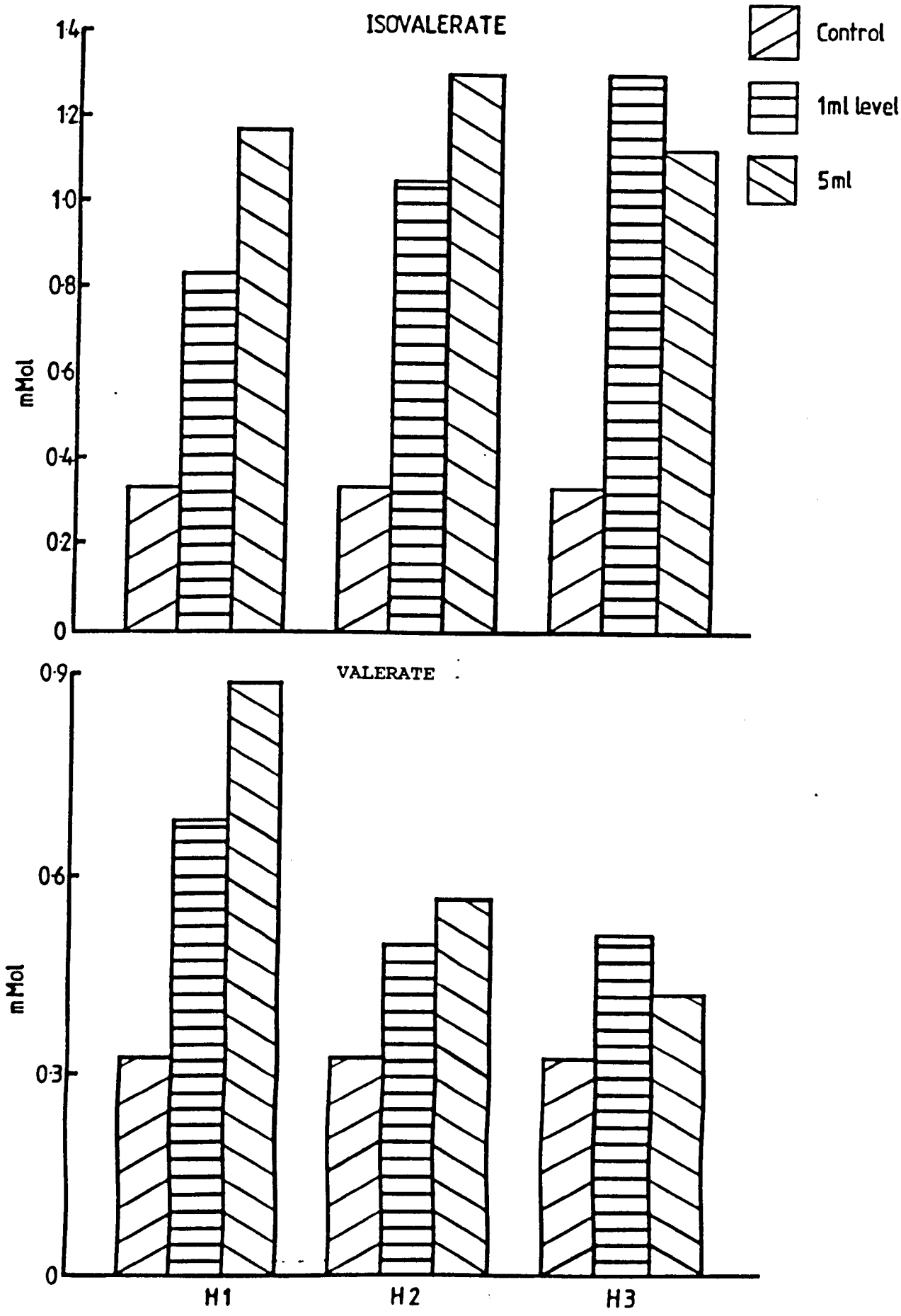


Fig. 8.7d Effect of A.flavus metabolites on the output of isovalerate and valerate during rumen saccharification of

Aflatoxin

Aflatoxin production in grain is influenced both by nutrients and environmental factors (Silman *et al.*, 1979; Faraj, 1990). Production of the mycotoxin in this project was likely to have been influenced by the high moisture content, relative humidity (98%) and period of incubation. Silman *et al.* (1979) demonstrated that aflatoxin production increased in parallel with substrate water content, and obtained 6- 7 ppm aflatoxin after 10 days at 20% substrate moisture content and lower values with decreasing levels of moisture. Faraj (1990) reported aflatoxin concentrations of 3717 ppb at 0.98 water activity and 50 ppb at 0.90 a_W in corn kernels fermented with *A. flavus*.

pH

The phenolic compounds at 0.05% - 0.1% had generally varying effects on rumen microbial activity as revealed by the reduction in pH to values compareable to control cultures. This was however not observed with the highest concentrations, 0.5%, of guaiacol, vanillin, Indulin and Klason lignin. Newbold and Wallace (1990) concluded there was a direct relationship between rumen pH values and bacterial population and recorded substantial decline in rumen bacterial populations at pH lower than 6.8. It is accepted that most rumen microbiota multiply most rapidly at pH values between 6.7 and 7.0. Chen *et al.* (1985) suggested that anaerobic degradation of lignin and

phenolic compounds would yield volatile acids and gases with a consequent reduction in pH.

Similar results were recorded when pure or crude toxin or fresh *A. flavus* fermented maize were added. No reduction in pH was observed when boiled or autoclaved *A. flavus* cells were added to cultures except on addition of 5 ml of autoclaved cells. This reduction in pH in the presence of fungal metabolites can be attributed to increased formation of volatile acids as a result of cellulose breakdown.

Bacterial population

Populations of rumen bacteria were influenced both by the presence of phenolic compounds and their concentration (Table 8.3). Phenolic compounds reduced bacterial populations either through lowering of pH by influencing volatile acid production or through specific toxicity of the compounds, particularly at the higher concentrations. Fungal metabolites had no marked effect on bacterial population and reductions below values for controls can be explained by the observed values for culture pH. Slight decreases were observed with pure aflatoxin but these can be ascribed to the toxic effect of the methanol supplied with the toxin (Sinha & Arora, 1982). Borneman *et al.* (1986) concluded that in general the phenolic compounds, in the absence of a source of carbohydrate, were unable to support microbial growth whereas Varel and Jung(1986) reported a 3 fold increase

in cellulolytic bacteria on supplementation of cultures with vanillin.

Soluble sugar

The observed accumulation of soluble sugar with high concentrations of either ferulic acid or guaiacol could be explained as either inhibition of hexose fermentation in cells or stimulation of cellulolytic activity in stressed cells. Borneman *et al.* (1986) also reported that cellulose depolymerisation by rumen microbes was enhanced in presence of p-coumaric and syringic acids. However, if soluble sugar accumulation is used as an index of fibre breakdown, it can be seen that these phenolic compounds may have an influence on fibre breakdown in the rumen. Akin *et al.* (1988) observed that the presence of certain phenolic substrates (coumaric and ferulic acid) resulted in reduced breakdown of Italian grass cell walls as determined by scanning electron microscopy and that fewer bacteria appeared to be associated with cell walls in the presence of the phenolic compounds. The inhibitory effect of the phenolic compounds was reduced if microbial suspensions were exposed to trans p-coumaric acid for 48 h prior to inoculation.

One explanation for the high concentrations of soluble sugar observed with cultures supplemented with fresh *A. flavus* fermented maize may be enhanced polysaccharide degradation products by enzymes secreted

by *A. flavus*. However, 5 ml aliquots of boiled or autoclaved *A. flavus* cells which had a similar effect on sugar accumulation demonstrate a possible probiotic effect of fungal cells in rumen fermentation, producing a stimulation of microbial flora as suggested by Newbold and Wallace (1990). This could explain increases in digestibility of forage feeds in the presence of yeast or *A. oryzae* cultures.

Cellulolytic enzymes

No consistent influence on cellulolytic activity was observed on supplementation with the phenolic compounds or fungal metabolites. Although moderate concentrations of ferulic acid enhanced total cellulase activity, endoglucanase activity was inhibited but less with 0.5% phenolic. Similar observations were recorded with p-hydroxybenzoic and t-cinnamic acid or guaiacol. Rumen cellulolytic enzyme activity can be related to accumulation of soluble sugar since the products of cellulose breakdown will be simultaneously metabolised into gaseous products and volatile acids. However, with the anaerobic fungus *Neocallimastix* MC-2 recent reports have suggested enhancement of release of fibre degrading enzymes when substrates contained higher levels of saponifiable ferulic and p-coumaric acids (Borneman *et al.*, 1990). Moreover, differences in cellulolytic enzyme activity obtained may also be explained in terms of catabolite repression of enzyme formation. Borneman *et*

al. (1989), in a study of the relationship between rumen fermentation products and cell wall degrading enzymes, concluded that depolymerisation of cellulosic substrates was not detected until after the disappearance of available reducing sugars. It was also found that increases in enzyme activity correlated with accumulation of fermentation products and substrate utilisation.

No significant differences in total cellulase and endoglucanase activity were observed in the presence of either crude or purified fungal toxins. It is possible that the toxin may be broken down in the rumen or that the concentration added was insufficient to have any adverse effect on the enzymic activities.

Rumen fermentation gases

In simulations of rumen fermentation, production of gases such as hydrogen, methane, and carbon dioxide and output of volatile acids are used as indices for assessing fibre or substrate breakdown in the rumen. The pathways for carbohydrate breakdown in the rumen are well documented. By contrast, information on anaerobic breakdown of lignin and lignin-related phenolic compounds in the rumen is very limited. Colberg and Young (1985) demonstrated that radioisotopically-labelled model lignin oligomers were metabolised in anaerobic cultures into monoaromatic compounds or gaseous products: a possible explanation for observed increases in methane and carbon dioxide output observed in cultures supplemented with the

cell wall-related phenolics. Such increases could be due to direct oxidation of alcohol and aldehyde groups of the phenolic monomers by anaerobic rumen bacteria to yield carbon dioxide and methane as reported by Zeikus *et al.* (1982) and Mountfort and Bryant (1983). In contrast, Martin (1988), in a study of the effects of p-coumaric acid and ferulic acid on methane production and fibre digestion by rumen microbes *in vitro*, concluded that digestibility of hay was reduced with addition of trans p-coumaric acid and trans -ferulic acid. Both phenolic compounds also appeared to inhibit methane production with p-coumaric acid having the greater effect. Chen *et al.* (1985) reported a novel pathway for anaerobic cleavage of β aryl ether bonds of the dimeric lignin model compound-Veratrylglycerol -β- guaiacyl ether(VGE) to yield vanillin, vanillic and guaiacoxylacetic acid (GAA) and also further metabolites, including guaiacol, phenol and phenoxyacetate. These compounds were finally mineralised into carbon dioxide and methane.

There were obvious differences in rumen gas output from cultures supplemented with boiled and autoclaved or fresh extracts of *A. flavus* cells. The slight increase in carbon dioxide release observed with autoclaved cells can possibly be attributable to stimulation of metabolism by fungal breakdown products. Marked differences in the output of end products of rumen fermentation were observed with fresh and killed *A. flavus* biomass.

Autoclaving denatures proteins and destroys biological activities of cultures (Newbold & Wallace, 1990) resulting in a very limited stimulation of rumen gas production. Fibre-degrading enzymes present in fresh *A. flavus* cultures would be active in the simulated rumen. Repeating the experiment with purified aflatoxin showed no distinct effect on methane and carbon dioxide output relative to controls.

Production of volatile acids

The phenolic compounds reduced values for acetate production to below those of controls which was in contradiction to the findings of Chen *et al.* (1985) who observed a 3.5 fold increase in acetate, propionate, isobutyrate and butyrate production in presence of guaiacoxyacetic acid. No effect was observed on valeric acid production. However, the experimental conditions described were markedly different in terms of incubation period and substrates from those in the present investigation. The observed differences could also arise from the mineralisation of volatile acids or utilisation of acids n syntheis of microbial biomass. Further possibilities are that phenolic compounds were either inhibitory to rumen microbes responsible for volatile acid formation or that the phenolics stimulated metabolism of acids thus reducing accumulation. Rumen microbes are known to differ in their sensitivity for toxic compounds (Harris *et al.*, 1987) with archaebacteria

being comparatively insensitive to compounds that inhibit the growth of eubacteria. Similarly, Stewart and Richardson (1989) in a study of the effect of monensin and lasalocid on formate production by the rumen bacterium *Methanobrevibacter smithii*), reported that formate did not accumulate under all experimental cultures indicating that the bacteria continued to metabolise formate in the presence of monensin.

In the present study, it was observed that purified aflatoxin slightly inhibited acetate and methane production which could be explained as inhibition of methanogenesis from acetate. However with fresh *A. flavus* extracts, acetate and other volatile acids accumulated, suggesting stimulation of growth of organisms involved in production of volatile acids by components of the fermented maize.

The manner in which cell wall-related phenolics inhibit fibre breakdown or influence the rumen fermentation is unclear. Rumen microbes would be predicted to tolerate low concentrations of these compounds but the results obtained in this study indicated that the modified lignin preparations had significant effects on fibre breakdown by rumen microbes. Recent reports have suggested that culture filtrates of anaerobic fungi are able to solubilise or degrade lignin in cell walls of bermuda grass releasing trans isomers of ferulic and p-coumaric acids (Borneman *et al.*, 1990). However these anaerobic fungi primarily degrade

unlignified cell walls in leaf blades and stems. Kiviasi *et al.* (1990) also reported the solubilisation of phenolic compounds during degradation of barley straw by rumen micro-organisms in a simulated rumen.

SECTION 9

9.1 GENERAL DISCUSSION

This study has investigated some of the limitations in upgrading agro-industrial plant residues for feeding of ruminants using fungal delignification. A novel feature is that it has highlighted the beneficial effect of using mixed fungal cultures in solid substrate fermentations to enhance rates of delignification. A number of cell wall-related phenolics have an influence on both hydrolysis of polysaccharides and oxidation of lignins by fungi and also an effect on microbial activity in a simulated rumen.

Biological delignification has been targeted towards production of animal feeds (Buswell & Odier, 1987), edible mushrooms (Tautorus, 1985) and composting processes (Peerally, 1981). The use of aerobic fungi to attack cell- walls of fibre and saccharification of cell wall carbohydrates has been demonstrated at a laboratory scale (Kirk *et al.*, 1976). However, limited data on the physiology of the process and effect of products on rumen fermentations have been published which may be a reflection of the lack of detailed information on the metabolism of lignin-attacking aerobic fungi and anaerobic rumen micro-organisms and their ability to modify, transform, degrade and, in many cases, mineralise

both recalcitrant plant polymers and xenobiotic organic compounds (Zadrazil, 1990; Bhatnagar & Fathepure, 1991; Bhatnagar *et al*, 1991). Mixed cultures of aerobic fungi are also known to be able to perform certain catabolic operations not known to occur with monocultures in laboratory fermentations.

Analyses of spruce sawdust in this study gave a composition that differed to some extent from the published findings of other workers. The Klason lignin content of 28.70% was outside the range 24.66 - 27.69% quoted by Effland (1977) for spruce woods. This can be ascribed to variations in age or variety of spruce or extraction procedures adapted. Although Effland did not state the age of the wood analysed in his work, in this study the originating tree was thought to be over 65 years of age. Moreover, Effland used benzene to remove tannins and other extractives from wood whereas in this study diethyl ether was employed. Higher values for lignin content were obtained using the methods described by Effland (28.7%) than with those for estimating detergent lignin (27.6%). Moreover the presence of cutins, carbohydrates and nitrogenous material in Klason lignin has been reported (Hartley & Ford, 1989).

Similar problems have been reported in quantification of the polysaccharides. Values for cell-wall polysaccharide, cellulose and hemicellulose for spruce sawdust were 68.69%, 43.21%, and 26.39% respectively

whereas Wayman and Parekh (1990) reported 72.3%, 47.4% and 24.9% for spruce. Under the assay conditions used, only 60 - 65% of glucose residues were recovered in acid hydrolysates of polysaccharides. Losses due to excessive or incomplete hydrolysis was compensated for by applying a factor 1.52 as described by Englyst and Cummings (1988).

It is clear that although spruce like other woods has a high lignin content, making it superficially unattractive as a ruminant feed, it represents both a major reservoir of total carbon fixed by photosynthetic plants and a resource of renewable raw material for use in new technologies.

The first objective in the project was to clarify the influence possible co-substrates might have on delignification in solid-substrate fermentations and whether cell-wall related phenolics might act as carbon sources for lignocellulose- degrading fungi. It became clear that fungi differed significantly in response to phenolic compounds in media. Vanillic acid was the least and phenol and coumarin the most inhibitory to a range of fungi and isolates. Addition of simple or complex carbohydrate enhanced production of fungal biomass but interestingly the modified cell wall polymer carboxymethyl-cellulose, a preferred soluble substrate for hydrolytic endoglucanases, was a poor carbon source for phenol oxidase secreting fungi. A further observation was that most fungi could produce significant amounts of

biomass in growth solely on minimal salts, including trace elements, and agar as reported by other workers (Ander & Eriksson, 1976; Eriksson *et al.*, 1983). Ander and Eriksson(1976) reported that 10^{-3} M vanillic, ferulic and p-hydroxybenzoic acid inhibited both mycelium, and with catechol, cellulase and xylanase production by *Sporotrichum pulverulentum*. Nazareth and Mavinkurve (1987) also concluded that many cell-wall related phenolics were inhibitory to fungal growth. Use of surface culture techniques in such studies of toxicity has been criticised although this strategy was also used by Ander and Eriksson (1976). Inhibition has also been studied in submerged liquid batch culture (Eriksson *et al.*, 1983) but such data is likely to be of limited value in clarifying the effect of such compounds on solid-substrate fermentations.

However submerged culture was used to determine the effect of varying concentrations of ferulic acid on the different enzymes involved in lignocellulose depolymerisation. The influence of this compound on enzyme activity in *P. chrysosporium* were similar to the published results of Ander and Eriksson (1976) for the imperfect form of this basidiomycete *Sporotrichum pulverulentum* (Table 9.1). The induction of laccase activity by ferulic acid has previously been reported in *C. versicolor* by Evans *et al.* (1984). There are no previous reports in the literature on inductive laccase production in *P. sajo-caju*.

Table 9.1 EFFECT OF PHENOLIC COMPOUNDS[a] ON FUNGAL METABOLITES

FUNGI	METABOLITES PRODUCED						
	SUGAR	MYCELIAL	LACCASE	B-GLUCOSIDASE	ENDOCELLULASE	TOTAL CELLULASE	AUTHOR
P.chrysosporium	+	++++	-	++++	++++	++++	This study
C.versicolor	+	+++	++/+++	+++	++	++	This study
P.sajo-caju	+	+	++	+++	+	++	This study
T.harzianum	+	++++	-	++++	+	++++	This study
C.cellulolyticum	+	++++	-	++++	++++	++++	This study
S.pulverulentum[b]	nd	++++	nd	nd	++++	++++	Ander & Eriksson (1976)
P. versicolor[a]	nd	nd	++	nd	nd	nd	Reinhammer (1984)

+ or - indicates the type of effect the phenolic compound had on the synthesis of these metabolite.

+ = synthesis of the metabolite was enhanced at high concentration of the phenolic.

++ = production of the metabolite was stimulated at low concentration of the phenolic

+++ = formation of the metabolite was enhanced at moderate concentration of the phenolic.

++++ = synthesis of the metabolite was inhibited at high concentration of the phenolic

[a] Phenolic substrate used was ferulic acid

[b] Phenolic substrate used includes vanillic acid & catechol

Although the strain of *P. chrysosporium* employed in this study was not observed to produce laccases, it was able to decolourise the polymeric dye Remazol brilliant blue, indicative of production of lignin peroxidases (Glenn & Gold, 1983). However the other two fungi *P. sajo-caju* and *C. versicolor* were also able to decolourise the dye, which has not previously been reported. It has not been clarified if the laccases in these organisms can also decolourise this dye, although *C. versicolor* has been reported to depolymerise complex lignins (Evans, 1985). This dye decolourisation activity in *P. sajo-caju* and *C. versicolor* was not influenced by exogenous nitrogen in growth media as in *P.chrysosporium*, a similar finding to that of Leatham and Kirk (1983).

Although the possibility that the phenol-oxidase laccase may also effect decolourisation of dyes has not been excluded, the role of laccase in lignin depolymerisation has not been clarified since Ishihara (1980), concluded this enzyme was catabolic and Haars and Hutterman (1980) biosynthetic. Laccases catalyse oxidation of both ortho and para -di phenols by removing an electron and a proton from hydroxyl radical groups to yield free radicals that can readily polymerise by coupling. However, Evans (1985) concluded there was no experimental evidence demonstrating that laccase alone can effect extensive lignin breakdown. Haars and Hutterman (1980), in studies of the laccase in the white-rot *Fomes annosus*, showed that thioglycolic acid, a

copper chelating agent, eliminated laccase activity but not breakdown of lignosulphonates. However in the presence of laccase, no low-molecular weight breakdown products from lignosulphonates were observed suggesting polymerisation of these components by this enzyme. Evans (1985) showed inhibition of laccase with specific antibodies had no effect on lignin depolymerisation by *C. versicolor* and that breakdown products detected during lignin breakdown by this fungus were similar to those reported for *Phanerochaete chrysosporium* suggesting a similar oxidative mechanism. Moreover lignin degradation in stationary cultures of *C. versicolor* was inhibited in the presence of catalase, which destroys hydrogen peroxide required for oxidation of lignin (Evans 1985). Evans *et al*, (1984) also isolated a haem protein from *C. versicolor*, similar to the lignin peroxidase of *P. chrysosporium*. It can thus be concluded there is a similar lignin oxidation activity in *P. sajo-caju*. It also seems reasonable to assume that fungi which were observed neither to secrete laccase nor decolourise the polymeric dye were unable to depolymerise lignins.

It can also be argued that results obtained from growth of fungi on synthetic media are unlikely to predict the patterns of lignocellulose degradation in solid-substrate fermentations with these fungi on natural substrates. However correlations were observed, for instance, the inhibitory effects of D(+)-glucosamine and ammonium dihydrogen phosphate on polymeric dye

decolourisation by *P. chrysosporium* were correlated with reduced rates of delignification in the presence of the amino sugar or $NH_4H_2PO_4$ in solid substrate fermentations. The stimulation of dye decolourisation in *C. versicolor* and *P. sajo-caju* with addition 0.05% ferulic acid had a parallel in enhanced rates of delignification in solid-substrate fermentations. Arora and Sandhu (1986) reported 14.13% to 14.91% lignin depletion by *Polyporous (Coriolus) versicolor* when gallic or tannic acid or orcinol was added to sawdust compared to 11.75% with controls. However, in contrast, the inhibitory effect of ferulic acid and yeast extract on dye decolourisation in surface culture of *P.chrysosporium* was not observed with solid substrate fermentations. No negative effect of exogenous nitrogen on delignification by *P.sajo-caju* and *C.versicolor* could be demonstrated either solid-substrate fermentations or with dye decolourisation in surface culture. Parallels between accumulation of solubles and synthesis of microbial protein were observed in solid-substrate and submerged fermentation.

Rates of depolymerisation of lignin and polysaccharides recorded in this investigation compare favourably with those reported by other workers (Table 9.2). Observed differences can be related to variations in culture condition and precise strain of white rot fungi employed in fermentations. The enhanced rates of delignification of birch and aspen wood over that observed with spruce, confirmed in this study (Table

Table 9.2 RELATIONSHIP BETWEEN LIGNIN AND POLYSACCHARIDE DEGRADATION (%) OBSERVED IN THIS STUDY WITH THOSE OF OTHER WORKERS.

Organism	Substrate	Incubation period(days)	Degradation (%)	Author
LIGNIN				
C.versicolor	spruce	42	14.86	This study
P.chrysosporium	spruce	42	8.28	This study
P.chrysosporium + C.versicolor + P.sajo-caju	spruce	21	16.24	This study
P.versicolor	angiosperm wood	60	23.72	Arora & Sanbhu (1986)
S.pulverulentum	spruce	46	10.0	Ander & Eriksson (1977)
S.pulverulentum	birch	46	25.0	Ander & Eriksson (1977)
C.versicolor	Aspen wood	56	24.1	Reid & Seifert (1982)
P.chrysosporium	Aspen wood	21	13.0	Reid & Seifert (1982)
Phlebia tremellosus	Aspen wood	42	34.0	Reid(1989)
Lentinus lepideus	Pine wood	56	9.2	Agosin *et al* (1989)
CELLULOSE				
S.pulverulentum	spruce	46	5.0	Ander & Eriksson (1977)
S.pulverulentum	birch	46	10.0	Ander & Eriksson (1977)
L.lepideus	pine	52	19.7	Agosin *et al* (1989)
C.versicolor	spruce	42	11.34	This study
P.chrysosporium	spruce	42	6.8	This study
P.chrysosporium + C.versicolor + P.sajo-caju	spruce	14	7.4	This study

9.2), suggest that hard wood lignins are more susceptible to fungal attack than soft woods as has been postulated by Buswell and Odier (1987). Spruce has a higher lignin content than birch wood and the intermonomer lignin bonds will differ as a function of guaiacyl:syringyl:hydroxycinnamic acid ratio as concluded by Sjostrom (1981). Sjostrom (1981) reported 48% arylglycerol-beta-aryl ether bonds in spruce lignin and 22- 28% in birch lignin. Similarly, about 9.5-11% biphenyl and 3.5 - 4% diaryl ether bond types are found in spruce lignin whereas birch lignin contains 4.5% of biphenyl and 1% of diaryl ether bond types. Moreover certain bonds such as glyceraldehyde-2-aryl ether(2%), non-cyclic benzyl aryl ether (6-8%), phenyl coumaran(9-12%), 1, 2 diarylpropane (7%) and beta- linked structures (2%) are found in spruce but not birch wood lignin. Such compositional differences may reduce susceptibility to attack by the oxidative lignin peroxidase enzymes of these fungi. The effect of this may be a limitation on the rate of solubilisation of cell wall polysaccharides by enzymes (Paterson, 1989).

Values for simulated rumen digestibility of spruce wood obtained correlated well with the findings of other workers (Table 9.3) on similar substrates (Reid, 1985, 1989 : Agosin *et al.*, 1989). The highest observed digestibility was 26.68% for a monoculture (*C. versicolor*) and 37.66% for mixed culture of the three fungi after 6 and 3 weeks of incubation, respectively.

TABLE 9.3 COMPARISON OF DIGESTIBILITY REPORTED IN THIS STUDY WITH THAT REPORTED BY OTHER WORKERS

Substrate	Fungi	Incubation period (d)	Digestibility (%) Before Trt	After Trt.	Author
Wheat straw	*C.versicolor*	120	40.0	62.0	Zadrazil & Brunnert (1981)
Aspen wood	*C. versicolor*	56	-	32.8	Reid & Seifert (1982)
Birch wood	*I. edodes*	69	20	60	Buswell & Odier (1987)
Aspen wood	*Fomes ulmarius*	77	46	64	"
Aspen wood	*P.tremellosus*	30	18.7	42.5	Reid (1989)
Beech	*A. aegerita*	60	6.0	3.0	Zadrazil (1980)
Beech	*P. cornucopiae*	60	6.1	17.6	Zadrazil (1980)
Spruce	*C.versicolor*	42	7.75	26.68	This study
Spruce	*C.versicolor + P.sajo-caju + P.chrysosporium*				
"	"	21	8.10	37.66	This study
Spruce + Bran	*C.versicolor*	42	23.58	46.36	This study
Birch	*P. chrysosporium*	42	13.79	32.24	This study

Agosin *et al.* (1989) using rumen cannula for *in vivo* quantification of dry matter digestibility obtained an estimate of 16% with radiata pine fermented with *Lentinus lepideus* for 8 weeks, whereas Reid (1985) reported *in vitro* digestibility of about 55% with aspen wood treated with *Merulius tremellosus* for 6 weeks. Reid (1989) also reported digestibility of 50.3% with aspen wood fermented with *Phlebia tremellosus* for 6 weeks. The difference between results obtained by Reid and his coworkers and in the present study may arise from differences in substrates, fungal strains, and method for digestibility quantification used. Reid (1985, 1989) quoted results based on crude breakdown of polysaccharides with cellulase enzymes whereas in this study RUSITEC was employed for all dry matter digestibility estimations of fermented woods. Variation in hemicellulose structure, phenolic content, cellulose order and age of wood will also influence comparison of results of different research groups (Hartley & Ford, 1989).

Differences between dry matter digestibilities observed with sound spruce and birch wood, barley straw, hay and rice bran may be explained in terms of variations in cell wall composition. Hartley and Ford (1989) concluded that an understanding of the relationships between cell wall constituents and wall biodegradation is vital to improve the economics of animal production, since enhancing digestibility of feedstuffs should mean better conversion ratios for

feeds. However cell wall constituents, notably phenolic constituents, polymeric lignin and highly ordered cellulose structure have all been suggested as factors limiting degradation of wall polysaccharides by rumen enzymes and microbes (Hartley, 1985). Studies in animal nutrition have revealed that the lignin content of graminaceous cell walls is inversely related to the digestibility in the ovine or bovine rumen. There have been recent reports that there is a correlation between alkali release of phenolic constituents of cell walls and biodegradability of walls measured *in vitro* with rumen liquor abstracted from sheep or cattle (Hartley & Ford, 1989). For instance, p-coumaric acid content of barley stem (3.42 mg g^{-1}) was markedly less than in maize stem (33.05 mg g^{-1}) but higher than in wheat bran (0.07 mg g^{-1}) as reported by Hartley and Keene (1984). In addition trans- dehydrodiferulic acid content of wheat bran (0.18mg g^{-1}) was higher than barley stem (0.13 mg g^{-1}) and maize stem (0.11 mg g^{-1}), whereas total ferulic acid content of barley stem, wheat bran and maize stem were quoted as 2.26, 5.60 and 3.78 mg g^{-1}, respectively. Such compositional factors may influence rumen digestibility. Certain monocotyledons and species of dicotyledon have similar contents of p-coumaric and ferulic acids to those of graminaceous plant residues like hay. It cannot be considered unexpected to have obtained maximal digestibility with rice bran (47 - 54%), followed by hay (37 - 43%), barley straw (31.6%), birch wood (13.8%) and

least with spruce wood at 7.8%. However, in this study the delignified substrates rice bran, had a digestibility of 51.3% (by *C. versicolor*), hay - 42.5% (*C. versicolor*), barley straw - 35.8% (*P. chrysosporium*), birch sawdust - 32.2% (*P. chrysosporium*), and spruce sawdust - 26.7% (*C. versicolor*)

The relationship between monomer composition of lignin and rumen digestibility reported in this study assists in the elucidation of factors limiting lignocellulose biodegradation. In this work vanillin was observed to be highly toxic to rumen bacteria, while t-cinnamic acid was inhibitory to methanogenesis and industrial indulin lignin strongly inhibitory to endoglucanase activity and acetate production in the simulated rumen. Other authors have investigated toxicity of monomeric phenolic acids and aldehydes of graminaceous cell walls to rumen microflora (Akin & Rigsby, 1987; Chesson *et al.*, 1982) and concluded that trans p-coumaric acid was the most toxic of the phenolic acids, decreasing the rate of digestion of cell walls by reducing the attachment of fibre-degrading bacteria (Akin *et al.*, 1988). However, as reported by Hartley and Keene (1984), only small amounts of ferulic acid are thought to be present in the cell walls of graminaceous plants (Hartley & Keene, 1984), and it seems unlikely that such concentrations are sufficient to limit degradation of cell walls substrates in hay, bran and barley straw. It is thought that the significantly higher proportion of β-

aryl ether and phenylcoumaran bonds may be responsible for the antinutritional property of the wood. Moreover, the presence of lignans and other extractives together with oxidation products from fungal degradation of spruce lignin may have altered the composition of microbial populations in the simulated rumen.

Wallace *et al.* (1981) earlier reported that the inclusion of monensin in ruminant diet affected the compostion of rumen microbial flora and recorded low levels of Gram positive cocci and increases in numbers of propionate producing Gram negative rods in the presence of the antibiotic monensin. Similarly they observed that the composition of ciliate population altered, since *Polyplastron*, was eliminated from "RUSITEC" vessels receiving monensin. In contrast Czerkawski (1984) noted that "RUSITEC" is capable of maintaining indefinitely a normal protozoal population when balanced solid food such as hay is used but much of the protozoa disappeared from the effluent when the food is supplied in solution. Czerkawski and Breckenridge (1979) also reported a considerable decline in protozoal the numbers in presence of heavily lignified inert matrix -as wood shavings. Supplying soluble nutrients to supplement the lignified material could not support the growth of protozoa in the liquid phase rather it encouraged good growth of bacterial species which increased when the protozoal numbers declined(Czerkaski & Breckenridge, 1979).

9.2 : IMPLICATIONS OF THE RESULT ; Efficiency and limitations of this study

Use of SSF in delignification

Aerobic oxidation of lignin and protein enrichment of plant residues by fermentation mycelial fungi can be achieved in both submerged or solid state cultures (Moo-Young *et al.*, 1978 ; Hesseltine, 1987). Enrichment of barley straw with protein and enhancement of digestibility value was achieved by Peitersen and Andersen (1978) in pretreatments with with 5 - 7% alkali solution followed by submerged fermentation with *Trichoderma viride* in a specialised bioreactor vessel. Although saccharification of cellulose to cellobiose was obtained, subsequent enzymatic hydrolysis of cellobiose was very low. The final product had crude protein contents of 21 to 24%, 30% lignin, and 67% digestibility. The most attractive system for protein enrichment of lignocelluloses is the Waterloo process developed by Moo-Young *et al.* (1979) who reported increases in protein content and specific growth rate of the thermotolerant fungus *Chaetomium cellulolyticum* on maize cob media.

Lobank *et al.* (1983) reported protein enrichment and reduction of lignin content of rye straw fermented with *Penicillium verruculosum* and animals (calves) fed with the bioconverted substrate showed no adverse effects, with increases in average daily weight gain of 32.9% compared to control animals. However, Golovleva *et al.*

(1983) studied solid state fermentation of birch sawdust with *Pholiota tuberculosa* and reported 5 - 6% increases in protein accumulation and extensive degradation of cellulose and lignin.

In this study increases in protein content of fungal treated spruce sawdust and moderate increases in digestibility and reduction in lignin content were obtained (Table 6.18 : Figs. 6.1 & 6.5). Supplementation of spruce sawdust with adjuncts such as rice bran or cow concentrate led to greater increases in digestibility, reductions in lignin content and more enhanced rumen fermentation characteristics (Tables 6.13 & 6.14). However, It is particularly difficult to assess the efficiency of feed bioconverted for ruminants consumption since higher protein content in bioconverted lignified substrate may imply loss of fermentable carbohydrates for the rumen fermentation. As a result a balance must be struck between net protein value (utilisation in the rumen) of bioconverted plant residue and reduction in lignin and polysaccharide content. Greater loss of fermentable carbohydrate fractions was observed in monoculture fungal fermentations (Table 6.21), especially when minimal medium contained mainly mineral nutrients and supplementation with such nutrients will not enhance substrate protein content, than with mixed cultures. This loss could be minimised by using adjuncts rich in soluble and fermentable carbohydrates. Increases in protein content may be higher in submerged fermentation as

compared to solid state cultures, reduction in lignin content may not be achieved. This may stem partly from the absolute requirement for oxygen by delignifying fungi: as oxygen can often be a limiting factor in liquid culture.

From the results of this study, using SSF as a delignification step for preparation of plant residues for animal feed was considered most suitable on the basis of cost - benefit ratio and product demand. The low capital investment, reduced energy requirement, low waste water output, improved product recovery, elimination of foam problems and less rigorous control of few parameters makes the SSF an attractive technology that can be applied by albeit technically advanced farmers with ease.

Manipulation of fungal physiology

Nitrogen is known to regulate synthesis of lignolytic enzymes in *P. chrysosporium* (Buswell & Odier, 1987). However, lignolytic enzyme activity in other basidiomycetes species was not influenced by concentration of nutrient nitrogen (Leatham & Kirk, 1983). A clear understanding of relevant fungal physiology together with careful manipulation of fermentation conditions would facilitate delignification processes. It is of particular interest to note that repression of lignolytic activity by nutrient nitrogen has only been reported among strains known not to secrete laccases: this was confirmed in this study. Furthermore,

studies in submerged culture showed that there was elevated production of cellulolytic enzymes and soluble sugar in presence of high concentration of ferulic acid (Figs. 4.3 & 4.6). At 0.5% ferulic acid, biomass accumulation and laccase synthesis were inhibited whereas at 0.1% increases in levels of laccase formation, solubilization of cellulose and repression of mycelial formation were observed. On the other hand, a major problem reported during fungal fermentation with some of the lignocellulosics was increased formation of mycelial biomass at expense of soluble sugar and polysaccharide fractions available for rumen fermentation (Table 6.2).

Consequently a number of factors must be considered when a lignified substrate is converted to animal feed. The required composition of feed for a monogastric will differ widely from that of a ruminant, since monogastrics do not have the microbial enzyme systems required to saccharify celluloses. The net protein value of their substrate will be critical in evaluating the benefit to the farmer. Ruminants with bacteria that secrete enzymes hydrolysing plant polysaccharides may not have a significant need for protein although nitrogen is often limiting in the diet. Therefore using mycelial fungi to improve nutritional value of lignocellulosics in terms of protein content, while conserving carbohydrate fraction for ruminant fermentation seems contradictory. To produce ruminant feeds of high protein content using mycelial fungi creates a dilemma. The extent to which fungal

protein will be digested by ruminant enzymes has not been clarified but the products of cell autolysis will enhance the rumen fermentation.

On the basis of results from this investigation, high quality ruminant feed could be produced from high fibre plant residue by fungal fermentation if the net protein content of the final product is less important than total carbohydrate. It is suggested that certain phenolic compounds in feeds will suppress mycelial biomass production while inducing high levels of cellulolytic and lignolytic enzymes leading to accumulation of degradation products. Such catabolic products can not be utilised by aerobic fungi but may enhance feed digestibility and rumen fermentation characteristics of bioconverted substrate. Obviously, fungi will differ in physiological response to substrate composition which should be optimised for each organism. An alternative approach would be the use of complex substrates like rice bran (with 18% protein) and cow concentrate as adjuncts in the fungal fermentation of lignocellulosics. Such adjuncts were found to be particularly effective in delignification with *C. versicolor* and could enable developments of commercial processes. A second option would be the use of mixed culture fermentation. This led to significant accumulation of solubles and delignification in the short period of time prior to the onset of production of significant mycelial biomass. The reduction in the period

of fermentation with mixed cultures increases the chances of adapting mixed culture technique for farm use and these will be more stable than monocultures.

RUSITEC as an adequate model system for fibre breakdown

Czerkaswki (1984), concluded that the rumen is a complex system with heterogeneous contents of liquid and solid digesta which are subject to differential flows and uneven distribution of micro-organisms. The rumen microbial system is maintained by the supply of food and conditions within the system are often regulated by well developed movement, the process of rumination, absorption of acids and the supply of saliva. In most artificial rumen systems, anaerobic fungi and protozoa appear to be lacking, simulating the action of only the fibre degrading bacteria. Since, fungi and protozoa have been observed to play active roles in cell wall degradation in the rumen (Stewart & Richardson, 1989), systems lacking such organisms will record a shortfall in the extent of fibre breakdown. Moreover, the absence of such organisms in simulated rumen fermentations of sawdust may explain the low output of methane and volatile acids (Bauchop & Mountfort, 1981). Bauchop and Mountfort (1981) reported that the anaerobic fungus *Neocallimastix frontalis* ferments plant cells with the formation of acetate, lactate and ethanol, together with formate and hydrogen from which methane may be formed by methanogens. Recent observations has shown that co-culture of *N. frontalis*

with methanogens enhanced plant cell wall degradation (Bauchop & Mountfort, 1981). A similar observation was made with another anaerobic fungus *Piromonas communis* (Citron *et al.*, 1987). Other problems likely to be encountered with simulated systems like RUSITEC, include increased dilution rate due to excessive infusion of artificial saliva especially when highly lignified feeds such as sawdusts are used.

Despite these limitations, RUSITEC has been found to be a more effective system compared than other fibre digestibility methods. Results obtained from these systems are used to predict rumen fermentation patterns and conversion of feed components that might occur *in vivo* in the rumen. Alternate methods such as crude cellulase enzyme assay and the cannula method lack these advantages. However, it would be possible to improve the effectiveness and performance of a RUSITEC system by deviating from the routine procedure. For instance, adequate microbial flora can be maintained if more food bags are used, half the number of bags containing a conventional feed like hay and the others roughage (test sample). Then the digestibility patterns of each food bag could be determined.

Secondly, continous addition of anaerobic fungi and protozoa could help to maintain stable fermentation patterns when unconventional substrates are used as feed source.

Net digestibility as an index for assessing bioconverted plant residues (ruminant feeds)

Marked increases in net digestibility values of fungal fermented spruce sawdusts reported in this study compare favourably with the findings of other workers on similar substrates (Tables 9.2 & 9.3). However, the typical rumen fermentation products with delignified lignocellulosics were present in considerably lower concentrations than those obtained with native hay. For fungal fermented plant residues to replace hay or serve as an alternate feed source for ruminants, it would probably have similar rumen fermentation characteristics to hay. In the present study it was not possible to achieve such comparable fermentation pattern with monocultures. Much better enhancements of rumen fermentation were observed when mixed cultures or complex carbohydrate adjuncts were used.

9.3 CONCLUSION

Detergent and colorimetric methods employed for the estimation of lignin and polysaccharide contents were particularly useful for composition determinations of native substrates. It was impossible to monitor changes in lignin and polysaccharide constituents of fungal fermented material using these methods, largely due to deposition of microbial biomass on the complex substrate. These problems could be alleviated if microbial biomass were selectively removed after fermentation by chemical or enzyme (chitinases or glucanases) treatments prior to analyses.

Using polymeric dye decolourisation as evidence of lignolytic activity, it was impossible to differentiate the three major lignolytic enzymes (laccases, ligninases & Mn- peroxidase). The evidence as to whether lignin peroxidases or laccases effect delignification of lignocelluloses is contradictory. However, it seemed that in both *C. versicolor* and *P. sajo-caju* the delignifying activity was not repressed by the presence of nitrogen. This implies that more than one enzyme system may be implicated in delignification in these fungi, thus enabling it to perform both polymerisation reaction and dye decolourisation. Elevated levels of β-glucosidase and endocellulase activity obtained in the presence of ferulic acid with laccase producing strains indicated that the latter enzyme could possess the capability of

detoxification of substrates. It was inferred that maximum delignification and saccharification of native lignocelluloses could be achieved, only with strains able to produce both peroxidase and laccases.

Marked delignification of sawdust was achieved in presence of carbohydrate adjuncts and with mixed cultures. Supplementation with simple sugars or water (alone) did not enhance delignification. A similar conclusion was obtained for digestibility. The net digestibility values obtained with mixed cultures or in presence of adjuncts were comparable to those reported for hay. Comparatively, there were distinct differences between rumen fermentation patterns (methane, CO_2, volatile acids) recorded for the two substrates (hay and spruce sawdust).

The high levels of fungal biomass deposited in polymeric substrate relative to soluble carbohydrate fractions will not enhance rumen digestibility. But accumulation of degradation products from aerobic oxidation of lignin together with cell autolysates had a beneficial effect on rumen fermentation as indicated by gas (carbon dioxide and methane) evolution.

9.4 SUGGESTIONS FOR FUTURE WORK

A number of topics, emerging from this study, would merit further attention:

(a) Application of mixed culture of lignolytic fungi in delignifications by solid-substrate fermentations.

(b) Transfer of genes coding for laccase and lignin peroxidase enzymes of *C. versicolor* or *P. sajo-caju* to thermophilic strains of *P. chrysosporium*. Production of mutants defective in polysaccharide degrading enzymes is likely to be time-consuming and requiring usage of a conventional genetic crossing programme.

(c) Use of specific antibodies to further clarify the role of individual enzymes involved in lignin biodegradation.

REFERENCES

ADLER, E. (1977). Lignin chemistry - past, present and future. *Wood Science and Technology* 11, 169 -218.

AGOSIN, E. & ODIER, E.(1985) . Solid state fermentation, lignin degradation and resulting digestibility of wheat straw fermented by selected white rot fungi. *Applied Microbiology and Biotechnology* 21, 397 - 403.

AGOSIN, E. ; TOLLIER, M.T. ; BRILLOUET, J.M.; THIVEND, P. & ODIER, E. (1986). Fungal pretreatment of wheat straw ; effects on the biodegradability of cell wall structural polysaccharide, lignin and phenolic acids by rumen micro-organisms. *Journal of the Science of Food and Agriculture* 37, 97 - 106.

AGOSIN, E. ; JARPA, S.; ROJAS, E. & ESPEJO, E. (1989). Solid state fermentation of pine sawdust by selected white rot fungi. *Enzyme and Microbial Technology* 11, 511 - 517.

AIDOO, K.E (1979). Studies on the koji process in soy-sauce fermentation with mechanised solid state fermenters- A scale up process. Ph.D thesis, *University of Strathclyde*, Glasgow, UK.

AIDOO, K.E. ; HENRY, R. & WOOD, B.J.B. (1982). Solid substrate fermentations. *Advances in Applied Microbiology* 28, 201 - 237.

AKIN, D.E. (1979). Microscopic evaluation of forage digestion by rumen micro-organisms - A Review. *Journal of Animal Science* 48, 701 - 710.

AKIN, D.E. & RIGSBY, L.L. (1987). Mixed fungal populations and lignocellulosic tissue degradation in the bovine rumen. *Applied and Environmental Microbiology* 53, 1987 - 1995.

AKIN, D.E. & BENNER, R. (1988). Degradation of polysaccharides and lignin by ruminal bacteria and fungi. *Applied and Environmental Microbiology* 54(5), 1117 - 1125.

AKIN, D.E ; RIGSBY, L.L. ; THEODOROU, M.K. & HARTLEY, R.D. (1988). Population changes of fibrolytic rumen bacteria in the presence of phenolic acids and plant extracts. *Animal Feed Science and Technology* 19(3), 261 - 275.

AL- AKBAR, M. ; SCAIFE, J.R. & TOPPS, J.H. (1987). The effect of casein, urea and dried poultry waste on the fermentation of starch or cellulose measured *in vitro*. *Journal of the Science of Food and Agriculture* 40, 105 - 118.

AL - ANI, F. (1985). - Delignification of sugar cane bagasse by chemical, physical and microbiological treatments. Ph.D Thesis, *University of Strathclyde*, Glasgow, UK.

AL - ANI, F. & SMITH, J.E. (1986). The effect of different nitrogen supplementation on fermentation of bagasse by *Sporotrichum pulverulentum* wild type and mutants.*Transactions of British Mycological Society* 86, 165 -168.

AL - ANI, F. & SMITH, J.E. (1988). Effect of chemical pretreatments on the fermentation and ultimate digestibility of bagasse by *Phanaerochaete chrysosporium*. *Journal of the Science of Food and Agriculture* 42, 19 - 28.

ALEXANDER, A. G. (1974). Sugarcane physiology. Elsevier Science Publishers, New York.

AMADO, R. & NEUKON, H. (1985). Minor constituents of wheat flour : The pentosans. In *New Approaches to Research on Cereal Carbohydrates* (R.D. Hill & L. Munck, eds.) Pp 241 - 251, Elsevier Publishers, Amsterdam.

ANDER, P. & ERIKSSON, K-E. (1975). Influence of carbohydrates on lignin degradation by white rot fungus *Sporotrichum pulverulentum*. *Svensk Papperstidn* 78, 643 - 652.

ANDER, P. & ERIKSSON, K.E. (1976). The importance of phenol oxidase activity in lignin degradation by the white rot fungus *Sporotrichum pulverulentum*. *Archives of Microbiology* 109, 1 - 8.

ANDER, P. & ERIKSSON, K.E. (1977). Selective degradation of wood components by white rot fungi. *Physiologia Plantarum* 41, 239 - 248.

ANDER, P. & ERIKSSON, K.E. (1978). Lignin degradation and utilisation by micro-organisms. *Progress in Industrial Microbiology* 14, 1 - 58.

ANDREASON, L.E. & REINHAMMAR, B. (1979). The mechanism of electron transfer in laccase catalysed reactions. *Biochimica et Biophysics Acta* 568, 145 - 156.

ANON (1975). Protein Advisory Group(PAG) adhoc working group meeting on clinical evaluation and acceptable nucleic acid levels of single cell protein(SCP) for human consumption. *Protein Advisory Group Bulletin* 54(3), 17.

A.O.A.C (1980). Official method of analysis of the Association of Official Analytical Chemists, 13th edition, Washington D.C.

A.O.A.C (1984). Fiber (acid detergent) and lignin in animal feed. *Official Methods of Analysis*, 14th edition. Pp 162 - 163.

ARIMA, K. (1964). Microbial enzyme production. In *Global Impact of Applied Microbiology*(M.P. Starr ed.). Pp 277 - 295. Wiley and Sons, New York.

ARORA, D.S. & SANDHU, D.K. (1986). Degradation of lignocellulosic residues by *Polyporus versicolor* and the effect of moisture contents and phenolic compounds. *Acta Biotechnology* 6(3), 293 - 297.

ATKINSON, B. (1974) Biochemical reactors. Pion Ltd, London. P.267.

ATKINSON, B.; BLACK, G. M. ; LEWIS, J.S.P. & PINCHES, A. (1979). Biological particles of given size, shape and density for use in biological reactors. *Biotechnology and Bioengineering* 21, 193 - 200.

BAILEY, R.W. ; CLARKE, R.T.J. & WRIGHT, D.E (1962); Carbohydrases of the rumen ciliate *Epidinium ecaudatum*. *Biochemical Journal* 83, 517 - 523.

BAKSHI, M.P.S ; GUPTA, V.K. & LANGAR, P.N. (1986). Fermented straw as a complete basal ration for ruminants. *Agricultural Wastes* 16, 37 - 46.

BAKSHI, M.P.S ; GUPTA, V.K. & LANGAR, P.N. (1987). Effect of moisture level on chemical composition and nutritive value of fermented straw. *Biological Wastes* 21, 283 - 289.

BALASUBRAMANYA, R.H.; KHANDEPARKAR, V.G.; BETRABET, S.M. & SUNDRARAM, V. (1981). Production of biogas from willow dust by a batch fermentation process. *Journal of The Textile Association (India)* 42, 145 - 149.

BALASUBRAMANYA, R.H.; BHARTAWDEKAR, S.P. & KHANDEPARKAR, V.G. (1987). Anaerobic digestion as a method of determining rumen digestibility of cellulosic materials. *Biological Wastes* 22, 229 - 232

BAR-LEV, S.S. & KIRK, T.K. (1981). Effects of molecular oxygen on lignin degradation by *Phanerochaete chrysosporium. Biochemistry and Biophysics Research Communications* 99, 373 - 378.

BAUCHOP, T. & MOUNTFORT, D.O. (1981). Cellulose fermentation by a rumen anaerobic fungus in both the absence and presence of rumen methanogens. *Applied and Environmental Microbiology* 42, 1103 - 1110.

BAVENDAMN, W. (1928). *Z. Pflanzenkrankh* 38, 357 . Cited by Harkin and Obst, 1973.

BEARDMORE, D.M.; FAN, L.T. & LEE, Y.H. (1980). Gamma Ray irradiation as a treatment for the enzymic hydrolysis of cellulose. *Biotechnology Letters* 2(10), 435 - 438.

BECKMANN, E. (1921). Conversion of grain straw and lupins into feeds of high nutrient value. *Festchr. Kaiser Welhelm Ges. Forderung Wiss. Zehnjahrigen Jubilaum* Pp 18 - 26.

BENNER, R. & HODSON, R.E. (1985). Thermophilic anaerobic biodegradation of (^{14}C) lignin , (^{14}C) cellulose and (^{14}C) lignocellulose preparations. *Applied and Environmental Microbiology* 50, 971 - 976.

BENNER, R. ; MAcCUBBIN, A.C. & HODSON, R.E. (1984). Anaerobic biodegradation of the lignin and polysaccharide components of lignocellulose and synthetic lignin by sediment microflora. *Applied and Environmental Microbiology* 47, 998 -1004.

BENTLEY, O.G. ; JOHNSON, R.R. ; HERSHBERGER, J.H.; CLINE, J.H. & MOXON, A.L. (1955). Cellulolytic factor activity of certain short chain fatty acids for rumen micro-organisms *in vitro. Journal of Nutrition* 57, 389 - 400.

BHATNAGAR, L. & FATHEPURE, B.Z. (1991). Mixed cultures in detoxification of hazardous waste, Chapter 11, In *Mixed cultures in Biotechnology* (J.G. Zeikus and Johnson, E. eds). McGraw -Hill New York. Pp 293 - 340.

BHATNAGAR, L.; MU, W.M. ; JAIN, M.K. & ZEIKUS, J.G. (1991). Design and function of biomethanation granules for harzadous waste treatment(H. Verachtert and W. Verstraete eds). Paper presented at the International

Symposium on Environmental Biotechnology 22 - 25 April Ostend, Belgium. Pp 1 -11.

BHATTACHARJEE, J.K. (1970). Micro-organisms as a potential source of food. *Advances in Applied Microbiology* 13, 139 - 161.

BIELY, P. (1985). Microbial xylanolytic systems. *Trends in Biotechnology* 3, 286 - 290.

BLANCHETTE, R.A. (1984). Screening wood decayed by white rot fungi for preferential lignin degradation. *Applied and Environmental Microbiology* 48, 647 - 653.

BLANCHAETTE, R.A. & ABAD, A.R. (1988). Ultrastructural localisation of hemicellulose in birch wood (*Betula papirifera*), decayed by brown and white rot fungi. *Holzforschung* 42, 393 - 398.

BLANCHETTE, R.A. & SHAW, C.G. (1978). Associations among bacteria, yeasts and basidiomycetes during wood decay. *Phytopathology* 68, 631 - 637.

BLOUIN, F.A. and ARTHUR, J.C. (1960). Degradation of cotton in an oxygen atmosphere by gamma radiation. *Journal of Chemical Engineering Data* 5, 470 - 475.

BONE, D.H. and LEVONEN-MUNOZ, E.(1984). Solid state fermentation of oat straw by *Polyporous sp.* A - 336 and the effect of added sugars. *Biotechnology Letters* 6, 657 - 662.

BORAN, N.M. and CZERKAWSKI, J.K. (1983). Incubation of rumen content in the mini artificial rumen response to increasing substrate concentration. In *Nitrogen Metabolism of Farm Animals* (K. Boda ed). Pp 471 -

478. Slovak Academy of Science, Institute of Animal Physiology, Kosice.

BORNEMAN, W.S. ; AKIN, D.E. & VAN ESELTINE, W.P. (1986). Effect of phenolic monomers on ruminal bacteria. *Applied and Environmental Microbiology* 52, 1331 - 1339.

BORNEMAN, W.S. ; AKIN, D.E. & LJUNGDAHL, L.G. (1989). Fermentation products and plant cell wall degrading enzymes produced by monocentric and polycentric anaerobic fungi. *Applied and Environmental Microbiology* 55, 1066 - 1073.

BORNEMAN, W.S.; HARTLEY, R.D. ; MORRISON, W.H. ; AKIN, D.E. & LJUNGDAHL, L.G.(1990). Ferulolyl and p-coumaroyl esterase from anaerobic fungi in relation to plant cell wall degradation. *Applied Microbiology and Biotechnology* 33, 345 - 351.

BRANDEN, R. MALMSTROM, B.G. & VANGARD, T. (1973). The effect of fluoride on the spectral and catalytic properties of three copper containing oxidases. *European Journal of Biochemistry* 36, 195 - 200.

BRUCE, E. L. & HUNT, J.R. (1988). Bioflocullation as a microbial response to substrate limitations. *Biotechnology and Bioengineering* 31, 91 - 101.

BRUNNER, M. ; SCHOBERT, S.M. & SAHM, H.(1987). Anaerobic microbial degradation of halogenated aromatics to form biogas. *Chemie Ingenieur Technik* 59, 55 - 57.

BRYANT, M.P. & SMALL, N. (1960). Observations on the ruminal micro-organisms isolated from inoculated

calves. *Journal of Dairy Science* 43, 654 - 667.

BUCKER, D. (1985). Determination of B-glucanase activity by the congo red method. *Journal of the Institute of Brewing* 91, 264 - 266.

BUSWELL, J.A. & ODIER, E. (1987). Lignin biodegradation. *CRC Critical Reviews in Biotechnology* 6(1), 1 - 63.

BUSWELL, J.A. ; MOLLET, B. & ODIER, E. (1984). Lignolytic enzyme production by *Phanerochaete chrysosporium* under conditions of nitrogen sufficiency. *FEMS Microbiology Letters* 25, 295 - 299.

BUTLER, J. H. & BUCKERFIELD, J.C. (1979). Digestion of lignin by termites. *Soil Biology and Biochemistry* 11, 507 - 513.

CALLEJA, G.B. (1984). Microbial aggregation. CRC Press, Boca Raton Florida.

CARRUTHERS, S.M. & RAYNER, A.D.M. (1979). Fungal communities in decaying hardwood branches. *Transactions of British Mycological Society* 72, 283 - 289.

CHAHAL, D.S. (1982). Bioconversion of lignocelluloses into food and feed rich in protein. In *Advances in Agricultural and Food Sciences*. (N. S. Subba Rao ed.). Butterworth Scientific Publishers. Pp 551 - 583.

CHAHAL, D.S. ; MOO-YOUNG, M. & DHILLON, G.S. (1979). Bioconversion of wheat straw and wheat components into single cell protein. *Canadian Journal of Microbiology* 25, 793 - 797.

CHAHAL, D.S. ; VLACH, D. & MOO-YOUNG, M. (1980). Upgrading the protein feed value of lignocellulosic materials using *Chaetomium cellulolyticum* in solid state fermentation. Paper presented at the 30th Annual meeting of the Canadian Society of Microbiologists, Guelph, Ontario, June 15 - 19.

CHANDRA, S. & JACKSON, M.G. (1971). A study of various chemical treatments to remove lignin from coarse roughages and increase their digestibility. *Journal of Agriculture and Science Cambridge* 77, 11 - 17.

CHAVEZ, E.R. ; TOUCHBOURN, A. & MOO-YOUNG, M. (1988a). Microbial biomass Protein from the fungus *Chaetomium cellulolyticum*. I. composition and nutritive evaluation. *Animal Feed Science and Technology* 22, 1 - 11.

_____ (1988b) . Microbial biomass Protein from the fungus *Chaetomium cellulolyticum* II. Effect of method of drying and response to amino acid supplementation. *Animal Feed Science and Technology 22, 15 - 21.*

_____(1988c). Microbial biomass protein from the fungus *Chaetomium cellulolyticum* III. Nutritive value for chicks and piglets. *Animal Feed Science and Technology* 22, 23 - 32.

CHEN, S.L. & PEPPLER, H.J.(1978). Single cell protein in food application. *Developments in Industrial Microbiology* 18, 79 - 94.

CHEN, W.; SUPANWONG, K.; OHMIYA, K. ; SHIMIZU, S. & KAWAKAMI, H. (1985). Anaerobic degradation of

veratarylglycerol-B-guaiacyl ether and guaiacoxyacetic acid by mixed rumen bacteria. *Applied and Environmental Microbiology* 50, 1451 -1456.

CHESSON, A.; STEWART, C.S. & WALLACE, R.J.(1982). Influence of plant phenolic acids on growth and cellulolytic activity of rumen bacteria. *Applied and Environmental Microbiology* 44, 597 -603.

CHUA, M.G.S & WAYMAN, M. (1979). Characterisation of autohydrolysis of aspen (*P.tremuloides*) lignins Part 3. Infra-red and ultra violet studies of extracted autohydrolysis lignin. *Canadian Journal of Chemistry* 57, 2603 - 2611.

CHUA, M.G.S. ; CHOI, S. & KIRK, T.K. (1983). Mycelium binding and depolymerisation of synthetic ^{14}C-labelled lignin during decomposition by *Phanerochaete chrysosporium* *Holzforschung* 37, 55 - 61.

CHURCH, M.B. (1923). Soy and related fermentations. *United States Department of Agriculture Bulletin* No. 1152. P.26.

CITRON, A.; BRETON, A. AND FONTY, G.(1987). Rumen anaerobic fungi. *Bulletin of the Institut Pasteur* 85, 329 - 343.

CLAEYSSENS, M. ; VAN TILBEURG, H.; TOME, P. & WOOD, T.M. (1989). Fungal cellulase systems of the specificities of the cellobiohydrolase isolated from *Penicillium pinophilum* and *Trichoderma reesei*. *Biochemical Journal* 261, 819 - 825.

COLBERG, P.J. & YOUNG. L.Y. (1982). Biodegradation of

lignin derived molecules under anaerobic conditions. *Canadian Journal of Microbiology* 28, 886 - 889.

COLBERG, P.J. & YOUNG, L.Y.(1985). Anaerobic degradation of soluble fractions of (^{14}C - lignin) lignocellulose. *Applied and Environmental Microbiology* 49, 345 - 349.

COLEMAN, G.S. (1964). The metabolism of *Escherichia coli* and other bacteria by *Entodinium caudatum*. *Journal of General Microbiology* 37, 209 - 223.

COMMANDY, F. & MACY, J.M. (1985). Effect of substrate nitrogen on lignin degradation by *Pleurotus ostreatus*. *Archives of Applied Microbiology* 142, 61 - 65.

COONEY, C. L. (1983). Bioreactors: Design and operation. *Science* 219, 728 - 733.

COUGHLAN, M.P. (1990). Symposium on Enzyme Systems for Lignocellulose Degradation. Applied Science Publishers, London. P.408.

COWLING, E.B. & KIRK, T.K. (1976). Properties of cellulose and lignocellulosic materials as substrates for enzymic conversion processes. *Biotechnology and Bioengineering Symposium* 6, 95 - 123.

CRAWFORD, R. L. (1981). Lignin Biodegradation and Transformation. John Wiley and Sons, Inc. New York. P.154.

CRAWFORD, R.L. & CRAWFORD, D.L. (1978). Radioisotopic methods for the study of lignin biodegradation. *Developments in Industrial Microbiology* 19, 35 - 49.

CRAWFORD, R.L. & CRAWFORD, D.L. (1984). Recent advances in studies of the mechanisms of microbial degradation of lignins. *Enzyme and Microbial Technology* 6, 434 - 442.

CUERO, R.G. ; SMITH, J.E. & LACEY, J. (1985). A novel containment system for laboratory scale solid particulate fermentation. *Biotechnology Letters* 7(7), 463 - 466.

CZERKASWKI, J.W. (1984). Degradation of solid feeds in the rumen; spatial distribution of microbial activity and it's consequences. Proceedings of Sixth International Symposium on Ruminant Phsiology pp 158 - 172.

CZERKAWSKI, J.W. & BRECKENRIDGE, G. (1977). Design and development of a long term rumen simulation technique (RUSITEC). *British Journal of Nutrition* 38, 371 - 384.

CZERKAWSKI, J.W. & BRECKENRIDGE, G. (1979a). Experiments with the long term rumen simulation technique (RUSITEC); response to supplementation of basal rations. *British Journal of Nutrition* 42, 217 -227.

CZERKAWSKI, J.W. & BRECKENRIDGE, G. (1979b). Experiments with the long term rumen simulation technique (RUSITEC): use of soluble food and inert solid matrix. *British Journal of Nutrition* 42, 229 -245.

CZERKAWSKI, J.W. & BRECKENRIDGE, G.(1985). Metabolism of protein supplements studied by the rumen simulation technique (RUSITEC). *Archiv Fuer Tierenaehrung, Berlin* 35, 261 - 277.

CZERKAWSKI, J. W. & CLAPPERTON, J. C. (1968). Analyses of gases produced by metabolism of micro-organisms. *Laboratory Practice* 17, 994 - 1012.

DAGLEY, S.(1978). Microbial catabolism, the carbon cycle and environmental pollution. *Naturwissenchatten* 65, 85 - 91.

DALE, B.E. & MOREIRA, M.J. (1982). A freeze explosion technique for increasing cellulose hydrolysis. *Biotechnology and Bioengineering Symposium* 12, 31 - 43.

DALE, B.E : HENK, L.L & SHIANG, M.(1985). Fermentation of lignocellulosic materials treated by ammonia freeze explosion. *Developments in Industrial Microbiology* 26, 223 - 233.

DALLYN, H. & FOX , H. (1980). Spoilage of materials of reduced water activity by xerophilic fungi. *In Microbial Growth and Extremes of Environment* (G.H Guld, and J.E.L. Corry eds). Academic Press, London. Pp 129 - 139.

DALY, H. W. & RUIZ, L.P. (1974). Reduction of RNA in single cell protein in conjunction with fiber formation. *Biotechnology and Bioengineering* 16, 285 - 287.

DALTON, H. & STIRLING, D.I. (1982). Co-metabolism. *In New Dimensions in Microbiology; Mixed Substrates, Mixed Cultures and Microbial Communities*(J.R. Quayl and A.T. Bull eds). The Royal Society of London. Pp 71 - 86.

DAVIDSON, R.W. ; CAMPBELL, W.A. & BLAISDELL, D.J. (1938). Differentiation of wood decaying fungi by their reactions on gallic or tannic acid medium. *Journal of Agricultural Research* 57, 683 - 695

DAVIS, C.L. & CLARK, J.H. (1981). Ruminant digestion and metabolism. In *Development in Industrial Microbiology* vol. 22 (L.A. Underkofler and M.C. Wulf eds). Pp 247 - 259. Society of Industrial Microbiology.

DEHORITY, B.A. (1965). Degradation and utilisation of isolated hemicellulose by pure cultures of cellulolytic rumen bacteria. *Journal of Bacteriology* 89, 1515 - 1520.

DESCHAMPS, A.M. ; MAHOUDEAU, G. & LEBEAULT, J.M. (1980). Fast degradation of kraft lignin by bacteria. *European Journal of Applied Microbiology and Biotechnology* 9, 45 - 51.

DETROY, R.W.; LINDEFELSER, L.A. ; JULIAN, G.S. Jr & ORTON, L.W. (1980). Saccharification of wheat straw cellulose by enzymatic hydrolysis following fermentative and chemical pre-treatment. *Biotechnology and Bioengineering Symposium* 10, 135 - 148.

DINSDALE, D. ; MORRIS, E.J. & BACON, J.S.D (1978). Electron microscopy of the microbial populations present and their modes of attack on various cellulosic substrates undergoing digestion in the sheep rumen. *Applied and Environmental Microbiology* 36, 160 - 168.

DONNELLY, P.K. & CRAWFORD, D.L. (1988). Production by *Streptomyces viridosporus* T7A of an enzyme which cleaves aromatic acids from lignocellulose. *Applied and Environmental Microbiology* 54, 2237 - 2244.

DOWDING, P.(1978). Methods for studying microbial interactions. *Annals of Applied Biology* 89, 166 - 171.

DUMLAO, C.T. AND PEREZ, Jr. C.B. (1976). Improving the feed value of rice straw by calcium oxide treatment. *Philippine Journal of Veterinary and Animal Science* 2, 95 - 110.

DUPIN, J.L. (1986). Proceedings, Stake - Technic, Technical conference, Soustons, France, 1 - 16, Toronto, Stake Technologies. Cited by Wayman and Parekh (1990).

DURAND, A. & CHEREAU, D. (1988). A new pilot reactor for solid state fermentation; Application to the protein enrichment of sugar beet pulp. *Biotechnology and Bioengnineering* 31, 476 - 486.

DURAN, N. MANSILLA, H. AND REYES, J.L. (1986). Biomass photochemistry X : Analysis of structural modifications in lignin under UV irradiation. *Journal of Photochemistry* 35, 209 - 217.

DURAN, N. ; FERRER, I. ; RODRIGUEZ, J. & MANSILLA, H. (1988). Biomass photochemistry XIII. Pre treated lignin from *Pinus radiata* and degradation by ligninase and horse radish peroxidase. *Journal of Photochemistry and*

Photobiology A; Chemistry 41, 267 - 273.

EATON, D. ; CHANG, H.M. & KIRK, T.K. (1980). Fungal decolorization of kraft lignin. *Tappi* 63, 103 - 106.

EDBERG, N. & HOFSTEIN, B. (1975). Cellulose degradation in waste water treatment. *Journal of the Water Pollution Control Federation* 47, 1012 - 1020.

EFFLAND, M.J. (1977). Modified procedure to determine acid insoluble lignin in wood and pulp. *Tappi* 60, 143 - 144.

ENGELS , F.M. & BRICE, R.E. (1985). A barrier covering lignified cell walls of barley straw that restricts access by rumen micro-organisms. *Current Microbiology* 12, 217 - 223.

ENGLYST, H.N. & CUMMINGS, J.H. (1988). Improved method for measurement of dietary fiber as non starch polysaccharides in plant foods. *Journal of the Association of Official Analytical Chemist* 71(4), 808 - 814.

ERDMAN, M.D. BERGEN, W.G. & REDDY, A. (1977). Amino acid profiles and presumptive nutritional assessment of single cell protein from certain lactobacilli. *Applied and Environmental Microbiology* 33, 901 - 905.

ERDMAN, M.D. & REDDY, C.A. (1987). Batch fermentation of cheese whey supplemented poultry swine and cattle waste filtrates. *Biological Wastes* 22, 23 - 37.

ERIKSSON, K-E. (1978). Enzyme mechanisms involved in cellulose hydrolysis by the rot fungus *Sporotichum pulverulentum*. *Biotechnology and Bioengineering* 20,

317 - 332.

ERIKSSON, K-E. (1981). Fungal degradation of wood components. *Pure and Applied Chemistry* 53, 33 - 43.

ERIKSSON, K-E. (1983). Advances in enzymatic degradation of lignocellulosic materials. In Proceedings of the *International Symposium on Ethanol from Biomass* , Winnipeg, Canada, October 13 - 15, 1982 (H.E Duckworth and E. A. Thompson eds.). Royal Society of Canada, Ottawa, Canada. Pp 345 - 370.

ERIKSSON, K-E. & LINDHOLM, U.(1971). Ligninets Mikrobiella Nedbrytning. *Svensk Papperstidning* 74, 701 - 704.

ERIKSSON, K-E. & GOODELL, E.W.(1974). Pleiotropic mutants of the wood rotting fungus *Polyporous adustus* lacking cellulase and xylanase. *Canadian Journal of Microbiology* 20, 371 - 378.

ERIKSSON, K-E ; JOHNSRUD, S.C. & VALLANDER, L.(1983). Degradation of lignin and lignin model compounds by various mutants of the white rot fungus *Sporotrichum pulverulentum*. *Archives of Microbiology* 135, 161 - 168.

ESLYN, W.E. ; KIRK, T.K. & EFFLAND, M.J. (1975). Changes in the chemical composition of wood caused by six soft rot fungi. *Phytopathology* 65, 473 - 475.

EVANS, S.C. (1985). Laccase activity in lignin degradation by *Coriolus versicolor in vivo* and *in vitro* studies. *FEMS Microbiology Letters* 27, 339 - 343.

EVANS, S.C. ; FARMER, J.Y. & PALMER, J.M.(1984). An

extracellullar haem protein from *Coriolus versicolor*. *Phytochemistry* 23, 1247 - 1250.

EVELEIGH, D.E (1987). Cellulase - A perspective. *Philosophical Transactions of Royal Society London.* A321, 435 - 447.

FAGERSTAM, L.G. & PETTERSSON, L.S. (1980). The 1,4 B - glucan cellobiohydrolase of *Trichoderma reesei*. A new type of synergism. *FEBS Letters* 119, 97 - 110.

FAHRAEUS, G.; TULLANDER, V. & LJUNGGREN, H.(1958). Production of high laccase yields in cultures of fungi. *Physiologia Plantarum* 11, 631 - 639.

FAISON, B.D & KIRK, T.K. (1985). Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* 49, 299 - 304.

FAN, L.T.; LEE, Y.H. & BEARDMORE, D.H.(1981a). The influence of major structural features of cellulose on rate of enzymatic hydrolysis. *Biotechnology and Bioengineering* 23, 419 - 424.

FAN, L.T. ; GHARPURAY, M.M. & LEE, Y.H. (1981b). Evaluation of pretreatments for enzymatic conversion of agricultural residues. *Biotechnology and Bioengineering Symposium* 11, 29 - 45.

FARAJ, M.K. (1990). Regulation of mycotoxin formation in *Zea mays* Ph. D Thesis, *University of Strathclyde*, UK.

FARR, D.F. (1983). Mushroom industry; Diversification with additional species in the United States. *Mycology*

75, 351 - 357.

FENN, P. & KIRK, K.(1979). Lignolytic system of *Phanerochaete chrysosporium* inhibition by o-phthalate. *Archives of Microbiology* 123, 307 - 309.

FIELD, J.A. & LETTINGA, G. (1989). The effect of oxidative coloration on the methanogenic toxicity and anaerobic biodegradability of phenols. *Biological Wastes* 29, 161 - 179.

FINA, L.R.; KEITH, C.L. ; BARTLEY, E.E ; HARTMAN, P.A. & JACOBSON, N.L. (1962). Modified *in vivo* artificial rumen (VIVAR) techniques. *Journal of Animal Science* 21, 930 - 934.

FLICKINGER, M.C. & TSAO, G.T. (1978). Fermentation substrates from cellulosic materials; fermentation products from cellulosic materials. In *Annual Reports on Fermentation processes* (D. Perlman ed.) vol.2 Pp 23 - 42. Academic press, London.

FOOD AND AGRICULTURAL ORGANISATION (FAO, 1965). Protein requirements. *FAO Nutrition Meetings Series* No. 37, Rome.

FORAGE, A.J. & RIGHELATO, R.C. (1978). Microbial protein from carbohydrate wastes. *Progress in Industrial Microbiology* 14, 59 - 95.

FORD, C.W. & HARTLEY, R.D.(1990). Cyclodimers of p-coumaric acid and ferulic acids in the walls of tropical grasses. *Journal of Science, Food and Agriculture* 50, 29 - 43.

FOUBERT, E.L. & DOUGLAS, H.C. (1948). Studies on the anaerobic micrococci. 1. Taxonomic considerations.

Journal of Bacteriology 56, 25 - 34.

FREER, S.N. & DETROY, R.W. (1982). Biological delignification of ^{14}C- labelled lignocellulose by basidiomycetes ; Degradation and solubilization of lignin and cellulose components. *Mycologia* 74, 943 - 951

FROEHNER, S.C. & ERIKSSON, K-E. (1974a). Induction of *Neurospora crassa* laccase with protein synthesis inhibitors. *Journal of Bacteriology* 120, 450 - 457.

_____ (1974b). Purification and properties of *Neurospora crassa* laccase. *Journal of Bacteriology* 120, 458 - 465.

GARTON, G.A. ; LOUGH, A.K. & VIOQUE, E. (1961). Glyceride hydrolysis and glycerol fermentation by sheep rumen contents. *Journal of General Microbiology* 25, 215 - 225.

GASCOIGNE, J.A & GASCOIGNE, M.M. (1960) Biological degradation of cellulose. Butterworths publishers, London. P.264.

GHARIB, F.H. ; GOODRICH, R.D. MEISKA, J.C. & EL SERAFY, A.M. (1975a). Effects of grinding and sodium hydroxide treatment on popular bark. *Journal of Animal Science* 40, 727 - 733.

GHARIB, F.H. ; GOODRICH, R.D. MEISKA, J.C. & EL SERAFY, A.M. (1975b). In vitro evaluation of chemically treated poplar bark. *Journal of Animal Science* 40, 734 - 742.

GIBSON, T. ; STIRLING, A.C. ; KEDDIE, R.M. & ROSENBERGER, R.F. (1958). Bacteriological changes in silage made at controlled temperatures. *Journal of General Microbiology* 19, 112 - 118.

GIBSON, T. ; STIRLING, A.C. ; KEDDIE, R.M. & ROSENBERGER, R.F. (1961). Bacteriological changes in silage as affected by laceration of the fresh grass. *Journal of Applied Bacteriology* 24, 60 - 67.

GIJZEN, H.J. ; ZWART, K.B.; VAN GERDER, P.T. & VOGELS G.D. (1986). Continous cultivation of rumen micro-organisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. *Applied Microbiology and Biotechnology* 25(2), 155 - 162.

GIOVANNOZI-SERMANNI, G.; BADIANI, M. & LUNA, M. (1982). Protein production and laccase activity in *Agaricus bisporus*. *Biotechnology Letters* 4, 507 - 512.

GIOVANNOZI - SERMANNI, G. BERTONI, G. & PORRI, A. (1989). Chemicals and Animal feeds. In *Enzyme systems for Lignocellulose Biodegradation* (M.P. Coughlan ed). Pp. 371 - 382 Elsevier Applied Science Publishers, London.

GLEGG, R.E. & KERTESZ, A.I. (1957). Effect of gamma radiation on cellulose. *Journal of Polymer Science* 26, 289 - 297.

GLENN, J.K. & GOLD, M.H. (1983). Decolourization of several polymeric dyes by the lignin degrading basidiomycete *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* 45, 1741 - 1747.

GLENN J.K. & GOLD, M.H. (1985). Purification and characterisation of an extracellular manganese dependent peroxidase from the lignin degrading basidiomycete *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics* 242, 329 - 341.

GOERING, H.K. ; SMITH, L.W. VAN SOEST, P.J. & GORDON, C.H. (1973). Digestibility of roughage materials ensiled with sodium chlorite. *Journal of Dairy Science* 56, 233 - 240.

GOLOVLEVA, L.A. ; GOLOVLEV, E.L.; CHERMENSKY, D.N.; OKUNEV, N. ; GOMBAROV, K.H. AND BRUTOVETSKAYA, T.A. (1983). Solid state fermentation of plant raw materials. Proceedings of the Finnish - Soviet Symposium held in Tuominnc, October 25 - 27, 1982 (T. Pirhonen and A. Hatakka eds). pp 21 - 41.

GONG, C.S. & TSAO, G.T. (1979). Cellulase biosynthesis and regulation. *Annual Report of Fermentation Processes* 3, 111 - 140.

GONZALEZ, A. GRINDERGS, J. & GRIVA, E. (1986). Biologische umwandlung von holz in rinderfutter - palo podrido. *Zentralblatt fur Mikrobiologie* 141, 181 - 186.

GONZALEZ, A.E. ; MARTINEZ, A.T. ALMENDROS, G. & GRINBERG, J. (1989). A study of yeasts during the delignification and fungal transformation of wood into cattle feed in Chilean rain forest. *Antonie Van Leeuwenhoek* 55, 221 - 336.

GORDON, G. L.R. & PHILIPS, M.W. (1989). Degradation and utilization of cellulose and straw by three different anaerobic fungi from the ovine rumen. *Applied and Environmental Microbiology* 55, 1703 - 1710.

GOTTLIEB, D. (1978). The germination of fungus spores. Meadowfield Press, Durham. UK.

GOULD, M.J. (1984). Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. *Biotechnology and Bioengineering* 26, 46 - 52.

GRACHECK, J.S. ; RIVERS, D.B.; WOODFORD, L.C.; GIDDINGS, K.E. & EMERT, G.H. (1981). Pretreatment of lignocellulosics to support cellulase production with *Trichoderma reesei* Qm 9414. *Biotechnology and Bioengineering Symposium* 11, 47 - 65.

GUNNISON, D. & ALEXANDER, M. (1975). Basis for the resistance of several algae to microbial decomposition. *Applied Microbiology* 29, 729 - 738.

HAARS, A. & HUTTERMANN, A. (1980). Function of laccase in white rot fungus- *Fomes annosus*. *Archives of Microbiology* 125, 233 - 237.

HAIDER, K. & TROJANOWSKI, J. (1975). Decomposition of specifically ^{14}C- labelled phenols and dehydropolymers. *Archives of Microbiology* 105, 33 -42.

HAN, Y .W. (1975). Microbial fermentation of rice straw. Nutritive composition and *in vitro* digestibility of the fermentation products. *Applied Microbiology* 29, 510 - 514.

HAN, Y.W. (1978). Microbial utilisation of straw (A review). *Advances in Applied Microbiology* 23, 119 - 153.

HAN, Y.W. & ANDERSON, A.W. (1975). Semi solid fermentation of rye grass straw. *Applied Microbiology* 30, 930 - 934.

HAN, Y.W. & CALLIHAN, D.C. (1974). Cellulose fermentations: effect of substrate pre treatment on microbial growth. *Applied Microbiology* 27, 159 - 165.

HAN, Y.W.; CHEEKE, P.R. ; ANDERSON, A.W. & LEKPRAYOON, A.C.(1976). Growth of *Aurobasidium pullulans* on straw hydrolysate. *Applied and Environmental Microbiology* 32, 799 -802.

HAO, L.C.; FULMER, E.I. & UNDERKOFLER, L.A. (1943). Fungal amylases as saccharifying agents in the alcoholic fermentation of corn. *Journal of Industrial and Engineering Chemistry* 35, 814 - 818.

HARKING, A.J. (1986). Economic Aspects of Biotechnology. In *Cambridge Studies in Biotechnology*(3) (J. Baddiley, N. H. Carey, J.F. Davidson , I.J. Higgins and W.G. Potter eds). Cambridge University Press, London. P. 306.

HARKING, J.M. & OBST, J.R. (1973). Syringaldazine an effective reagent for detecting laccase and peroxidase in fungi. *Experientia* 29, 381 - 508.

HARKING, L. & ANAGNOSTAKIS, S.L. (1977). Solid medium containing carboxymethylcellulose to detect C_x cellulase activity of micro-organisms. *Journal of General Microbiology* 98, 109 - 115.

HARMAN, L.S.; CARVER, D..L.; SCHREIBER, J. & MANSON, R.P.(1986). One -and two- electron oxidation of reduced glutathione by peroxidases. *Journal of Biological Chemistry* 261, 1642 - 1648.

HARRIS, C.E.; RAYMOND, W.F. & WILSON, R.F. (1966). The voluntary intake of silage. Proceedings of 6th International Grassland Congress, Helsinki, Pp 564.

HARRIS, J.E. ; EVANS, D.M. ; KNOX, M.R. & ARCHER, D.B. (1987). Genetic approaches with methanogens important in mesophilic anaerobic digestion. *In Recent Advances in Anaerobic Bacteriology.* (S. P. Borriello and E. J. M. Hard eds). Pp 123 - 137. Dorddrecht Martinus Nijhoff.

HART, M. R; WALKER,Jr H.G. ; GRAHAM, R.P. ; HANNI, P.J.; BROWN, A.H. & KOHLER, G.O. (1980). Steam treatment of crop residues for increased ruminant digestibility. I. Effect of process parameters. *Journal of Animal Science* 51, 402 - 408.

HARTLEY, R.D. (1985). In *Improved Utilisation of Lignocellulosic Materials in Animal feed* OECD Paris, 1985. Pp 10 - 30. Cited by Hartley & Ford (1989).

HARTLEY, R.D. & FORD, C.W. (1989). Phenolic constituents of plant cell walls and wall biodegradability. In *Plant cell wall polymers : Biogenesis and Biodegradation* (N.G. Lewis and M.G. Paice eds) Pp 138 - 145. American Chemical Society Washinghton, D.C.

HARTLEY, R.D. & KEENE, A.S. (1984). Aromatic aldehydes constituents of graminaceous cell walls. *Phytochemistry* 23, 1305 - 1307.

HATTAKA, A.I. & UUSI-RAUVA, A.K. (1983). Degradation of ^{14}C- labelled popular wood lignin by selected white rot fungi. *European Journal of Applied Microbiology and Biotechnology* 17, 235 - 242.

HATTAKA, A.I. ; BUSWELL, J.A.; PIRHONEN, T.I. & UUSI - RAUVA, A.K. (1983). Degradation of ^{14}C-labelled lignins by white rot fungi. In *Recent Advances in Lignin Biodegradation Research* (T. Huguchi, T. Chang and T.K. Kirk eds).Pp 176 - 187. UNI Publication co. Ltd, Tokyo, Japan .

HAYES, W.A. & NAIR, N.G. (1975). The cultivation of *Agaricus bisporus* and other edible mushrooms. In *The filamentous fungi* (J.E. Smith and D. R. Berry eds.) vol. 1, pp 212 - 248 Edward Arnold, London.

HAZLEWOOD, G.P.; DAVIDSON, K.; LAURIE, J.; ROMANEC, M. & GILBERT, H.J. (1990). Cloning and sequencing of the gene encoding endoglucanase of *Butyrivibrio fibrisolvens* strain A46. *Journal of General Microbiology* 136, 2089 -

2097.

HEALY, J.B. ; YOUNG, L.Y. & REINHARD, M. (1980). Methanogenic decomposition of ferulic acid - a model lignin derivative. *Applied and Environmental Microbiology* 39, 436 - 441.

HERMANS, P.H. (1949). Physics and Chemistry of Cellulosic Fibres. Elsevier, New York. P. 534.

HESSELTINE, C.W. (1972). Solid state Fermentation. *Biotechnology and Bioengineering* 14, 517 - 532.

HESSELTINE, C.W. (1977). Solid state fermentation. *Process Biochemistry* 12, 24 - 27 , 30 - 32.

HESSELTINE, C.W. (1987). Solid state fermentation- An overview. *International Biodeterioration* 23, 79 - 89.

HESSELTINE, C.W. ; SHORTWELL, O.L.; SMITH, M.C. SHANNON, G.M. VANDEGRAFT, E.E. & GOULDEN, M.L. (1968). Laboratory studies on the formation of aflatoxin in forages. *Mycologia* 60, 304 - 312.

HIGUCHI, T. (1953). Biochemical study of wood rotting fungi ; Studies on the enzymes which cause Bavendamm's reaction. *Journal of Japanese Forestry Society.* 35, 77 - 85.

HIGUCHI, T.M. ; SHIMADA, F. ; NAKATSUBO, M & TANAHASHI, Y.(1977). Differences in biosynthesis of guaiacyl and syringyl lignins in woods. *Wood Science and Technology* 11, 153 - 167.

HOBSON, P.N. (1971). Rumen micro-organisms. *Progress in Industrial Microbiology* 9, 42 - 77.

HOBSON, P.N. & MANN, S.O. (1961). The isolation of

glycerol fermenting and lignolytic bacteria from the rumen of the sheep. *Journal of General Microbiology* 25, 227 - 240.

HOFFMAN, R.M. & WOOD, T.M. (1985). Isolation and partial characterisation of mutants of *Penicillium funiculosum* for the saccharification of Straw. *Biotechnology and Bioengineering* 27, 81 - 85.

HOMB, T. (1956). Norwegische erfahrungenbei der strohaufschliessung nach dem beckmannschen verfahren. *Futterkoservierung (Frankfurt -Am - Main).* 2, 129 - 146.

HOWARD, B.H. (1959). The biochemistry of rumen protozoa 2. some carbohydrates in cell free extracts of *Dasytrichia* and *Isotrichia*. *Biochemical Journal* 71, 675 - 680.

HOWARD, B.H. (1963). Metabolism of carbohydrates by rumen protozoa. *The Biochemical Journal* 89, 89(abstract).

HRUBAN, T.G.R. ORTON, W.L. & RHODES, G. (1976). In American Society of Microbiology Abstract No. 182 cited from Hesseltine (1977).

HUANG, N.L. (1982). Studies on chemical constituents of *Volvariella volvaceae* and other edible species. M.Phil thesis Dept of Biology, *The University of Hong Kong*, Hong Kong.

HUMPHREY, A.E. (1975). Production outlook and technical feasibility of single cell protein. In *Single Cell Protein II* (S.R. Tannenbaum and D.I.C Wang eds). MIT Press Cambridge, Massachussetts. Pp 1 - 23.

HUMPHREY, A.E.; MOREIRA, A. ; ARMIGER, W. & ZABRISKIE, D. (1977). Production of single cell protein from cellulose wastes. *Biotechnology Bioengineering Symposium* 7, 45 - 64.

HUNGATE, R.E. (1943). Further experiments on cellulose digestion by the protozoa in the rumen of cattle. *Biological Bulletin* 84, 157 - 163.

HUNGATE, R.E. (1966). The Rumen and it's Microbes. Academic Press, London. P. 533.

HUNT, W.H. & PIXTON, S.W. (1974). Moisture : It's significance and measurement. *In Storage of Cereal Grains and their Products* (C.M. Christiansen ed.). Published by American Association of Cereal Chemists Minnesota. Pp 1 - 55.

HUYNH, V. B & CRAWFORD, L. (1985). Novel extracellular (ligninases) of *Phanerochaete chrysosporium. FEMS Microbiology Letters* 28, 119 -123.

IBRAHIM, M.N.M & PEARCE, G.R. (1983). Effects of chemical pretreatments on the composition and *in vitro* digestibility of crop by- products. *Agricultural Wastes* 5, 135 - 156.

IMRIE, F.K.E & RIGHELATO, R.C. (1976). Production of microbial protein from carbohydrate wastes in developing countries. In *Food from Wastes* (G.G. Birch, K.J.Parker and J. T. Worgan eds.) Pp. 79 - 94. Applied Science Publishers, London.

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY (IUPAC), (1974). Proposed guidelines for testing single

cell protein destined as major protein sources for animal feed. Information Bulletin and Technical report No. 12.

ISHIHARA, T. (1980). Lignin Biodegradation: Microbiology, Chemistry and Potential Applications (T.K. Kirk, T. Higuchi and H. M. Chang eds). Volume 2, 17 - 31. CRC Press Boca Raton, Florida.

ISHIHARA, T. & MIYAZAKI, M. (1972). Oxidation of milled wood lignin by fungal laccase. *Mokuzai Gakkaishi* 18, 415 - 422.

ISHIHARA, T. & MIYAZAK, M. (1974). Demethylation of lignin and lignin models by fungal laccase. *Mokuzai Gakkaishi* 20, 39 - 45.

ISHIHARA, T. & ISHIHARA, M. (1975). Estimation of ortho - quinone structure and methanol formed from lignin models by fungal laccase. *Mokuzai Gakkaishi* 21, 323 - 329.

ISHIKAWA, H.W.; SCHUBERT, J. & NORD, F.F (1963). Investigations on lignins and lignification xxvii. The enzymatic degradation of soft wood lignin by white rot fungi. *Archives of Biochemistry and Biophysics* 100, 131 - 139.

JACKSON, M.G. (1977). The alkali treatment of straws. *Animal Feed Science and Technology* 2, 105 - 130.

JANSHEKAR, H. & FIECHTER, A. (1988). Cultivation of *Phanerochaete chrysosporium* and production of lignin peroxidases in submerged stirred tank reactors. *Journal of Biotechnology* 8, 97 - 112.

JAYASURIYA, M.C.N. & H.G.D. PERERA (1982). Urea ammonia treatment of rice straw to improve it's nutritive value for ruminants. *Agricultural Wastes* 4, 143 - 150.

JEFFRIES, G.A. (1948). Mold enzymes produced by continuous tray method. *Food Industry* 20(1) 688 - 690, 825 - 826.

JOBLIN, K.N. & NAY, G.E. (1989). Fermentation of woods by rumen anaerobic fungi. *FEMS Microbiology Letters* 65(1 - 2), 119 - 122.

JOBLIN, K.N. ; NAYLOR, G.E. & WILLIAMS, A.G.(1990). Effect of *Methanobrevibacter smithii* on xylanolytic activity of anaerobic ruminal fungi. *Applied and Environmental Microbiology* 56(8), 2287 - 2295.

JOHNS, A.J. (1953). Fermentation of glycerol in the rumen of sheep. *New Zealand Journal of Science and Technolology* A35, 262 - 269.

JOHNSRUD, S.C. & ERIKSSON, K.E. (1985). Cross breeding of selected and mutated homokaryotic strains of *Phanerochaete chrysosporium* K-3 ; New cellulase deficient strains with increased ability to degrade lignin. *Applied Microbiology and Biotechnology* 21, 320 - 327.

JONES, D.H. & HAYWARD, M.V. (1973). A cellulase digestion technique for predicting the dry matter digestibility of grasses. *Journal of the Science of Food and Agriculture* 24, 1419 - 1426.

JONES, D.H. & HAYWARD, M.V. (1975). The effect of pepsin

pretreatment of herbage on the prediction of dry matter digestibility from solubility in fungal cellulase solutions. *Journal of the Science of Food and Agriculture* 26, 711 - 718.

JOUANY, J.P & SENAUD, J. (1982). Effect of rumen ciliates on the digestion of different carbohydrates in sheep. I. Utilisation of cell wall carbohydrates (cellulose and hemicellulose) and of starch. *Reproduction, Nutrition and Development* 22, 735 - 752.

JULIANO, B.O.(1985). Rice Chemistry and Technology. American Association of Cereal Chemists, Inc. P.774.

JUNG, H.J. & SHALITA - JONES, S.C. (1990). Variations in the extractability of esterified p-coumaric and ferulic acids from forage cell walls. *Journal of Agricultural Food Chemistry* 38(2), 397 - 402.

JURASEK, L. (1979). Enzymic hydrolysis of pretreated aspen wood. *Developments in Industrial Microbiology* 20, 177 - 183.

JURASEK, L.(1990). Biological bleaching. Paper presented at the Fourth International Mycological Congress Regensburg, Germany, 28th August - 3rd September, 1990.

KAJI, A. (1984). L-Arabinosidases. *Advances in Carbohydrate Chemistry and Biochemistry* 42, 383 - 394.

KAMRA, D.N. & ZADRAZIL, F.(1988). Microbiological improvement of lignocellulosics in animal feed

production. In *Treatment of Lignocellulosics with White Rot Fungi* (F. Zadrazil and P. Reininger eds.) Pp 50 - 63. Elsevier Publishers, London.

KARAPINAR, M. & WORGAN, J.T. (1983). Bioprotein production from the waste products of olive oil extraction. *Journal of Chemical Technology and Biotechnology* 33B(3), 185 - 188.

KARASAWA, Y.; TOSHIMA, Y. & KIBE, K. (1983). Effects of carbohydrates on cellulolytic activity of rumen micro-organisms in vitro. *Shinshu Daigaku Nogakubu Kiyo*, 20(2), 155 - 165.

KARKALAS, J. (1985). An improved enzymic method for the determination of native and modified starch. *Journal ofof the Science of Food and Agriculture* 36, 1019 - 1027.

KASSIM, E.A. & GHAZI, L.M. (1987). Microbial protein from cellulolytic fungi. *Zentralblatt Mikrobiologie* 142, 257 -261.

KEYS, J.E.Jr ; VAN SOEST, P.J. & YOUNG, E.P. (1969). Comparative study of the digestibility of forage cellulose and hemicellulose in ruminants and non ruminants. *Journal of Animal Science* 29, 11 - 15.

KEYSER, P. ; KIRK, T.K. & ZEIKUS, J.G. (1978). Ligninolytic enzyme system of *Phanerochaete chrysosporium* synthesised in the absence of lignin in response to nitrogen starvation. *Journal of Bacteriology* 135, 790 - 794.

KHAN, W.A. ; LABRIE, J.P. & McKEOWN, J. (1987). Electron beam irradiation pre-treatment and enzymatic

saccharification of used newsprint and paper mill wastes. *Radiation Physics and Chemistry* 29(2), 117 - 120.

KHARATYAN, S.G. (1978). Microbes as food for humans. *Annual Review of Microbiology* 32, 301 - 327.

KIRK, T.K. (1971). Effects of micro-organisms on lignin . *Annual Review of Phytopathology* 9, 185 - 210.

KIRK, T.K. (1973). Polysaccharide integrity as related to the degradation of lignin in wood by white rot fungi. *Phytopathology* 63, 1504 - 1507.

KIRK, T.K. (1983). Degradation and conversion of lignocelluloses. In *The Filamentous Fungi volume iv. Fungal Technology* (J.E. Smith, D.R. Berry and B. Kristiansen eds). Pp 266 - 289. Edward Arnold Publishers London.

KIRK, T.K. & ADLER, E. (1970). Methoxy deficient structural elements in lignin of sweetgum decayed by a brown rot fungus. *Acta Chemica Scandinavica* 24, 3379 - 3390.

KIRK, T.K. & CHANG, H.M. (1975). Decomposition of lignin by white rot fungi II. Characterisation of heavily degraded lignins from decayed spruce. *Holzforchung* 29, 56 - 64.

KIRK, T.K. & CHANG, H.M. (1981). Potential applications of biolignolytic systems. *Enzyme Microbiology and Technology* 3, 189 - 196.

KIRK, T.K. & MOORE, W.E. (1972). Removing lignin from wood with white rot fungi and digestibility of resulting

wood. *Wood Fiber* 4, 72 - 80.

KIRK, T.K. & REID, I.D. (1990). Biological pulping. Paper presented at the Fourth International Mycological congress, Regensburg, Germany, 28th August - 3rd September.

KIRK, T.K.; CONNORS W.J. & ZEIKUS, J.G. (1976). Requirement for a growth substrate during lignin decomposition by two wood rotting fungi. *Applied and Environmental Microbiology* 32, 192 - 194.

KIRK, T.K.; SCHULTZ, E.M.; CONNORS, W.J. ; LORENZ, L.F. & ZEIKUS, J.G. (1978). Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. *Archives of Microbiology* 117, 277 - 285.

KIVIASI, A.K.; OP DEN CAMP, H.J.M. ; LUBBERDING, H.J.; BOON, J.P & VOGELS, G.D.(1990). Generation of soluble lignin derived compounds during degradation of barley straw in artificial rumen reactor. *Applied Microbiology and Biotechnology* 33, 93 - 98.

KLING, S.H.; NETO, C.C. ; FERRARA, M.A. ; TORRES,J.C.R ; MAGALHAES, D.B. & RYU, D.D.Y.(1987). Enhancement of enzymatic hydrolysis of sugarcane bagasse by steam explosion pretreatment. *Biotechnology and Bioengineering* 29, 1035 -1039.

KLYOSOV, A. A. ; SINITYSIN, A.P. & RABINOVITCH, M.L. (1980). The comparative role of exoglucosidase and cellobiase in glucose formation from cellulose. In *Enzyme Engineering 5* (H.H. Weetail and G. P. Royer eds.)

KNAPP, J.S. (1985). Biodegradation of celluloses and lignins. *In Comprehensive Biotechnology 4. The Principles, Applications and Regulations of Biotechnology in Industry, Agriculture and Medicine* (M. Moo-Young, W. Campbell and J. A. Howell eds.) pp 835 - 847. Pergamon Press, Oxford.

KNAPPERT, D. GRETHLEIN, H. & CONVERSE, A.(1981). Partial acid hydrolysis of popular wood as a pretreatment for enzymatic hydrolysis. *Biotechnology and Bioengineering Symposium* 11, 67 - 77.

KNOCHE, W.E. ; CRUZ - COKE, E. & PACOTET, M. (1929). Cited by Gonzalez *et al*, 1989.

KOMISARCZUK, S.; DURAND, M.; DUMAY, C. & MOREL, M.J. (1986). Use of semi- continous culture system (RUSITEC) to study the effect of phosphorus defficiency on rumen microbial digestion. *Progress in Biotechnology* 2, 47 - 53.

KOMISARCZUK, S.; MERRY, R.J. & McALLAN, A.B. (1987). Effect of different levels of phosphorous on rumen microbial fermentation and synthesis determined using a continous culture technique. *British Journal of Nutrition* 57(2), 279 - 290.

KOPECNY, J. (1985). Cellulolytic enzymes of Butyrivibrio fibrisolvens XIV. International Congress of Microbiology *Abstract P(B)*, 17 - 25. P. 104.

KRISHNA, G.K. CZERKAWSKI, J.W. & BRECKENRIDGE G. (1986). Fermentation of various preparations of spent hops (*Humulus lupulus*) using the rumen simulation

technique(RUSITEC). *Agricultural Wastes* 17, 99 - 117.

KUMAH, R. & SINGH, M. (1984). Tannins ; their adverse role in ruminant nutrition. *Journal of Agriculture and Food Chemistry* 32, 447 - 453.

KUWAHARA, M. ; GLENN, J.K. ; MORGAN, M.A. & GOLD, M.H. (1984). Separation and characterisation of two extracellular H_2O_2 - dependent oxidases from lignolytic cultures of *Phanerochaete chrysosporium FEMS Letters* 169, 247 - 250.

KWAI, S. ; UMEZAWA, T.; SHUMADA, M. & HIGUCHI, T.(1986). C-C cleavage of -1 lignin substructure models by the laccase of *Coriolus versicolor*. In *Biotechnology in the Pulp and Paper Industry*. The Third International Conference, Stockholm 16 -18th June.

LADISCH, M.R. (1979). Fermentable sugars from cellulosic residues. *Process Biochemistry* January, 21 - 25.

LANGSTON, C.W. & BOUMA, C. (1960). A study of the micro-organisms from grass silage II. The lactobacilli. *Applied Microbiology* 8 , 223 -234.

LAUKEVICS, J.J. APISTE, A.F. VIESTURS, U.E. & TENGERDY, R.P. (1984). Solid substrate fermentation of wheat straw to fungal protein. *Biotechnology and Bioengineering* 26, 1465 - 1474.

LAW, D.J. & TIMBERLAKE, W.E. (1980). Developmental regulation of laccase levels in *Aspergillus nidulans*. *Journal of Bacteriology* 144, 509 - 512.

LAWTON, E.J. ; BELLAMY, W.D; HUNGATE, R.E; BRYANT, M.P & HALL,E. (1951). Some effects of high velocity

electrons on wood. *Science* 113, 380 - 383.

LEATHAM, G.F. (1986). The lignolytic activities of *Lentinus edodes* and *Phanerochaete chrysosporium. Applied Microbiology and Biotechnology* 24(1), 51 - 58.

LEATHAM, G.F. & KIRK, T.K. (1983). Regulation of lignolytic activity by nutrient nitrogen in white rot basidiomycetes. *FEMS Microbiology Letters* 16, 65 - 71.

LEE, B. (1979). M.S Thesis, *University of Washington*, Seatle Washington. Cited by Sarkanen (1980).

LEISOLA, M.S.A AND FIECHTER, A. (1985). New trends in lignin biodegradation. *Advances in Biotechnology Processes* 5, 59 - 89.

LEISOLA, M. ; ULMER, D. & FIECHTER, A. (1983). Problem of oxygen transfer during degradation of lignin by *Phanerochaete chrysosporium. European Journal of Applied Microbiology and Biotechnology* 17, 113 - 116.

LEISOLA, M.S.A ; ULMER, D.C. & FIECHTER, A. (1984). Factors affecting lignin degradation in lignocellulose by *Phanerochaete chrysosporium. Archives of Microbiology* 137, 171 - 175.

LEISOLA, M. ; BROWN, C.; LAURILA, M. ; ULMER, D. & FIECHTER, A. (1982). Polysaccharide synthesis by *Phanerochaete chrysosporium* during degradation of kraft lignin. *European Journal of Applied Microbiology and Biotechnology* 15, 180 - 184.

LEONHARDT, J.W. ; BAER, M. ; HUEBNER, G.; HENNIG, A. &

NEHRING, K. (1983). Gamma and electron radiation effects of straw. *Radiation Physics and Chemistry* 21, 397 -400.

LEONOWITZ, A. & TROJANOWSKI, J. (1978). Induction of laccase in basidiomycetes. The laccase coding messenger. *Acta Biochemica Polonica* 25, 147 - 153.

LEVONEN -MUNOZ, E. ; BONE, D.H. & DAUGULIS, A.J. (1983). Solid state fermentation and fractionation of oat straw by basidiomycetes. *European Journal of Applied Microbiology and Biotechnology* 18, 120 - 123.

LI, G.S.F & CHANG, S. (1982). The nucleic acid content of some edible mushrooms. *European Journal of Applied Microbiology and Biotechnology* 15, 237 - 240.

LILLEHOJ, E.B. & HAN, Y.W. (1983). Chemical and gamma ray modified bagasse as substrates for bioproduction of cellullases and protein. *Biotechnology and Bioengineering* 25, 2077 - 2084.

LINDEBERG, G. & HOLM, G. (1952). Occurrence of tyrosinase and laccase in fruit bodies and mycelia of some hymenomycetes. *Physiologia Plantarum* 5 , 100 - 114.

LINDEN, J.C. ; HENK, L.C. ; MURPHY, V.G. ; SMITH, D.H.; GABRIELSEN, B.C.; TENGERDY, R.P. & CZAKO, L. (1987). Preservation of potential fermentables in sweet sorghum by ensiling. *Biotechnology and Bioengineering* 30, 860 - 867.

LINDENFELSER, L.A. & CIEGLER, A. (1975). Solid state fermentor for ochratoxin A production. *Applied Microbiology* 29, 323 -327.

LIWICKI, R. ; PATERSON, A.; MACDONALD, M.J. & BRODA, P. (1985). Phenotypic classes of phenol oxidase negative mutants of the lignin degrading fungus *Phanerochaete chrysosporium. Journal of Bacteriology* 162(2), 2467 - 2473.

LOBANOK, A.G. ; STAKHEEV, I.V. & BABITSKAYA, V.G. (1983). Bioconversion of straw into protein using mycelial fungi. Proceedings of the Finnish - Soviet Symposium held in Tuaminnc, October 25 - 27, 1982 (T. Pirhonen and A. Hatakka eds). pp 3 - 12.

LONSANE, B.K. GHILDYAL, N.P. BUDIATMAN, S. & RAMAKRISHNA, S.U. (1985). Engineering aspects of solid state fermentation. *Enzyme and Microbial Technology* 7, 258 - 265.

LUNDQUIST, K. & KIRK, T.K (1978). De novo synthesis and decomposition of veratryl alcohol by a lignin degrading basidiomycete. *Phytochemistry* 17, 1676 - 1682.

LYR, H. (1958). Uberden nachweis von oxydases und peroxidasen rei holheren puzen und ue bedentung dieser enzyme fur die Bavendamm reaction. *Planta* 50, 359 - 370.

MACKENZIE, C.R. ; BILOUS, D. ; SCHNEIDER, H. & JOHNSON, K.G.(1987). Induction of cellulolytic and xylanolytic enzyme systems in *Streptomyces* spp. *Applied and Environmental Microbiology* 53, 2835 - 2839.

MAGAN, N. & LACEY, J. (1984a). Effect of temperature and pH on water relations of field and storage fungi.

Transactions of British Mycological Society 82(1), 71 - 81.

MAGAN, N. & LACEY, J. (1984b). Effect of water activity , temperature and substrate on interactions between field and storage fungi. *Transactions of British Mycological Society* 82(1), 83 - 93.

MAHASNETH, A.M. & STEWART, P. (1980). A medium for detecting B(1,3) glucanase activity in bacteria. *Journal of Applied Bacteriology* 48, 457 - 458.

MANDELS, M. ; HONZT, L. & NYSTROM, J. (1974). Enzymatic hydrolysis of waste cellulose. *Biotechnology and Bioengineering* 26, 1471 - 1493.

MANDELS, M. ; ANDREOTTI, R. & ROCHE, C. (1976). Measurement of saccharifying cellulase. *Biotechnology and Bioengineering Symposium* 6, 21 - 33.

MARTIN, S.A. (1988). Effects of p-coumaric acid and ferulic acid on methane production and fiber digestion by mixed rumen micro-organisms in vitro. *Letters in Applied Microbiology* 7(4), 113 - 114.

MARTIN, S.M. & SO, V. (1969). Solubilization of autoclaved feathers and wool by myxobacteria. *Canadian Journal of Microbiology* 15, 1393 - 1397.

MARTINEZ, A.T.; BARRASA, J.M.; ALMENDROS, G. & GONZALEZ, A.E. (1990) ; Fungal transformation of lignocellulosics as revealed by chemical and ultrastructural analyses. In *Advances in Biological Treatment of Lignocellulosic Materials* (M.P Coughlan and M. T. Amaral - Collaco eds). Pp 129 - 149. Elsevier Applied Science,

London.

MAYER, A.M. & HAREL, E.A. (1968). A laccase like enzyme in peaches. In *Phytochemistry* vol.7. Pergamon press Ltd. P. 1253.

McBEE, R.H. (1953). Manometric method for the evaluation of microbial activity of rumen with application to utilisation of cellulose and hemicellulose. *Applied Microbiology* 1, 106 - 110.

McCARTHY, A.J. (1987). Lignocellulose degrading actinomycetes. *FEMS Microbiology Review* 46, 145 - 163.

McCARTHY, A.J. & BRODA, P. (1984). Screening for lignin degrading actinomycetes and characterisation of their activity against ^{14}C- labelled wheat lignocellulose. *Journal of General Microbiology* 130, 2905 - 2913.

McCARTHY, A.J. ; MACDONALD, M.J. ; PATERSON, A. & BRODA, P. (1984). Degradation of ^{14}C - lignin labelled wheat lignocellulose by white rot fungi. *Journal of General Microbiology* 130, 1023 - 1030.

McCARTHY, A.J. ; PATERSON, A. & BRODA, P.(1986). Lignin solubilization by *Thermomonospora mesophila*. *Applied Microbiology and Biotechnology* 24, 347 - 352.

McCRAE, S.I.; FALCONER, D.J. & WOOD, T.M.(1989). Hydrolysis of the polysaccharide of straw by enzymes produced by mutant strain of the fungus *Penicillium pinophilum*. *Biotechnology and Bioengineering* 33(4), 422 - 427.

McDONALD, P. (1976). Trends in silage making. In *Microbiology in Agriculture; Fisheries and Food* (J.C. Carr ed.). Pp 109 -123. Academic Press, London.

McDONALD, P. & WHITTENBURY, R. (1973). Ensilage. In *The Chemistry and Biochemistry of Herbage*(G.W. Butler and B. W. Bailey eds). Academic Press, London.

McDONALD, P. ; STIRLING, A.C. ; HENDERSON, A.R. & WHITTENBURY, R. (1964). Fermentation studies on inoculated herbages. *Journal of the Science of Food and Agriculture* 15, 429 - 435.

McDOUGAL, E.I. (1948). Studies on ruminant saliva. The composition and output of sheep's saliva. *Biochemical Journal* 43, 99 - 109.

McLEOD, D.S. ; WILKINS, R.J. & RAYMOND, W.F. (1970). The voluntary intake by sheep and cattle of silages differing in free acid content. *Journal of Agricultural Science Cambridge* 75, 311 - 316.

METZ, B.; KOSSEN, W.F. & VAN SUIJDAM, J.C. (1979). The biology of mould suspensions. *Advances in Biochemical Engineering* 11, 103 - 156.

MIALL, L.M. (1975). Historical development of the fungal fermentation industry. In *The Filamentous Fungi* Vol. 1 (J.E. Smith and D. R. Berry eds.). Pp 104 -119 Edward Arnold Publishers, London.

MILLET, M.A. ; BAKER, A.J. & SATTER, L.D. (1975). Pre treatments to enhance chemical, enzymatic and microbiological attack of cellulosic materials. *Biotechnology and Bioengineering Symposium* 5, 193 -

219.

MILSTEIN, O.; BECLIAR, A.; SHRAGINA, L. & GRESSEL, J. (1987). Solar pasteurisation of straw for nutritional upgrading and as substrate for lignolytic organisms. *Biotechnology Letters* 9, 269 - 274.

MOEBUS, O. & TEUBER, M. (1983). General aspects of production of biomass by yeast fermentation from whey permeate. In *Production and Feeding of Single Cell Proteins* (H.P. Ferranti and A. Fiechter eds.). Pp 124 - 135. Applied Science Publishers, London.

MOIR, R.J. & MASSON, M.J. (1952). An illustrated scheme for the microscopic identification of the rumen organisms of sheep. *Journal of Pathology and Bacteriology* 64, 343 - 350.

MONTENECOURT, B.S. & EVELEIGH, D.E. (1979). Cited by Enari, T.M. (1989). Microbial cellulases. In *Microbial Enzymes and Biotechnology* (W.M. Forgarty ed.) Pp 183 - 223. Applied Science Publishers, London.

MOO-YOUNG, M. & BLANCH, H.W. (1981). Design of biochemical reactors - Mass transfer criteria for simple and complex systems. *Advances in Biochemical Engineering* 19, 1 - 69.

MOO - YOUNG, M.; CHAHAL, D.S. ; SWAN, J.E. & ROBINSON, C.W. (1977). Single cell protein production by *Chaetomium cellulolyticum*, A new thermotolerant cellulolytic fungus. *Biotechnology and Bioengineering* 19, 527 -538.

MOO - YOUNG, M.; CHAHAL, D.S. & VLACH, D. (1978). Single cell protein from various chemically pre treated wood

substrates using *Chaetomium cellulolyticum*. *Biotechnology and Bioengineering* 20, 107 - 118.

MOO - YOUNG, M. ; DAUGULIS, A.J. ; CHAHAL, A.S. & MACDONALD, D.G.(1979a). The Waterloo process for SCP production from waste biomass. *Process Biochemistry* 14(10), 38 - 40.

MOO - YOUNG, M.; VAN DEDEM, G. & BINDER, A. (1979b). Design of scraped tubular fermentors. *Biotechnology and Bioengineering* 21, 593 - 607.

MOO - YOUNG, M.; MOREIRA, A.R. & TENGERDY, R.P. (1983). principles of solid substrate fermentation. In *The Filamentous Fungi, vol.4* (J.E. Smith ; D.R. Berry and B. Kristiansen eds). pp 117 - 144. Erdward Arnold Publishers, Ltd, London.

MORRIS, E.J. & BACON, S.D. (1977). The fate of acetyl groups and sugar components during the digestion of grass cell walls in sheep. *Journal of Agricultural Science Cambridge* 89, 327 -340.

MORRISON, I.M. (1980). Hemicellulose contamination of acid detergent residues and their replacement by cellulose residues in cell wall analyses. *Journal of the Science of Food and Agriculture* 31, 639 - 645.

MORRISON, I.M. (1986). Factors affecting the breakdown of dietary fibre in the rumen. *Hannah Research*, 89 - 96.

MORRISON, S.M. ; ELMUND, G.K. ; GRANT, D.W. & SMITH, V.J.(1977). Protein production from feedlot waste. *Developments in Industrial Microbiology* 18, 145 -

155.

MOUNTFORT, D.O. & BRYANT, M.P. (1983). Isolation and characterisation of an anaerobic syntrophic benzoate degrading bacterium from sewage sludge. *Archives of Microbiology* 133, 249 - 256.

MOYSON, E. ; DE SMET, K. & VERACHTERT, H. (1991). Biodegradation of wheat straw by higher fungi. Paper presented at the International Symposium on Environmental Biotechnology 22 -25 April, Ostend Belgium. Pp 539 - 543.

MUDGET, R.E. & PARADIS, A.J. (1985). Solid state fermentation of natural birch lignin by *Phanerochaete chrysosporium. Enzyme and Microbial Technology* 7, 150 - 154.

MUELLER, J.C. & GAWLEY, J.R. (1983). Cultivation of phoenix mushrooms on pulp mill sludges. *Mushroom Newsletter Tropical* 4, 3 - 10

MULLER, H.W.; TROSCH, W. & KULBE, K.D. (1988). Effect of phenolic compounds on cellulosic degradation by some white rot basidiomycetes. *FEMS Microbiology Letters* 49, 87 - 93.

MULLER - HARVEY, I. ; HARTLEY, R.D.; HARRIS, P.J & CURZON, E.H.(1986). Linkage of p-coumaryl and ferulolyl groups to cell wall polysaccharides of barley straw. *Carbohydrate Research* 148, 71 - 185.

NAZARETH, S. & MAVINKURVE, S. (1987). Isolation of potential lignolytic organisms. *International Biodeterioration* 23, 271 - 280.

NEELY, W.C. (1984). Factors affecting the pre treatment of biomass with gaseous ozone. *Biotechnology and Bioengineering* 26, 59 - 65.

NELSON. N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry* 153, 375 - 380.

NEUHAUSER, E. & HARTENSTEIN, R. (1976a). Degradation of phenol, cinnamic acid and quinic acid in the terrestial crustacean *Oniscus asellus*. *Soil Biology and Biochemistry* 8, 95 - 98.

_____ (1976b). On the presence of O- demethylase activities in invertebrates. *Comparative Biochemistry & Physiology* 53, 37 - 39.

NEUHAUSER, E. ; HARTENSTEIN, R. & CONNORS, W.J. (1978). Soil invertebrates and the degradation of vanillin, cinnamic acid and lignins. *Soil Biology and Biochemistry* 10, 431 - 435.

NEWBOLD, C.J. & WALLACE, R.J. (1990). Yeast as a probiotic for ruminants. Paper presented at the Symposium on Industrial Application of Yeast held at Heriot Watt University, Edinburgh, June, 1990.

NICOLINI, L.; HUNOLSTEIN, V.C. & CARILLI, A. (1987). Solid state fermentation of orange peel and grape stalks by *Pleurotus ostreatus*, *Agrocybe aegerita* and *Armillaria mellea*. *Applied Microbiology and Biotechnology* 26, 95 - 98.

NIGAM, P. ; PANDEY, A. & PRABHU, K.A. (1987). Lignolytic activity of two basidiomycetes cultures in the

decomposition of bagasse. *Biological Wastes* 21, 1 - 10.

NORKRANS, B. (1950). Studies in growth and cellulolytic enzymes of Tricholoma. *Symbolae Botanicae Upsalienses.* 11, 1 - 126.

ODIER, E. & MONTIES, B. (1978). Biodegradation of wheat lignin by Xanthomonas 23. *Annales de Microbiologie* 129A, 361 - 377.

ONG,E. ; GREENWOOD, J. M.; GILKES, N.R. ; KILBURN, D.G.; MILLER, R.C. & WARREN, R. A. J. (1989). The cellulase binding domains of cellulases: tools for biotechnology. *Trends in Biotechnology* 7, 239 - 243.

ONODERA, R. ; YAMASKI, N. & MURAKAMI, K. (1988). Effect of inhabitation by ciliate protozoa on the digestion of fibrous materials *in vivo* in the rumen of goats and *in vitro* rumen microbial system. *Agricultural and Biological Chemistry* 52, 2635 - 2637.

ORPIN, C.G. (1981). Isolation of a cellulolytic phycomycete fungi from the caecum of the horse. *Journal of General Microbiology* 123, 287 - 296.

ORPIN, C.G. & LETCHER, A.J. (1978). Some factors controlling the attachment of the rumen holotrich protozoa, *Isotrichia intestinalis* and *I.prostoma* to plant particles *in vitro*. *Journal of General Microbiology* 106, 33 - 40.

ORPIN,C.G.; MATHIESEN, S.D.; GREENWOOD, Y. & BLIX, A.S. (1985). Seasonal changes in the ruminal microflora of the high artic Svalbard reindeer (*Rangifer taranchus*

platyrhynchus). *Applied and Environmental Microbiology* 50, 144 - 151.

ORTH, G.O. JR. & ORTH, R.D. (1977). US patent 4, 017, 642. Cited by Sarkanen (1980).

OTJEN, L. ; BLANCHETTE, R ; EFFLAND, M. & LEATHAM, G.(1987). Assessment of 30 white rot basidiomycete for selective lignin degradation. *Holzforschung* 41(6), 343 - 349.

PALMER, J.M. ; HARVEY, P.J & SCHOEMAKER, H.E. (1987). The role of peroxidases, radical cations and oxygen in degradation of lignin. *Philosophical Transactions of the Royal Society, London* A321, 495 - 505.

PAMMENT, N. ; ROBINSON, C.W. & MOO-YOUNG, M. (1979). Pulp and paper mill solid waste as substrates for single cell protein production. *Biotechnology and Bioengineering* 211, 561 - 573.

PASZCZYNSKI, A. ; HUYNH, V.B. & CRAWFORD, R.L. (1986). Comparison of ligninase 1 and peroxidase M2 from the white rot fungus *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics* 244, 750 - 765.

PATERSON, A. (1989). Biodegradation of lignin and cellulose materials. In *Biotechnology for Livestock Production.* Food and Agricultural Organization pp 245 - 261.

PEERALLY, A. (1981). Sugar cane by products as bulk ingredients in mushroom compost. *Mushroom Science* 11, 293 - 297.

PEITERSEN, N. (1975a). Production of cellulase and protein from barley straw by *Trichoderma viride*. *Biotechnology and Bioengineering* 17, 361 - 374.

_____(1975b). Cellulase and protein production from mixed cultures of *Trichoderma viride* and a yeast. *Biotechnology and Bioengineering* 17, 1291 - 1300.

PEITERSEN, N. & ANDERSEN, B. (1978). Fermentation of barley straw by *Trichoderma viride* recovery and nutritive value of the fermentation product. *AICHE Symposium Series* 77 (172), 100 - 104.

PELCZAR, M.J.; REID, R.D.& CHAN, E.C.S. (1977). Microbiology. 4th edition. Mcgraw hill, Inc. New York Pp 716 - 718.

PELMONT, J. ; TOURNESAC, C. MLIKI, A. BARRELLE, M. & BEGUIN, C.(1989). A new bacterial alcohol dehydrogenase active on degraded lignin and several low molecular weight aromatic compounds. *FEMS Microbiology Letters* 57(1), 109 - 113.

PHILIPI, F. (1893). Die pilze chiles. Soweit dieselben als nahrungsmittel gebraught werden hedwigia(dresden) 115 - 118. Cited from Gonzalez *et al* 1989.

PHILIPS, J.A. & HUMPHREY, A.E (1983). Process biotechnology for the conversion of biomass into liquid fuels. In *Liquid Fuel Development* (D.L. Wise ed.) Pp 65 - 95. Florida CRC Press, Boca Raton.

PITT, J.I. (1975). Xerophilic fungi and the spoilage of foods of plant origin. In *Water Relations of Food*

(R.B. Duckworth ed). Pp 273 - 307. Academic Press London.

PITT, J.I. (1979). Genus Penicillium and it's teleomorphic states Eupenicillium and Talaromyces. Academic Press London, Pp 17 - 24.

POUTANEN, K. ; RATTO, M.; PULS, J. & VIIKARI, L. (1987). Evaluation of different microbial xylanolytic systems. *Journal of Biotechnology* 6, 49 - 60.

PRESTON, R.D. & CRONSHAW, J. (1958). Constitution of the fibrillar components of the walls of *Valonia venticosa*. *Nature* 181, 248 - 250.

PRINCE, C.R. & STIEFEL, E.I. (1987). Lignin degradation. *Trends in Biochemical Sciences* 12, 136 - 137.

PROTEIN ADVISORY GROUP(PAG) OF UNITED NATIONS SYSTEM (1970). Guideline No. 6 for preclinical testing of novel sources of protein.

_____ (1972). Guideline No. 12 for the production of single cell protein for human consumption.

_____(1974) Guideline No. 15 on nutritional safety aspects of novel protein sources for animal feeding.

PULS, J. & POUTANEN, K. (1990). Mechanisms of enzymic hydrolysis of hemicelluloses (xylans) and procedures for determination of the enzyme activities involved. In *Enzyme Systems For Lignocellulose Degradation* (M.P. Coughlan, ed.) pp 151 - 165. Elsevier Applied Science, London.

PURI, V.P (1984). Effect of crystallinity and degree of polymerisation of cellulose on enzymatic

saccharification. *Biotechnology and Bioengineering* 26, 1219 - 1222.

PURI, V.P & MAMERS, H.(1983). Explosive pre treatment of lignocellulosic residues with high pressure carbon dioxide for the production of fermentation substrates. *Biotechnology and Bioengineering* 25, 3149 - 3161.

RAI, S.N. & MUDGAL, V.D. (1986). Effect of alkali and steam pressure treatment on compositional changes and *in vitro* fibre digestibility of rice straw. *Indian Journal of Animal Nutrition* 3, 95 - 101.

RAI, S.N. & MUDGAL, V.D. (1987). Effect of sodium hydroxide and steam pressure treatment on the utilisation of wheat straw by rumen micro-organisms. *Biological Wastes* 21, 203 - 212.

RAIMBAULT, M. & ALZARD, D. (1980). Culture method to study fungal growth in solid state fermentation. *European Journal of Applied Microbiology and Biotechnology* 9, 199 - 209.

RANADE, D.R.; YEOLE, T.Y. & GODBOLE, S.H. (1987). Production of biogas from market waste. *Biomass* 13, 147 - 155.

RASMUSSEN, M.A.; HESPELL, R.B. ; WHITE, B.A. & BOTHAST, R.J.(1988). Inhibitory effects of methylocellulose on cellulose degradation by *Ruminococcus flavefaciens*. *Applied and Environmental Microbiology* 54(4), 890 - 897.

REDDY, C.A. & ERDMAN, M.D. (1977). Production of a ruminant protein supplement by anaerobic fermentation of feedlot waste filtrate. *Biotechnology and Bioengineering Symposium* 7, 11 - 22.

REDDY, C.A.: HENDERSON, H.E. & ERDMAN, M.D. (1976). Bacterial fermentation of cheese whey for production of ruminant feed supplement rich in crude protein. *Applied and Environmental Microbiology* 32, 769 - 776.

REESE, E.T. ; SIV, R.G.H & LEVINSON, H.S. (1950). Biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *Journal of Bacteriology* 59, 485 - 497.

REID, I.D. (1983). Effects of nitrogen supplements on degradation of Aspen wood lignin and carbohydrate components by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* 45, 830 - 837.

REID, I.D.(1985). Biological delignification of Aspen wood by solid state fermentation with the white rot fungus *Merulius tremellosus*. *Applied and Environmental Microbiology* 50, 133 - 139.

REID, I.D. (1989a). Solid state fermentations for biological delignification. *Enzyme and Microbial Technology* 11, 786 - 803.

REID, I.D. (1989b). Optimisation of solid state fermentation for selective delignification of Aspen wood with *Phlebia tremellosa*. *Enzyme and Microbial Technology* 11, 804 - 809.

REID, I.D. & SEIFERT, K.A. (1982). Effect of an atmosphere of oxygen on growth, respiration and lignin degradation by white rot fungi. *Canadian Journal of Botany* 60, 252 - 260.

REIHAMMAR, B. (1970). Purification and properties of laccase and stellacyanin from *Rhus vernicifera*. *Biochimica et Biophysica ACTA* 205, 35 - 47.

REIHAMMER, B. (1984). Laccase. In *Copper Proteins and Copper Enzymes Vol. 3* (R. Lonte ed.) CRC Press, Boca Raton, Florida.

RIVERS, B.D. & EMERT, G.H. (1988). Factors affecting the enzymic hydrolysis of municipal solid waste components. *Biotechnology and Bioengineering* 31, 278 - 281.

ROBBINS, C.T.; VAN SOEST P.J. ; MAUTZ, W.A. & MOEN, A.N.(1975). Feed analysis with reference to white tailed deer. *Journal of Wildlife Management* 39, 67 - 69.

ROBBINSON, J.E. & CRAWFORD, R.L. (1978). Degradation of ^{14}C - labelled lignins by *Bacillus megaterium*. *FEMS Microbiology Letters* 4, 301 - 302.

ROBERTSON, J.B. & VAN SOEST, P.J.(1981). The detergent system of analysis and its application to human foods. In *The Analysis of Dietary Fiber in Food* (W.P.T. James and O. Theander eds.) pp 123 - 157. Marcel Dekker, Inc., USA.

ROLZ, C. (1984). Microbial biomass from renewables ; A second review of alternatives. *Annual Report on Fermentation Processes* 7, 213 - 356.

ROSENBERG, R.F. (1976). The cell wall. *In The Filamentous Fungi Vol II* (J.E. Smith and D. R. Berry eds).Pp 328 - 344. Edward Arnold Publishers, London. Pp 328 - 344.

ROTSTEIN, O.D.; KAY, R.M. & STRASBERG, S.M. (1981). *Gastroenterology* 81, 1098 - 1103. Cited by Wayman and Parekh (1990).

RUEL, K. (1990). Ultrastructural alterations of wood cell walls during degradation by fungi. In *Advances on Biological Treatment of Lignocellulosic Materials* (M.P. Coughlan and M. T. Amaral -Collaco eds). Pp 117 - 129. Elsevier Science Publishers, London.

RYU, D.Y.D. (1989). Enhancement of nutritional value of cellulosic feed resources by pre treatment and bioconversion. In *Biotechnology for Livestock Production.* Pp 223 - 243. Food and Agricultural Organisation, New York.

SANNI, S.O. (1981). Garbage as energy and agricultural resource with special bias to biofertilisers. In *Global Impacts of Applied Microbiology* (S.O. Emejuaiwe, O. Ogumbi and S. O. Sanni eds.) Sixth International Conference . Pp 227 - 232. Academic press, London.

SARBOULOUKI, M.N. & MOACANIN, J. (1980). Chemicals from biomass-United States prospects for the turn of the century. *Solar Energy* 25, 303 - 315.

SARKANEN, K.V. (1980). Acid catalysed delignification of lignocellulosics in organic solvents. In *Progress in Biomass Conversion* (K.V. Sarkanen and A. Tillman eds). Pp 127 - 143. Academic Press Inc. New York.

SAVORY, J.G. & PINION, L.C. (1958). Chemical aspects of decay of beech wood by *Chaetomium globosum*. *Holzforschung* 12, 99 -103.

SCHULZE, K.C. (1962). Continous thermophilic composting. *Applied Microbiology* 10, 108 - 122.

SCHINK, B.(1988). Fermentative degradation of nitrogenous aliphatic and aromatic compounds. In *Anaerobic Digestion. Proceedings of International Symposium* (E. R. Hall and P. N. Hobson eds.) Pp 459 - 464. Pergamon Press Oxford, UK.

SCRIMSHAW, N.S. (1985). Acceptance of single cell protein for human food applications. In *Comprehensive Biotechnology vol.4; The Principles, Applications and Regulations of Biotechnology in Industry, Agriculture and Medicine* (M. Moo- Young, C.W. Robinson and J. A. Howell eds). Pp 673 - 683. Pergamon Press, Oxford.

SCRIMSHAW, N.S. & DILLON, S. (1979). Allergic responses to some single cell proteins in human subjects. In *Single Cell Protein - Safety for Animal and Human Feeding* (S. Garattini,; S. Pagalialunga and N. S. Scrimshaw eds.) P.171. Pergamon Press Oxford.

SEHGAL, S.N.; SINGH, K. & VEZINA, C.(1966). Process for producing spores. United States patent 3, 294, 647.

SENEZ, J.C. (1987). Single cell protein; past and present developments. In *Microbial Technology in the Developing World* (E.J. Dasilva, Y.R. Dommrgues, E.J. Nyns & C. Ratledge eds.) Pp 226 - 238. Oxford University, Press, Oxford.

SENIOR, E. (1989). Renewable energy sources. A paper presented to Microbiology Society,14th April, University of Strathclyde, UK.

SHARMA, S.D. & JACKSON, M.G. (1975). The effect of sodium hydroxide treatment on the composition and digestibility of cell walls. In *Improved Utilisation of Agricultural Waste Materials and Industrial by Products as Livestock Feed.* Research Progress Report 1969 - 1974. G.B. Pant University, Pantnagar, Pp 16 - 25.

SIHTOLA, H. & NEIMO, L. (1975). The structure and properties of cellulose. In *Symposium on Enzymatic Hydrolysis of Cellulose* (M. Bailey, T.M. Enari and M. Linko eds.) Pp 9 -21. SITRA, Helsinki.

SILMAN, R.W.; CONWAY, H.F.; ANDERSON, R.A. & BAGLEY, E.B. (1979). Production of aflatoxin in corn by a large scale solid substrate fermentation process. *Biotechnology and Bioengineering* 21, 1799 - 1808.

SINHA, R.R.P. & ARORA, S.P.(1982). Influence of different levels of aflatoxins on rumen fermentation (*in vitro*). *Journal of Nuclear Agriculture and Biology* 11, 87 - 99.

SJOSTROM, E. (1981). Wood Chemistry; Fundamentals and Applications. Academic Press, London.

SLONEKER, J.H. (1976). Agricultural residues, including feedlot wastes. *Biotechnology and Bioengineering symposium* 6, 235 - 250.

SMITH, J.E. (1985). Aspects of Microbiology II. Biotechnology Principles. Van Nostrand Reinhold Ltd., England. P.119.

SMITH, R.E. (1977). Rapid method for detecting fungal cellulase production. *Applied and Environmental Microbiology* 33, 980 - 981.

SMITH, P.H. & HUNGATE, R.E. (1958). Isolation and characterisation of *Methanobacterium ruminantium*. *Journal of Bacteriology* 75, 713 - 718.

SMITH, A.M. ; MORRISON, W.L. & MILHAM, P.J. (1982). *Biochemistry, Washington* 21, 4414 - 4419. Cited by Palmer *et al.* (1987)

SMITH, J.E. ; ANDERSON, J.G.; SENIOR, E. & AIDOO, K.(1987). Bioprocessing of lignocelluloses. *Philosophical Transactions of Royal Society , London.* A321, 507 - 521.

SMITH, J.F.; CLAYDON , N.; LOVE, M.E. ; ALLAN, M. & WOOD, D.A.(1989). Effect of substrate depth on extracellular endocellulase and laccase production of *Agaricus bisporus*. *Mycological Research* 93(3), 292 - 296.

SMITH, L.W. ; GOERING, H.K. & GORDON, C.H. (1970). *In vitro* digestibility of chemically treated faeces.

Journal of Animal Science 31, 1205 - 1209.

SOLOMONS, G.L. (1986). Microbial proteins and regulatory clearance for RHM mycoprotein. In *Microbial Biomass Proteins* (M. Moo-Young & F.K. Gregory eds). Pp 19 - 26. Elsevier Applied Science Publishers, London.

SOMOGYI, M.J. (1952). Notes on sugar determination. *Journal of Biological Chemistry* 195, 19 - 23.

SOUTHGATE, D.A.T. (1976). Determination of food carbohydrates. Applied Science Publishers, London.

SPENCER, P.A. & TOWER, G.H.N (1989). Virulence inducing phenolic compounds detected by *Agrobacterium tumefaciens*. In *Plant Cell Wall Polymers ; Biogenesis and Biodegradation* (N.G. Lewis and M.G. Paice eds) Pp 383 - 398. American Chemical Society, Washington, D.C.

STEWART, C .S. & RICHARDSON, A.J. (1989). Enhanced resistance of anaerobic rumen fungi to the ionophores monensin and Lasalocid in the presence of methanogenic bacteria. *Journal of Applied Bacteriology* 66, 85 - 93.

STEWART, C. ; AKOYO J.A. & ZADRAZIL, F. (1990). Enhanced degradability of wheat straw following fermentation with *Pleurotus ostreatus* and the contribution of rumen fungi and bacteria to straw degradation *in vitro*. In *Advances in Biological Treatment of Lignocellulosic Materials* (M.P. Coughlan and M.T. Amaral Collaco eds). Pp 269 - 281. Elsevier Applied Science, London.

STRINGER, D.A. (1985). Acceptance of single cell protein

for animal feeds. In *Comprehensive Biotechnolgy vol.4; The Principles, Applications and Regulations of Biotechnology, Agriculture and Medicine* (M. Moo-Young, C.W. Robinson and J.A. Howell eds.).Pp 685 - 694 Pergamon Press Oxford.

SUHASUKAN, S. AND YASIN, B.A. (1986). SCP production from citrus wastes by using constituted mixed fungal inoculum. *Process Biochemistry* 21(2), 50 - 53.

SUMNER, J.B. (1925). The estimation of sugar in diabetic urine using Dinitro- salicyclic acid. *Journal of Biological Chemistry* 62, 287 - 290.

SZTEJNBERG, A. ; AZAIZIA, H. & LISKER, N. (1989). Effect of tannins and phenolic extracts from plant roots on the production of cellulase and polygalacturonase by *Dematophora necatrix*. *Phytoparasitica* 17, 49 - 53.

TAKAGI, M. (1987). Pretreatment of lignocellulosic materials with hydrogen peroxide in presence of manganese compounds. *Biotechnology and Bioengineering* 29, 165 - 170.

TAKAISHI, M. (1970). Influence of level of initial air inclusion in ensiling on quality of silage. *Journal of Japanese Grassland Science* 16, 98 - 100.

TAMIR, M. & ALUMOT, E. (1969). Inhibition of digestive enzymes by condensed tannins from green and ripe carobs. *Journal of the Science of Food and Agriculture* 20, 199 - 205.

TASSINARI, T. & MACY, C. (1977). Differential speed two roll mill pre-treatment of cellulosic materials for

enzymatic hydrolysis. *Biotechnology and Bioengineering* 19, 1321 - 1330.

TAUTORUS, T.E. (1985). Mushroom fermentation . In *Advances in Biotechnological Processes* 5(A. Mizrai and A. L. Van Wezel eds). pp 227 - 273. Alan R. Liss Inc.

TEATHER, R.M. & WOOD, P.J. (1982). Use of Congo red polysaccharide interactions in enumeration and characterisation of cellulolytic bacteria from the bovine rumen. *Applied and Environmental Microbiology* 43, 777 - 780.

THEANDER, O. & AMAN, P. (1980). Chemical composition of some forages and various residues from feeding value determinations. *Journal of the Science of Food and Agriculture* 31, 31 - 37.

TIEN, M. & KIRK, T.K. (1983). *Science Washington* 221, 661 - 663. Cited by Palmer *et al.* (1987).

TIEN, M. & KIRK, T.K. (1984). *Proceedings of National Academy of Science USA* 81, 2280 - 2284. Cited by Kirk (1987). Lignin degrading enzymes. *Philosophical Transactions of Royal Society London* A321, 461 - 474.

TILLEY, J.M.A. & TERRY, R.A. (1963). A two stage technique for *in vitro* digestion of forage crops. *Journal of British Grassland Society* 18, 104 - 111.

TIMELL, T.E. (1967). Recent progress in the chemistry of wood hemicelluloses. *Wood Science and Technology* 1, 45 - 70.

THOMAS, J.W. ; MOORE, L.A. ; OKAMOTO, M. & SYKES, J.F. (1961). A study of factors affecting rate of intake of heifers fed silage. *Journal of Dairy Science* 44, 147 - 153.

TOYAMA, N. (1976). Feasibility of sugar production from agricultural and urban cellulosic wastes with *Trichoderma viride* cellulase. *Biotechnology and Bioengineering Symposium* 6, 207 - 219.

TROWELL, H. (1972). Perspectives in nutrition ;Ischemic heart disease and dietary fiber. *American Journal of Clinical Nutrition* 25, 926 - 932.

TSAO, G.T. & CHIANG, L.C. (1983). Cellulose and hemicellulose technology. In *The Filamentous Fungi* (J.E. Smith; Berry, D.R. and B. Kristiansen eds.). vol. 4. Pp 296 - 326. Edward Arnold Publishers Ltd, London.

UDALL, J.N. & SCRIMSHAW, N.S. (1986). Evaluating the safety and nutritional value of microbial biomass. In *Microbial Biomass Protein* (M. Moo - Young and K. F. Gregory eds). Pp 97 - 103. Elsevier Applied Science Publishers, London.

ULMER, D.C.; TENGERDY, R.P. & MURPHY, V.G. (1981). Solid state fermentation of steam treated feedlot waste fibers with *Chaetomium cellulolyticum. Biotechnology and Bioengineering Symposium* 11, 449 - 461.

ULMER, D. ; LEISOLA, M. ; PUHAKKA, J. & FIECHTER, A. (1983). *Phanerochaete chrysosporium;* Growth pattern and lignin degradation. *European Journal of Applied Microbiology and Biotechnology* 18, 153 - 157.

UMEZAWA, T. & HIGUCHI, T. (1989). Cleavages of aromatic ring and beta 0-4 bond of synthetic lignin (DHP) by lignin peroxidase. *FEBS Letters* 242(2), 325 - 329.

UNDERKOFFLER, L.A. ; FULMER, E.I. & SCHOENE, L. (1939). Saccharification of starchy grain mashes for the alcoholic fermentation industry. Use of mould amylase. *Journal of Industrial and Engineering Chemistry* 31, 734 - 738.

UNDERKOFFLER, L.A. ; SEVERSON, G.M. & GOERING, K.J. (1946). Saccharification of grain mashes for alcoholic fermentation; plant scale use of mold amylase. *Journal of Industrial Engineering and Chemistry* 38, 980 -985.

UNDERKOFLER, L.A. ; SEVERSON, G.M.; GOERING, K.G. & CHRISTENSEN, L.M. (1947). Commercial production and use of mold bran. *Cereal Chemistry* 24, 1 - 22.

USHIDA, K. ; KANEKO, T. & KOJIMA, Y. (1987). Effect of large Entodiniomorphid protozoa on the rumen bacterial flora. *Nippon Chikusan Gakkaiho* 58, 893 - 902.

VAN DER MEER, J.M. (1990). Applications of enzymes-Chairmans report. In *Enzyme Systems for Lignocellulose Degradation* (M.P. Coughlan ed.). Pp 397 - 398. Elsevier Applied Science Publishers, London.

VAN DER MEER, J.M. & KETELAAR, R. (1990). Evaluation of enzymically changed feeds. In *Enzyme Systems for Lignocellulose Degradation* (M.P. Coughlan ed.). Pp 383 -396. Elsevier Applied Science Publishers, London.

VAN DER MEER, J.M. & VAN ES, A.J.H. (1987). Optimal degradation of lignocellulosic feeds by ruminants and *in vitro* digestibility tests. In *Degradation of Lignocellulosics in Ruminants and in Industrial Processes* (J.M. Van der Meer, B.A. Rijkens and M.P. Ferranti eds.) Pp 21 - 34. Elsevier Applied Science Amsterdam.

VAN DER MEER, J,M.; WEVER, G. & SEYOUM, BEDIYE (1988). Rumen bacteria for evaluation of enzymically changed animal feeds and genetic varieties of fodder plants. *Analytica Chimica Acta* 213, 177 - 185.

VAN SOEST, P.J. (1963a). Use of detergents in the analyses of fibrous feeds. 1. Preparations of fibre residues of low nitrogen content. *Journal of the Association of Official Analytical Chemists* 46, 825 - 829.

VAN SOEST, P.J. (1963b). Use of detergents in the analysis of fibrous feeds II. A rapid method for the determination of fibre and lignin. *Journal of the Association of Official Analytical Chemists* 46, 829 - 835.

VAN SOEST, P.J. (1965). Use of detergents in the analysis of fibrous feeds III. Study of effect of heating and drying on yield of fibre and lignin in forages. *Journal of Association of Official Analytical Chemists* 48, 785 - 790.

VAN SOEST, P.J. (1967). Use of detergents in the analysis of fibrous feeds iv. Determination of plant cell wall constituents. *Journal of the Association of Official Agricultural Chemists* 50, 50 - 55.

VAN - VUUREN, A.M. & SPOELSTRA, S.F. (1987). Effect of cellulase treatment on sacco degradation of grass silage. In *Summary of papers* (C. Thomas ed.). 8th Silage Conference P. 109. Institute of Grassland and Animal Production, Hurley, Maidenhead, UK.

VAREL, V & JUNG, H.J. (1986). Influence of forage phenolics on ruminal fibrolytic bacteria and *in vitro* fibre degradation. *Applied and Environmental Microbiology* 52, 275 - 280.

VIESTURS, U.E. ; APISTE, A.F. ; LAUKEVICS, J.J. ; OSE, V.P. & BEKERS, S.M. (1981). Solid state fermentation of wheat straw with *Chaetomium cellulolyticum* and *Trichoderma lignorum*. *Biotechnology and Bioengineering Symposium* 11, 359 - 369.

VIESTURS, U.E.; STRIKAUSKA, S.V. ; LEITE, M.P. ; BERZINS, A.J. & TENGERDY, R. (1987). Combined submerged and solid substrate fermentation for the bioconversion of lignocellulose. *Biotechnology and Bioengineering* 30, 282 -288.

VON SCHELHORN, M. (1950). Untersuchungen uber den verberd wasserarmer lebensmittle durch osmophile mikro-organismen II. Grenkonzentrationen fur den osmophile. Schimmelpilz *Aspergillus glaucus* in

Abhangigkeit vom pH wert des substrates. *Zeitschrift fur Lebensmittelunter-Schung und Forschung* 91, 338 - 342.

WALKER, G.J. & HOPE, P.M. (1964). Degradation of starch granules by some amylolytic bacteria from the rumen of sheep. *Biochemical Journal* 90, 398 - 408.

WALLER, J.C. & KLOPFESTEIN, T.J. (1975). Hydroxides for treating crop residues. *Journal of Animal Science* 41, 424.

WALLACE, R.J. ; CHENG, K.J. & CZERKAWSKI, J.W. (1980). Effect of Monensin on fermentation characteristics of the artificial rumen. *Applied and Environmental Microbiology* 40(1), 672 - 674.

WALLACE, R.J. CZERKAWSKI, J.W. & BRECKENRIDGE, G. (1981). Effect of Monensin on the fermentation of basal rations in the rumen simulation technique (RUSITEC). *British Journal of Nutrition* 46, 131 - 148.

WALLACE, L.; PATERSON, A.; McCARTHY, A.J.; RAEDER, U. ; RAMSEY, L. MACDONALD, M. ; HAYLOCK, R. & BRODA, P. (1984). The problem of lignin biodegradation. *Biochemical Society Symposium* 48, 87 - 95.

WATSON-CRAIK, I. A. (1987). Landfill as an anaerobic filter for the co-disposal of phenolic wastewater. PhD Thesis University of Strathclyde, UK.

WAYMAN. M. & PAREKH, S.R. (1990). Biotechnology of Biomass conversion. Open University Press Milton, Keynes. P.278.

WEDIG, C.L.; JASTER, E.H. & MOORE, K. J. (1987). Hemicellulose monosaccharide composition and *in vitro* disappearance of orchard grass and alfalfa hay. *Journal of Agriculture and Food Chemistry* 35(2), 214 - 218.

WEILAND, P. (1988). Principles of solid state fermentation In *Treatment of Lignocellulosics with White Rot Fungi* (F. Zadrazil and P. Reiniger eds.) Pp 64 - 77. Applied Science, London.

WESTERMARK, U. & ERIKSSON, K.E. (1974). Cellobiose : quinone oxidoreductase. A new wood degrading enzyme from white rot fungi. *Acta Chemica Scandinavica* B28, 209 - 214.

WHITAKER, D.R.; COLVIN, J.R. & COOK, W.H. (1954). The molecular weight and shape of *Myrothecium verrucaria* cellulase. *Archives of Biochemistry and Biophysics* 49, 257 - 262.

WILKINS, R.J. ; HUTCHINSON, K.J.; WILSON, R.F. & HARRIS, C.E. (1971). The voluntary intake of silage by sheep 1. Interrelationships between silage composition and intake. *Journal of Agricultural Science Cambridge* 77, 531 - 537.

WILSON, R.K. & PIDGEN, W.J. (1964). Effect of a sodium hydroxide treatment on the utilisation of wheat straw and popular wood by rumen micro-organisms. *Canadian Journal of Animal Science* 441, 122 - 123.

WOOD, D.A. (1980). Production, purification and

properties of extracellular laccase of *Agaricus bisporus*. *Journal of General Microbiology* 117, 327 - 338.

WOOD, T.M. (1980). Co-operative action between enzymes involved in the degradation of crystalline cellulose. Paper presented at a conference on "Cellulolyse Microbienne" at Centre National de la Kecherche Scientifique, 23 - 24th May, France Pp 167 -176.

WOOD, T.M. (1985). Properties of cellulolytic enzyme systems. *Biochemical Society Transactions*. 13, 407 - 410.

WOOD, T.M. & McCRAE, S.I. (1979). Synergism between enzymes involved in the solubilization of native cellulose. *Advances in Chemistry series* 181, 181 - 209.

WOOD, T.M.; McCRAE S.I. & BHAT, K.M. (1988). The mechanism of fungal cellulase action; synergism between enzyme components of *Penicillium pinophilium* cellulase in solubilizing hydrogen bond ordered cellulose. *Biochemical Journal* 260, 7 - 43.

WOODWARD, J. (1987). Utilisation of cellulose as a fermentation substrate; problems and potential. In *Carbon Substrates in Biotechnology*. Symposium of the Fermentation Physiology and Biochemistry Groups of Society of General Microbiology, UK , 21; 45 - 65. IRL Press Oxford.

YADAV, J.S. (1987). Influence of nutritional supplementation on solid substrate fermentation of

wheat straw with an alkaliphilic fungus (*Coprinus sp*). *Applied Microbiology and Biotechnology* 26, 474 - 478.

YADAV, J.S. (1988). SSF of wheat straw with alcaliphilic fungus *Coprinus sp.* 386. *Biotechnology and Bioengineering* 31, 414 - 417.

YAMANOBE, T. ; MITSUICHI, Y. & TAKASAKI, Y. (1987). Isolation of cellulolytic enzyme producing micro-organism, culture conditions and some properties of the enzymes. *Agricultural Biological Chemistry* 51, 65 - 74.

YAMAZAKI, I. & YOKOTA, K.N. (1973). *Molecular and Cellular Biochemistry* 2, 39 - 52. Cited by Palmer *et al.*, 1987.

YAMIN, M.A. & TRAGER, W. (1979). Cellulolytic activity of an axenically cultivated termite flagellate, *Trichomitopsis termopsidis*. *Journal of General Microbiology* 113, 417 - 420.

YOUNG, R.A. (1986). Structure, swelling and bonding of cellulose fibres. In *Cellulose Structure, Modification and Hydrolysis* (R.A. Young and R. M. Rowell eds.) Pp 91 - 128. Wiley Interscience, New York.

YOSHIHARA, K. & KOBAYASHI, Y. (1981). Bacterial retting of non woody bast fibres for paper making. *Biotechnology Letters* 3, 113 - 118.

YU, Y. ; THOMAS, J.W. & EMERY, R.S. (1975). Estimated value of treated forages for ruminants. *Journal of Animal Science* 41(6), 1742 - 1751.

ZADRAZIL, F. (1980). Conversion of different plant waste into feed by basidiomycetes. *European Journal of Applied Microbiology and Biotechnology* 9, 243 - 248.

ZADRAZIL, F. (1984). Microbial conversion of lignocellulose into feed . In *Straw and other Fibrous By-products as Feed* (F. Sundstol, F. and E. Owen eds.) Pp 276 - 292. Elsevier Applied Science, Amsterdam.

ZADRAZIL, F. (1985). Screening of fungi for lignin decomposition and conversion of straw into feed. *Angew Botanik* 59, 433 - 452.

ZADRAZIL, F. (1990). Selection of white rot fungi for delignification and their utilisation under industrial conditions. Paper Presented at the Fourth International Mycological Congress, Regensburg, Germany, 28th Aug. - 3rd September.

ZADRAZIL, F. & BRUNNERT, H. (1980). The influence of ammonium nitrate supplementation on degradation and in vitro digestibility of straw colonised by higher fungi. *European Journal of Applied Microbiology and Biotechnology* 9, 37 - 44.

ZADRAZIL, F. & BRUNNERT, H. (1981). Investigation of physical parameters important for solid state fermentation of straw by white rot fungi. *European Journal of Applied Microbiology and Biotechnology* 11, 183 - 190.

ZADRAZIL, F. & KAMRA, D.N. (1989). Influence of air and oxygen supplies on lignin degradation and its relation with *in vitro* digestibility of wheat straw fermented with *Stropharia rugosoanulata*, *Pleurotus eryngii* and *Pleurotus sajo-caju*. *Mushroom Journal of Tropics* 9, 79 - 88.

ZADRAZIL, F.; DIEDRICHS, M. ; JANSEN, H.; SCHUCHARDT, F. & PARK, J.S. (1990). Large scale solid state fermentation of cereal straw with *Pleurotus* spp . In *Advances in Biological Treatment of Lignocellulosic Materials* (M.P. Coughlan and M. T. Amaral-collaco eds.) Pp 43 - 59. Elsevier Applied Sciences, London.

ZEIKUS, J.G (1980). Fate of lignin and related aromatic substrates in anaerobic environments. In *Lignin Biodegradation* (T. K. Kirk, T. Higuchi and H. M. Chang, eds.) Pp 101 - 109. CRC Press, Boca Raton Florida.

ZEIKUS, J.G. (1981). Lignin metabolism and the carbon cycle; Polymer biosynthesis, biodegradation and environmental recalcitrance vol. 5. In *Advances in Microbial Ecology* Vol. 5 (M. Alexander ed.) Pp 211 - 243. Plenum Press New York .

ZEIKUS, J.G.; WELLSTEIN, A.L. & KIRK, T.K. (1982). Molecular basis for the biodegradative recalcitrance of lignin in anaerobic environments. *FEMS Microbiology Letters* 15, 193 - 200.

ZIMBO, M. & TIMELL, T.E. (1967). Studies on a native xylan from Norway spruce (*Picea abies*). *Svensk*

Papperstidung 70, 597 - 606.

ZIMMERMANN, W. (1990). Hemicellulolytic enzyme systems from actinomycetes. In *Enzyme Systems for Lignocellulose Degradation* (M.P Coughlan ed.) Pp 167 - 182 . Elsevier Science Publishers, London.

ZIMMERMANN, W. ; WINTER, B. & BRODA, P. (1988). Xylanolytic enzyme activities produced by mesophilic and thermophilic actinomycetes grown on graminaceous xylan and lignocellulose. *FEMS Microbiology Letters* 55, 181 - 186.

Appendix

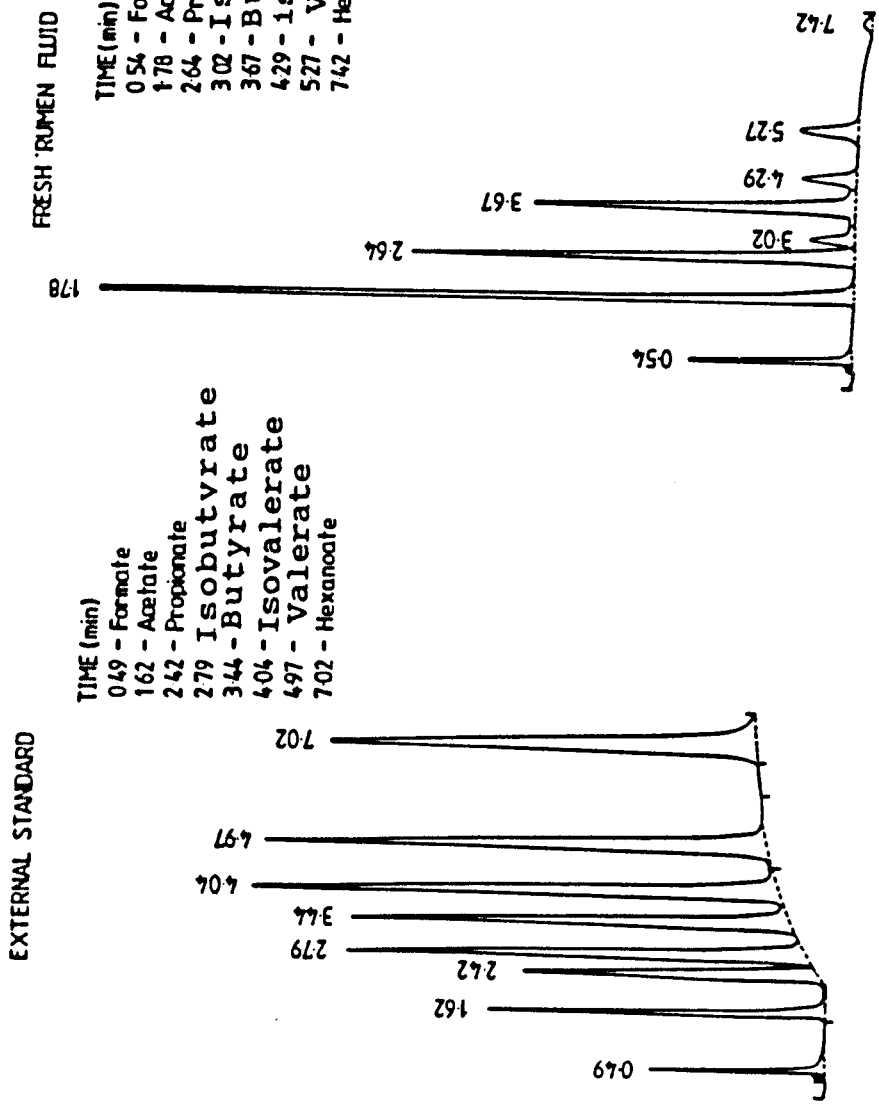


Fig. A1 Chromatogram illustrating the elution of rumen volatile acids.

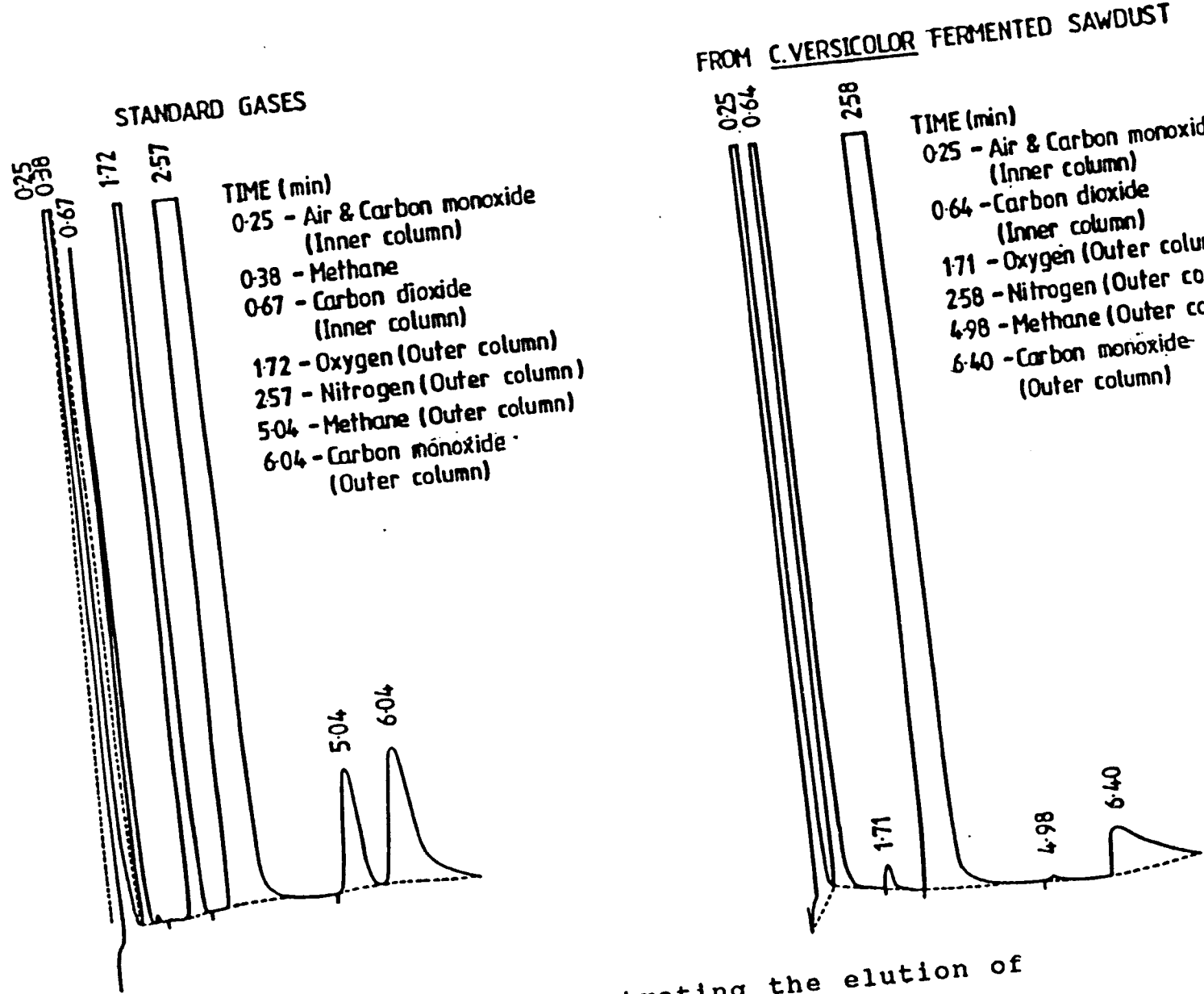


Fig. A2 Chromatograms illustrating the elution of fermentation gases.

Appendix 3 DESCRIPTION AND NUMERICAL VALUES USED TO CLASSIFY INTERACTIONS BETWEEN FIELD AND STORAGE FUNGI ON AGAR MEDIA.

CLASSIFICATION DESCRIPTION	NUMERICAL VALUE
Mutual intermingling	1
Mutual inhibition on contact or space between colonies small(<2mm).	2
Mutual inhibition at a distance(>2mm)	3
Inhibition of one organism on contact, the inhibitor species continues to grow unchnaged or at a reduced rate through the inhibited colony.	4
Inhibition of one organism at a distance, the inhibitor species then continuing to grow through the resulting clear zone and the inhibited colony, perhaps at a reduced rate.	5

FROM: MAGAN & LACEY (1984).

Appendix 4; Extraction and quantification of aflatoxin

Step 1 ; (a) Filter paper and filter funnel were assembled over a clean beaker.

(b) The cap on top of aflatest affinity column(Rhone Poulenc, Diagnostics Ltd, Glasgow) was removed, the sealed end cut off and replaced on the affinity column.

(c) The column was attached firmly to a 10ml glass syringe barrel and placed in a stand (Fig. A3).

Step 2 - sample extraction

(a) Aliquots (250 ml) of 60 % methanol was put into 500 ml solvent resistant metal blender jars.

(b) *A.flavus* fermented maize grain (50 g) was added to jars, the blender was covered and blended at high speed for 1 min.

(c) After blending, extracts were diluted with 250 ml distilled water, mixed well by swirling and filtered through a filter funnel assembly.

Step 3 Adsorption on to affinity column

(a) filtrate was diluted several times with 30% methanol.

(b) Aliquots (10ml) of filtrate diluted as appropriate were transferred into glass syringe barrels.

(c) Bottom plug of the affinity columns were removed.

(d) A hand pump was placed on top of glass syringe while the rubber connector attached to the pump was firmly placed to ensure a good seal.

(e) By a slow steady pressure(drop rate of 3 ml min^{-1}),

the 10ml portions of extract were pushed through the affinity column.

(f) aflatoxin in the extract will be adsorbed by monoclonal antibody within the column whereas other metabolites will pass through the column.

Step 4 Washing

(a) Distilled water (10 ml) was added into the glass syringe barrel and the column was washed by passing the water through using a steady pressure.

(b) Washing was repeated to ensure that non specific antigenic particles were expelled.

Step 6a Elution Fluorosil - UV detection

(a) Fluorosil tip was attached to the lower end of the affinity column.

(b) HPLC grade methanol (1 ml) was added to the syringe barrel.

(c) The pump unit was replaced and methanol was eluted through affinity column and fluorosil tip to waste by applying steady pressure with the hand pump.

(d) The fluorosil tip was removed from the column and externally cleaned with methanol to remove extraneous material.

(e) The fluorosil tip was then placed in a UV light box and illuminated at 366 nm.

(f) The blue fluorescence emitted was compared with the Fluorescent comparitor.

(g) This is a semi- quantitative method for estimating aflatoxin concentration.

Step 6b Fluorimetric determination

(a) Aflatest developer was prepared by diluting concentrated aflatest developer with water (1 : 9). The solution was prepared fresh and kept in an amber bottle.

(b) A clean glass fluorimeter curvette was placed under the affinity column.

(c) The hand pump was removed and 1 ml HPLC grade methanol was put into the glass syringe barrel.

(d) With aid of hand pump the methanol was passed through the column and eluate containing toxin was collected in the cuvette.

(e) Diluted aflatest developer (1.0 ml) was added to the eluate and solution mixed by vortex mixing.

(f) The outside of the cuvette was cleaned, placed in a calibrated fluorimeter (calibrated with standards(0 - 20 ug kg^{-1} aflatoxin) and the reading taken.

Usually the fluorimeter reading should range between 0 - 100. Any reading outside this range indicates that the monoclonal antibodies have been saturated and some of the toxin have been expelled. In such circumstances, the original sample must be diluted in 30% methanol and the dilution factor noted.

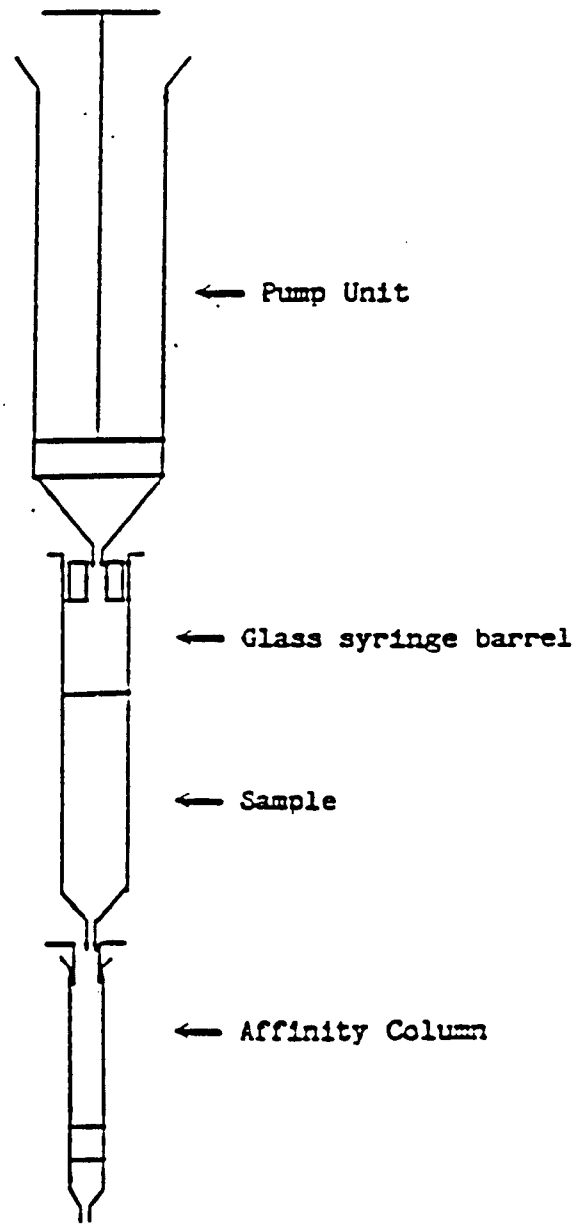


Fig. A3 The Aflatest system unit