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BIOLOGICAL SULPHATE REDUCTION USING LIGNOCELLLOSE HYDROLYSIS BY-PRODUCTS PRODUCED BY FUNGAL HYDROLYSIS OF *CENCHRUS CILIARIS* CV. MOLOPO (BUFFELSGRASS)

by

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Submitted in the partial fulfillment
of the requirements for the degree

Master of Science

In the Faculty of Biological, Agricultural and Information Sciences
Department of Microbiology and Plant Pathology
University of Pretoria
Pretoria
South Africa

October 2000

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"Try not to become a man of success
but rather a man of value."

Albert Einstein

I certify that the thesis hereby submitted, and the work presented therein, to the University of Pretoria for the degree of M.Sc. has not been previously submitted by myself in respect of a degree at any other University.

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Degree : M.Sc. (Microbiology)

SUMMARY

The biological treatment of acid mine drainage using sulphate reducing bacteria (SRB) is an alternative to chemical treatment. However, substrate availability normally becomes the limiting factor for sustaining sulphate reduction. Biological pretreatment of *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) by white rot fungi was investigated to enhance the biodegradability of this lignocellulose substrate. *In vitro* dry matter digestibility was used to measure the effectiveness of fungal delignification. None of the treatments increased the digestibility of the natural substrate. Only *Pleurotus ostreatus* was capable of improving the initial digestibility of steam pasteurized grass by 7% over 6 weeks. A brown leachate was produced in all treatments over the experimental period. Literature indicated that such a leachate could contain inhibitors or stimulants of bacterial growth. An antimicrobial activity assay indicated that the leachate did not inhibit aerobic Gram negative or Gram positive bacteria. The effect of the leachate on sulphate reduction by SRB was investigated at 3% and 6% concentrations, using 3% preconditioned inoculum. Overall sulphate reduction varied from 27.7% to 44.9% and long-term sulphate reduction rates of 9.4 mg/l/d to 15 mg/l/d were observed. Sulphate removal efficiencies (in terms of mg SO_4^{2-} reduced / mg COD consumed) of 27% to 52% were obtained. No biological sulphate reduction was observed in the control reactors. Reactors amended with 3% *P. ostreatus*, *S. commune* and *P. chrysosporium* leachate

performed better compared to other leachate treatments. Sulphate removal was comparable to literature values, but sulphate reduction rates were considerably lower in this study. Near-neutral pH was maintained in all leachate amended reactors, unlike the controls. Therefore, leachate buffering capacity could enhance SRB survival and consequently expedite biological sulphate reduction.

BIOLOGIESE SULFAATREDUKSIE DEUR GEBRUIK TE MAAK VAN LIGNOSELLULOSE AFBRAAK PRODUKTE GEPRODUSEER VANAF DIE AFBRAAK VAN *CENCHRUS CILIARIS* CV. MOLOPO (BUFFELSGRAS) DEUR SWAMME

deur

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OPSOMMING

Die gebruik van sulfaatreduserende bakterieë vir die behandeling van suurmyn dreinering is 'n altenatief tot chemiese behandeling. Volgehoude sulfaatreduksie word normaalweg beperk deur die beskikbaarheid van die substraat. Wit-vrot swamme is gebruik om die bioafbreekbaarheid van *Cenchrus ciliaris* cv. Molopo (Buffelsgras) te verbeter. Die effektiwiteit van lignien afbraak deur die wit-vrot swamme is gemeet in terme van *in vitro* droë materiaal verteerbaarheid. Geeneen van die behandelings kon die verteerbaarheid van natuurlike Buffelsgras verbeter nie. Die verteerbaarheid van stoom-gepasteuriseerde Buffelsgras is deur *Pleurotus ostreatus* met 7% oor 6 weke verbeter. Tydens die eksperimentele tydperk is 'n bruin vloeistof in al die reaktors geproduseer. Volgens die literatuur kon hierdie uitloogmateriaal chemiese verbindings bevat wat bakteriële groei inhibeer of stimuleer. 'n Filtreerpapierskyfie tegniek het getoon dat beide Gram negatiewe en Gram positiewe aerobe bakterieë nie deur die uitloogmateriaal geinhibeer word nie. Die uitwerking van die uitloogmateriaal op biologiese sulfaatreduksie is ondersoek deur gebruik te maak van 3% of 6% van die uitloogmateriaal en 3% van 'n voorafbehandelde inokulum. Die totale sulfaatreduksie het gewissel tussen 27.7% en 44.9%, terwyl langtermyn sulfaatreduksie tempo's van 9.4 mg/l/d tot 15 mg/l/d aangeteken is. Die effektiwiteit van sulfaatverwydering (in terme van mg SO_4^{2-} gereduseer / mg chemiese suurstofbehoefte verbruik) het gewissel

tussen 27% en 52%. Geen biologiese sulfaatreduksie het in die kontrole reaktors plaasgevind nie. In vergelyking met die kontrole reaktors, was die sulfaatreduksie beter in die reaktors met 3% uitloogmateriaal van *P. ostreatus, S. commune* en *P. chrysosporium.* Alhoewel die sulfaatreduksietempo's baie laag was, vergelyk die totale sulfaatreduksie goed met dié wat in die literatuur beskryf is. 'n Neutrale pH is nie in die kontrole reaktors gehandhaaf nie, maar wel in al die reaktors wat uitloogmateriaal bevat het. Dus kan die bufferkapasiteit van die lignosellulose uitloogmateriaal sulfaatreduserende bakterieë se oorlewingsvermoë verbeter en gevolglik biologiese sulfaatreduksie voorthelp.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
ADF	acid detergent fiber
AMD	acid mine drainage
APHA	American Public Health Association
APPL	acid-precipitable polymeric lignin
ATP	adenosine triphosphate
AWWA	American Water Works Association
BOD_5	5 day biochemical oxygen demand
cfu	colony forming units
cfu/ml	colony forming units per milliliter
cm	centimeter
COD	chemical oxygen demand
CoA	coenzyme A
CSL	corn steep liquor
cv.	cultivar
d	days
°C	degrees Celsius
dH_2O	distilled water
EMP	Embden-Meyerhof-Parnas
FNC	fine natural control
FNPc	fine natural *Phanerochaete chrysosporium*
FNPo	fine natural *Pleurotus ostreatus*
FNSc	fine natural *Schizophyllum commune*
g	gram
g/l	gram per liter
h	hour
HPLC	high performance liquid chromatography
INRA	Institut de la Recherche Agronomique
IR	infrared

IVD	*in vitro* digestibility
IVDMD	*in vitro* dry matter digestibility
IVRD	*in vitro* rumen digestibility
k	specific rate constant
LCCs	lignin-carbohydrate complexes
LiP	lignin peroxidase
l	liter
ME	malt extract
Mn	manganese
MnP	manganese-dependent peroxidase
$\mu g.g^{-1}$	microgram per gram
μm	micrometer
mg/l	milligram per liter
mg/l/d	milligram per liter per day
ml	milliliter
ml/l	milliliter per liter
mm	millimeter
min	minutes
M	molar
NDF	neutral detergent fiber
NMR	nuclear magnetic resonance
o	ortho
p	para
P. chryso	*Phanerochaete chrysosporium*
PCP	pentachlorophenol
%	percent
rpm	revolutions per minute
s	seconds
SEM	scanning electron microscopy
SF	submerged fermentation
spp.	species
SRB	sulphate reducing bacteria

SSF	solid-state fermentation
TAPC	total anaerobic plate count
TCA	tricarboxylic acid cycle
TEM	transmission electron microscopy
UV	ultraviolet
V	volt
WPCF	Water Pollution Control Federation

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CHAPTER 1

INTRODUCTION

Acid mine drainage (AMD) is a man-made phenomenon that represents a persistent and destructive environmental problem. The heavy metal concentrations present in the effluent are toxic to all biota and the acidic pH disturbs the ecological systems maintained in the receiving waters (Figure 1.1 and 1.2) (Gray, 1997). If left untreated, the acidity and metal content of AMD flowing into the environment can have a devastating effect on terrestrial and aquatic ecosystems. Therefore, AMD is categorized as a multifactor pollutant (Figure 1.3) (Gray, 1997). AMD is caused by chemical and microbiological factors (Atlas and Bartha, 1993; Brierly and Brierly, 1997). The product is a leachate resulting from the oxidation of sulfide containing minerals exposed to water, air and bacteria.

Because of the biological entities involved in AMD formation, preventative control efforts have been developed that specifically target this component (Ledin and Pedersen, 1996). Surfactants and slow release biocides become diluted after time and thus proved ineffective. The financial costs involved in controlling the microbiological origin of the problem are daunting. The only really effective means of preventing AMD will be to separate the sulfide-bearing rocks from air and water (Brierly and Brierly, 1997; Ledin and Pedersen, 1996). Although this seems impossible to achieve, new approaches toward waste management have been implemented. These include covering the rocks with air and water repulsing clay, vegetating waste piles, and placing the rocks on specially engineered sites that allow capture and treatment of the effluent (Brierly and Brierly, 1997).

Sulphate is an important component of acid mine drainage because it causes the formation of sulphuric acid in the receiving waters. The chain of chemical reactions that

lead to the formation of sulphuric acid can be interrupted with the removal of sulphate. Financial constraints prevent the widespread application of demineralization processes such as reverse osmosis and electrodialysis that may be applied for sulfate removal (Maree and Strydom, 1987). The drainage water itself can be treated by the addition of alkaline chemicals or by constructing artificial wetlands (Ledin and Pedersen, 1996). An inexpensive, low maintenance, on-site treatment process is greatly desired because of the large volumes of AMD that must be treated. The biological treatment of AMD using sulfate-reducing bacteria is a possible alternative to chemical treatment.

Tuttle *et al.* (1969) studied a stream that was impeded by a dam wall composed primarily of wood dust, a waste product from a small log-cutting mill. The stream contained ferric, sulfite and hydrogen ions that were produced from pyritic minerals associated with coal. The retarded flow of water resulted in a pond behind the dam (the upper pond) and another pond (the lower pond) was formed due to uneven terrain downstream. The porous quality of the wood dust allowed the water to permeate through at a very low rate, thereby enriching it in organic nutrients as the water entered the lower pond. The degradation of wood dust was required to establish an anaerobic microflora in order for sulfate reducing bacteria to utilize the sulfate present in the medium. It appeared as if the rate of wood dust degradation determined the initiation and rate of sulfate reduction. Partially degraded wood dust contained fermentation products that could be utilized by the sulfate reducing bacteria.

Previous studies have indicated that substrate availability became the limiting factor in terms of sulfate reduction in pilot plants operated at an AMD affected site after a period of approximately 9 months. A similar observation was made during the development of a mixed aerobic-anaerobic microbial treatment process for acid-mine drainage using straw as a substrate (Béchard *et al.*, 1994). The authors assumed that the biodegradation of the straw would provide organic carbon necessary to sustain the treatment process. However, the long-term stability of their bioreactors could not be maintained and supplementation

with urea and sucrose was required. Carbon was the primary limiting factor in the treatment process.

Lignocelluloses are the building blocks of all plants. Physical barriers and the chemical recalcitrance of the lignocellulose substrate can prevent its complete utilization by microorganisms. Exposing complex lignocellulose materials to white-rot fungi facilitates preferential delignification of the lignocellulose matrix (Lee, 1997). The result is the release of cellulose and hemicellulose fibers previously shielded by lignin polymers. This increased digestibility provides organic carbon that can be fermented to organic acids in an anaerobic environment (Müller and Trösch, 1986). If the purpose of biological pretreatment of lignocellulose is to obtain a cellulose-enriched substrate, then the ideal microorganism will cause accurate lignin degradation without severe polysaccharide loss. Therefore, pretreatment of the complex carbon sources by white-rot fungi may be necessary to enhance the biodegradability of the lignocellulose substrate. The biologically modified carbon source, or its by-products, should be able to enhance sulfate-reducing bacterial activity.

The objectives of this study were to investigate:

1. The biological pretreatment of *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) with *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Schizophyllum commune* as well as the fungal community structure of the decaying grass.

2. The effect of lignocellulose leachate, produced during hydrolysis of Buffelsgrass by *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Schizophyllum commune* and natural fungi, on sulphate reduction by sulphate reducing bacteria.

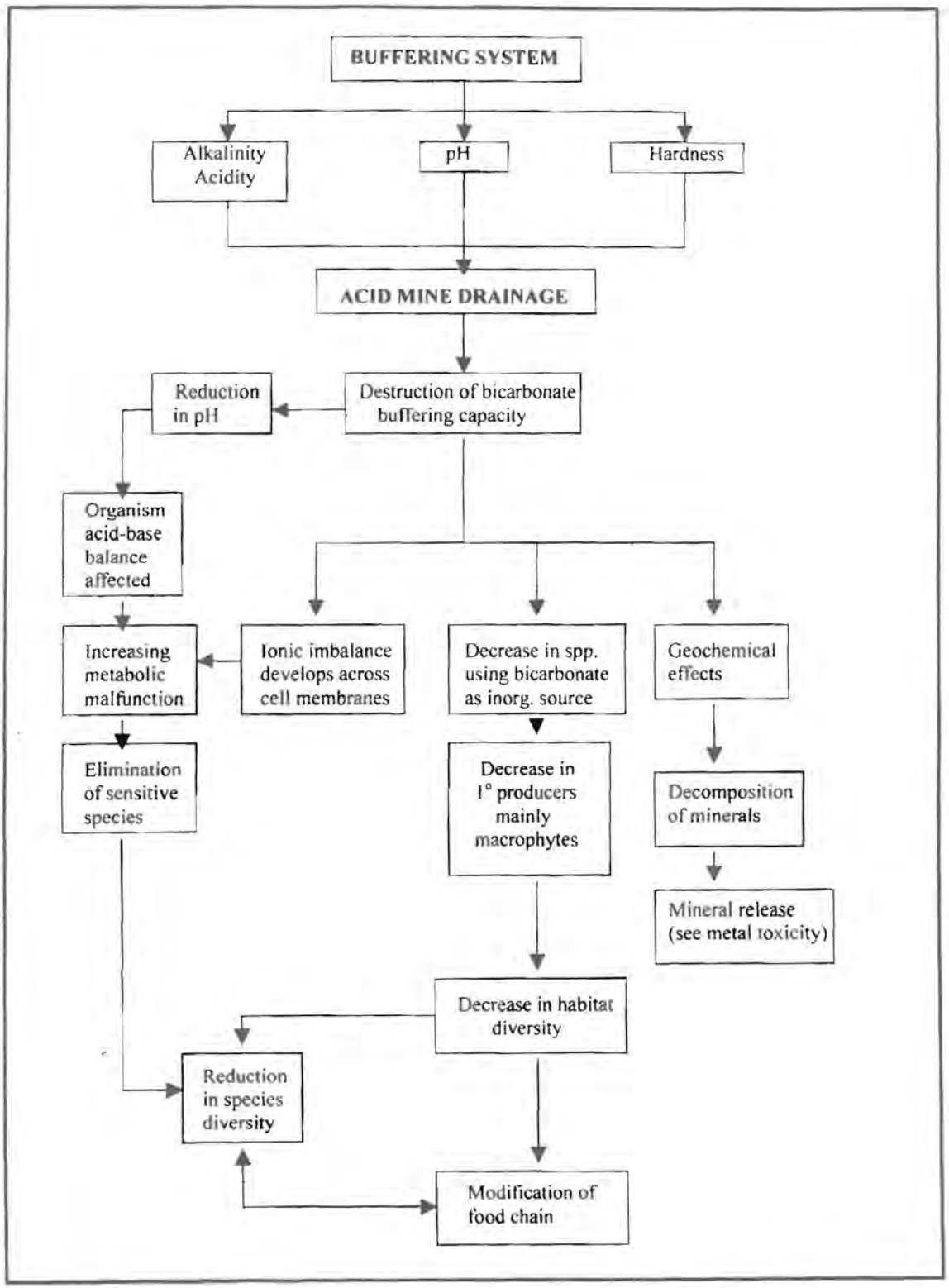


Figure 1.1: Effects of acidity originating from AMD in river systems (Gray, 1997).

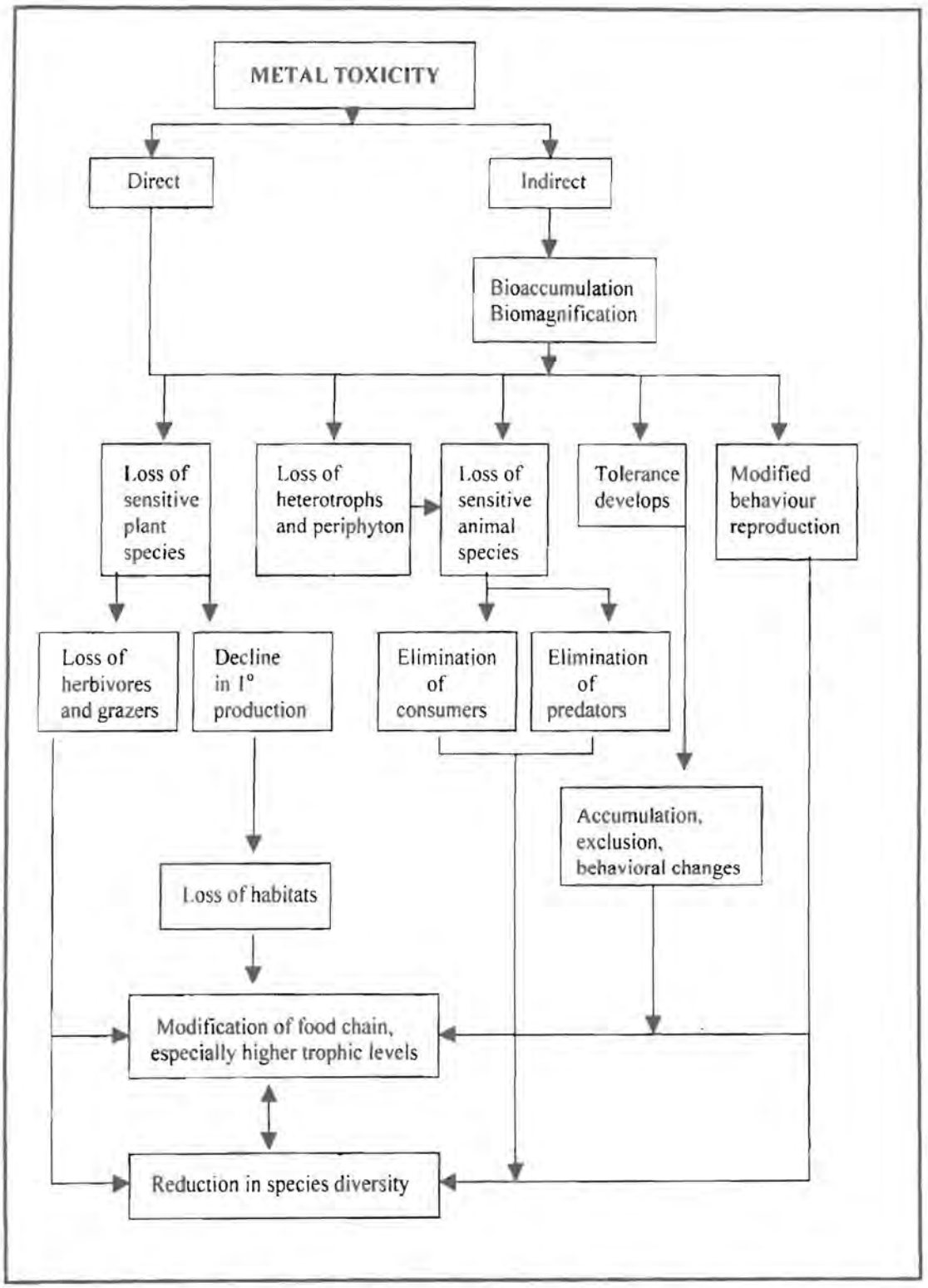
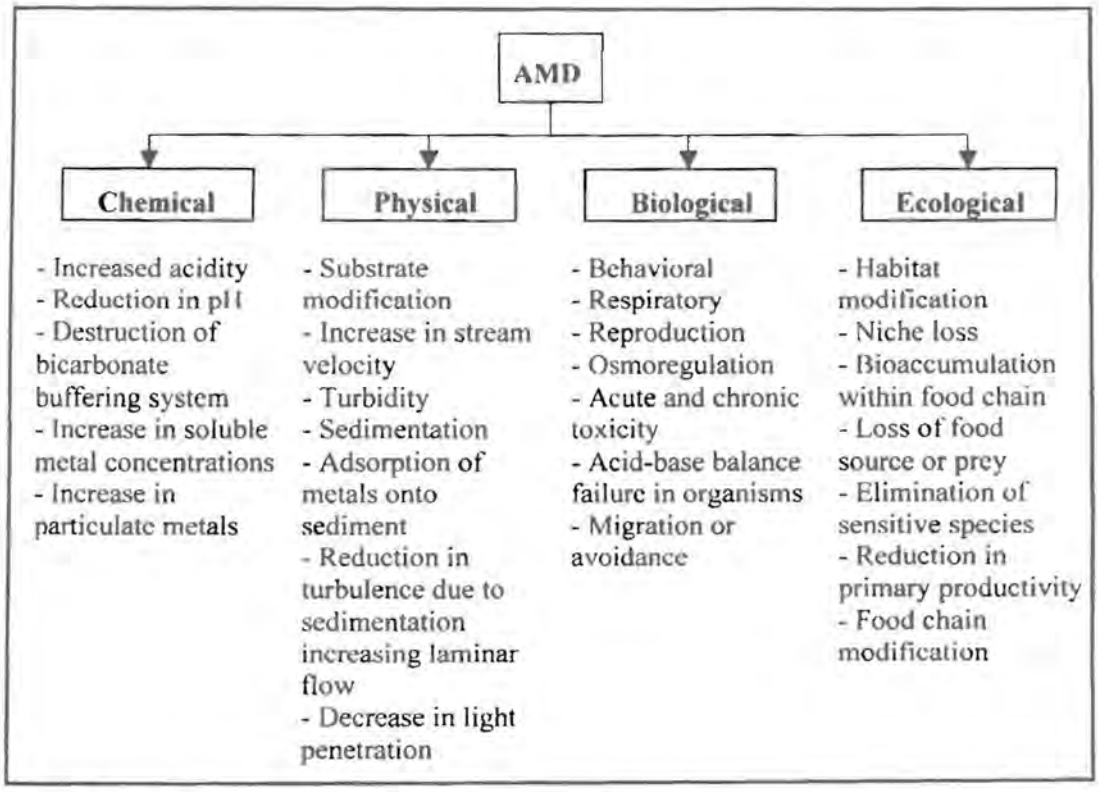


Figure 1.2: Effects of heavy metals originating from AMD in river systems (Gray, 1997).



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CHAPTER 2

LITERATURE REVIEW

1. Lignocellulose composition

1.1 Chemical composition

Lignocellulose is the term used to describe the composite of the predominant polymers of plants. Cellulose, hemicellulose, and lignin are the main constituents of lignocellulosic materials (Deobald and Crawford, 1997; Kuhad *et al.*, 1997; Tuor *et al.*, 1995). These components are present in different quantities in different plant species (Table 2.1). Cellulose is a linear polymer of glucose linked through α-1,4 linkages and is usually arranged into microcrystalline structures, which is very difficult to dissolve or hydrolyze (Figure 2.1). Hemicellulose is a heteropolysaccharide composed of different hexoses, pentoses and glucoronic acid (Figure 2.2). Hemicellulose is more soluble than cellulose and is frequently branched. Xylan is the most common hemicellulose component of grass and wood. In plants they are complexed with substances like lignin and cellulose that may complicate their hydrolysis (Jeffries, 1990). Lignin is a highly irregular and insoluble polymer made up of phenylpropanoid subunits, namely *p*-coumaroyl, coniferyl, and sinapyl alcohols (Figure 2.3). These phenyl moieties differ in the hydroxy and methoxy substituents and are called *p*-hydroxyphenyl (H-type), guaiacyl (G-type) and syringyl (S-type) units. Unlike cellulose or hemicellulose, no chains containing repeating subunits are present, thereby making the enzymatic hydrolysis of this polymer extremely difficult (Figure 2.4).

Table 2.1: Typical chemical composition of various lignocellulosic materials (Betts *et al.*, 1991).

Raw material	Lignin (%)	Cellulose (%)	Hemicellulose (%)
Hardwoods	18-25	45-55	24-40
Softwoods	25-35	45-50	25-35
Grasses	10-30	25-40	25-50

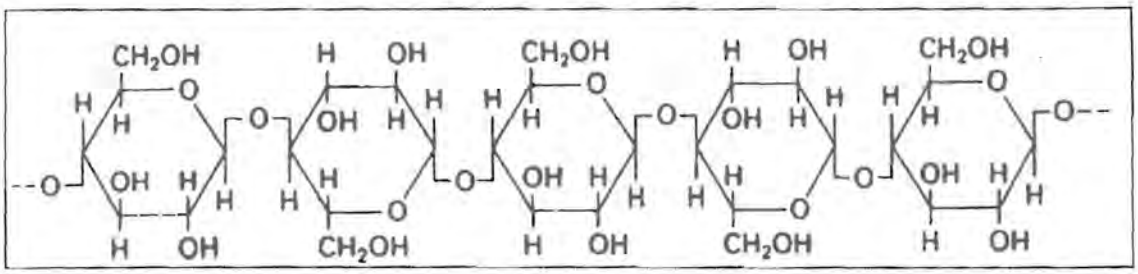

Figure 2.1: Structure of cellulose showing α-1,4-linkages (Dix and Webster, 1995).

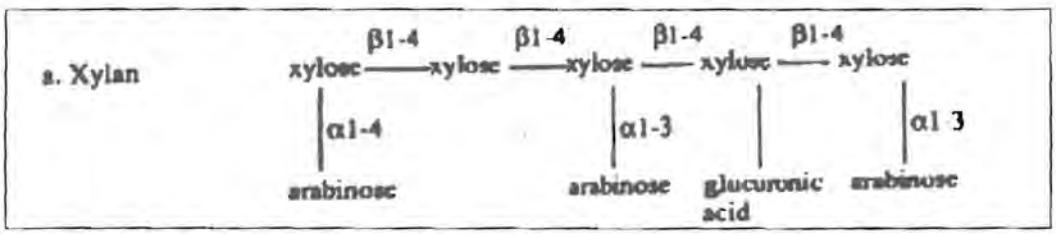

Figure 2.2: Schematic representation of xylan hemicellulose (Betts *et al.*, 1991).

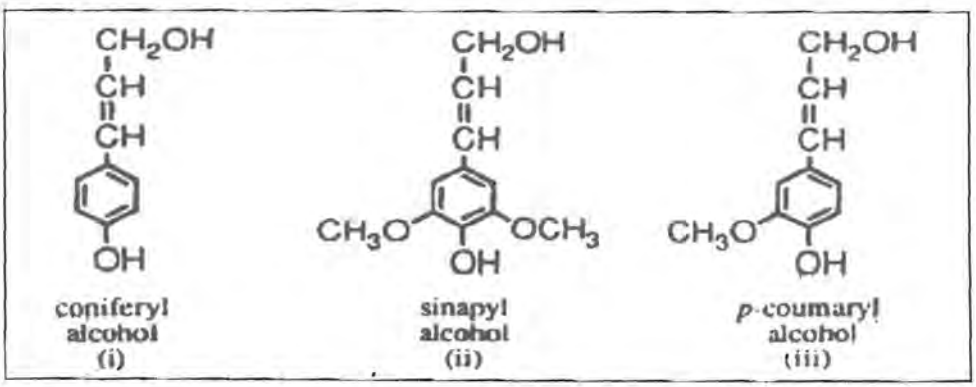

Figure 2.3: Hydroxycinnamyl alcohol subunits of lignin (Betts *et al.*, 1991).

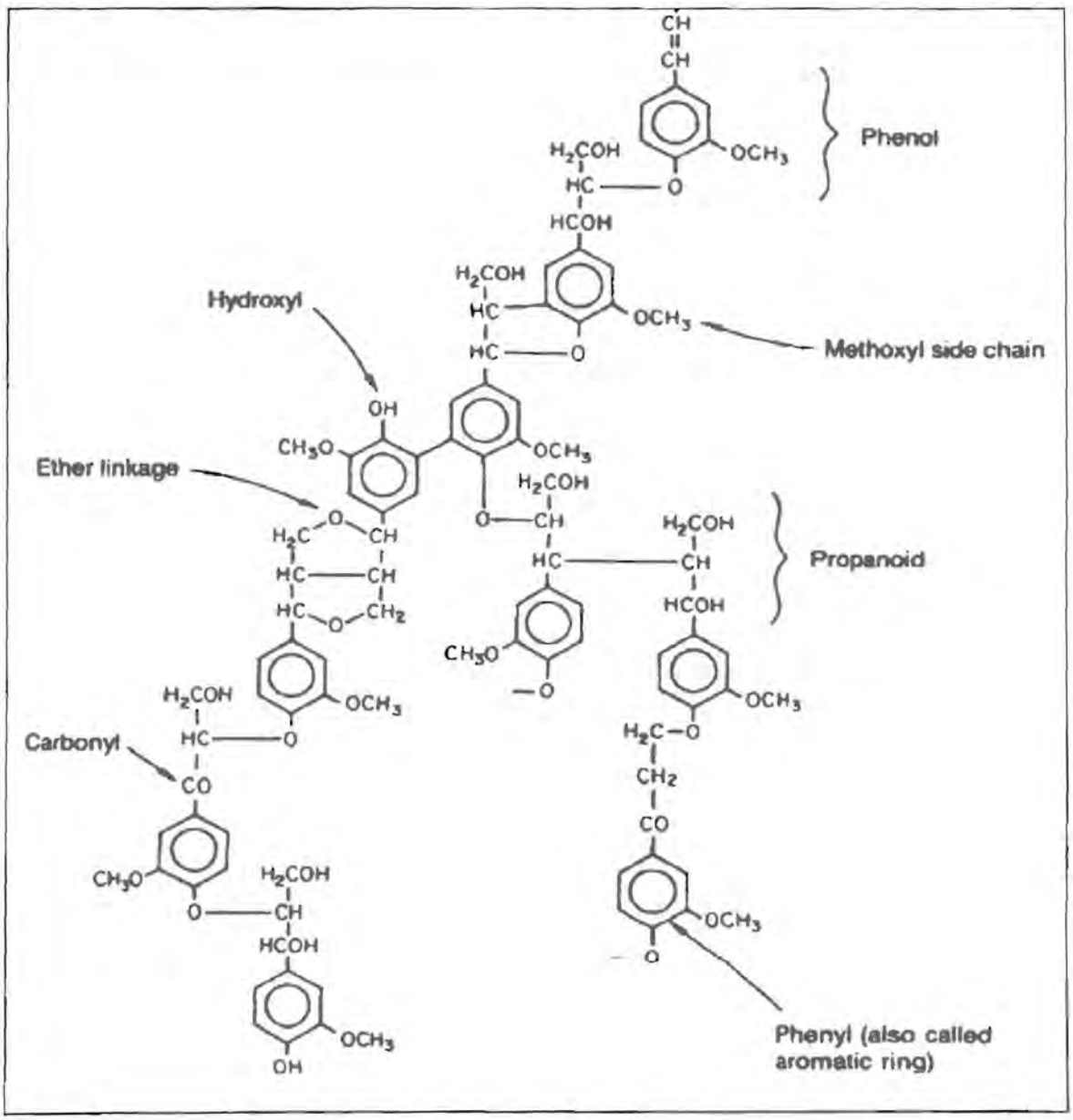

Figure 2.4: General structural formula of a softwood lignin (Paul and Clark, 1989). Important chemical functional groups are indicated.

1.2 Tertiary architecture of the lignocellulose matrix

Lignin-carbohydrate complexes (LCCs) are recognized as key structures determining forage digestibility (Hatfield *et al.*, 1999). The accessibility of cell wall polysaccharides from the mature plant to microbial enzymes is dictated to a large extent by the degree to which they are associated with phenolic polymers (Chesson, 1981). Insight into the three-dimensional architecture of the lignocellulose matrix might answer the question of how a small amount of lignin can have a negative effect on cell wall digestibility

Cellulose microfibrils are embedded in an amorphous matrix, similar to reinforced concrete (Leonowicz *et al.*, 1999; Tomme *et al.*, 1995). These microfibrils have a structural function in the cell wall, imparting strength and rigidity. The matrix materials consist of hemicelluloses that are further associated with pectins and proteins in primary cell walls and with lignin in secondary cell walls (Tomme *et al.*, 1995). In secondary cell walls the hemicelluloses consist mainly of xylans and to some degree mannans and glucomannans. Xylans in grasses are generally characterized by the presence of L-arabinose linked as a single unit substituent to a D-xylose backbone (Kuhad *et al.*, 1997).

In grasses, ferulic and *p*-coumaric acids are esterified to hemicellulose and lignin (Jeffries, 1990; Mueller-Harvey and Hartley, 1986). Xylans appear to be a major interface between lignin and other carbohydrate components in many isolated phenolic-carbohydrate complexes (Cornu *et al.*, 1994; Jeffries, 1990). Ferulic acid anchors hydrophobic lignins to hydrophilic polysaccharides via alkali sensitive ester bonds (Figure 2.5). Intramolecular lignin bonds are usually of the alkali-resistant ether type. This intricate association with lignin shields hemicellulose from direct enzymatic hydrolysis (Sun *et al.*, 1996).

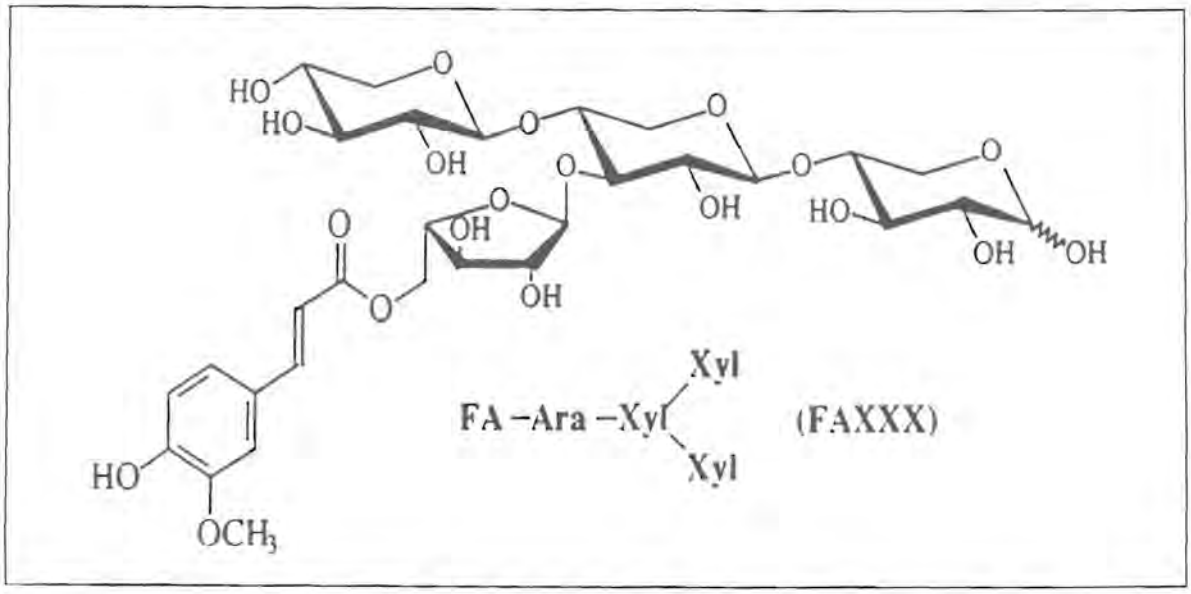

Figure 2.5: Incorporation of ferulic acid into cell wall matrices via attachment to structural polysaccharides (Hatfield *et al.*, 1999).

1.3 Physical structure of grass cell walls

The cell wall structure of cereal straw has been less studied than that of wood. Compared to wood, straw is a much more heterogeneous raw material. Straw fibers that are principally derived from cells and internodes, however, are fairly long and slender with sharply pointed ends (Fan *et al.*, 1982). In addition to these fibers, however, straw also contains short non-fibrous cells consisting of epidermal cells, platelets, serrated cells, and spirals. Whereas 96% of the cells in wood can be considered as fibers, only about 35-39% of the cells in straw is fibers (Fan *et al.*, 1982). Straw species are more uniform in composition than wood species. Generally, straw has lower cellulose content than wood, but has higher proportions of hemicellulose and lignin compared to wood. The ash content is greater in straw than in wood (Fan *et al.*, 1982).

Buffelsgrass (*Cenchrus ciliaris* cv. Molopo) is a perennial grass adapted to hot dry climate conditions and is commonly found in Southern Africa (Russell *et al.*, 1990) (Figure 2.6). This grass was compared to other European forages and it did not have thicker cell walls or larger cells (Moghaddam and Wilman, 1998). It does have a larger proportion of thick walled cells in the leaf blades and leaf sheaths. However,

digestibility was not especially low, as one would expect (Wilman and Moghaddam, 1998). The high proportion of thick walled cells and the associated closeness of the veins, presumably help to support the leaf blades when growing in hot, dry conditions, but are a considerable disadvantage if forage of high digestibility and high intake potential is required (Moghaddam and Wilman, 1998; Wilman *et al.*, 1996).

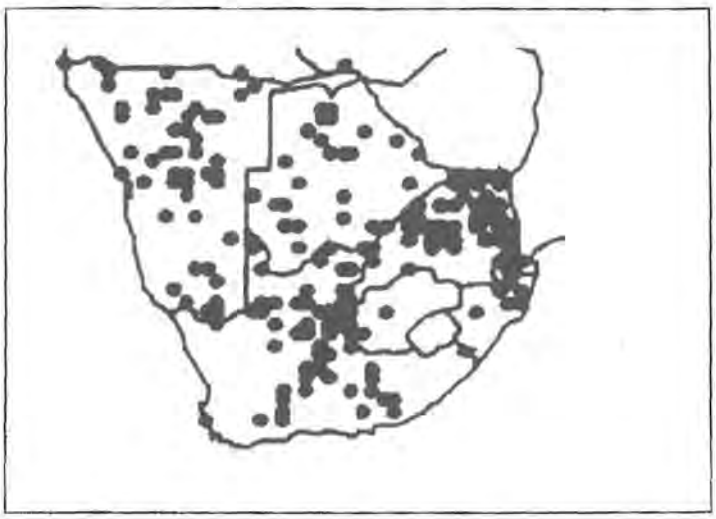

Figure 2.6: Distribution of *Cenchrus ciliaris* (Buffelsgrass) in Southern Africa (Russell *et al.*, 1990).

The constraints imposed by these physiological traits become more severe as the plant ages. Cellulose and lignin contents of switchgrass increased during spring and fall from 40.7% and 5.5% to 44.9% and 12.0% respectively (Dale *et al.*, 1996). Hemicellulose and ash contents decreased by 4% and 1% over the same time period.

2. Degradation of plant organic matter in soil

The reactions responsible for the conversion of exogenous plant material to humic substances and the mineralization of organic matter are the result of soil biotic activity. Both soil microfauna (for example ants and earthworms) and microflora (soil microorganisms) facilitate these conversion reactions, but the soil microbial complement is responsible for the most degradation and mineralization activity

(Figure 2.7). The specific metabolic activities of these microbes on the various plant constituents will be discussed in much detail elsewhere.

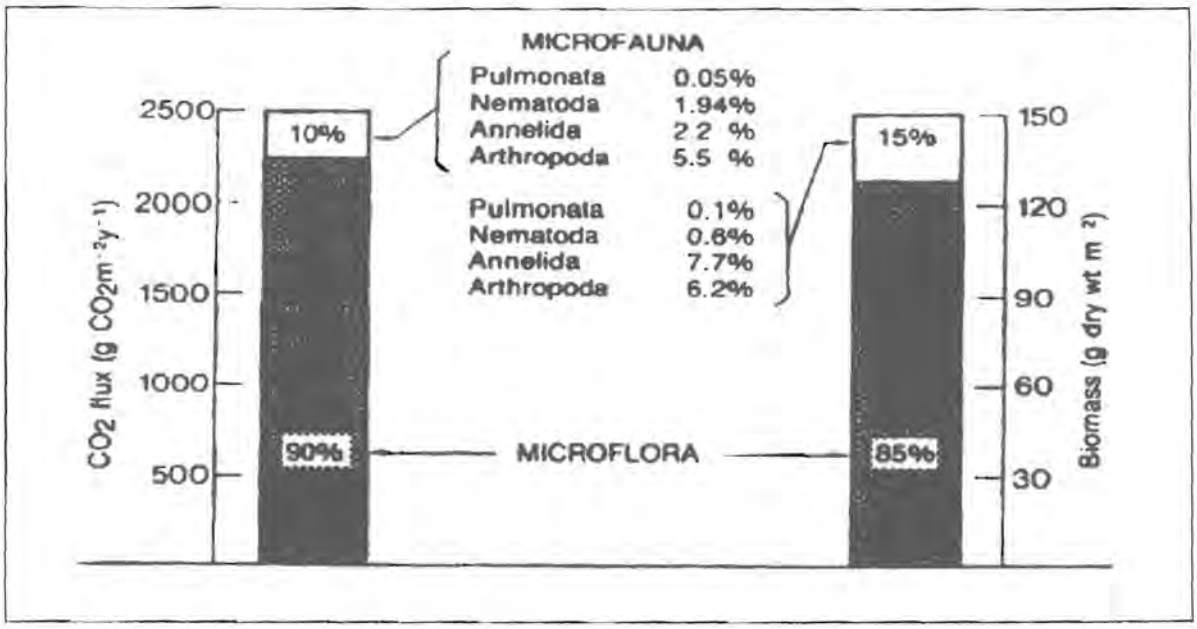

Figure 2.7: Comparative biomass and CO_2 flux for the microflora and faunal decomposers in forest litter (Paul and Clark, 1989).

2.1 Role of soil microfauna

Soil microfauna has a dramatic effect on mineralization of plant material entering the soil ecosystem (Table 2.2). Any process increasing the surface area of the debris before incorporation into the soil increases the decomposition rate. In tropical regions, the microfauna play a lesser part in plant material decomposition and primarily microbes execute this function.

These organisms also play a more direct role in mineralization and biochemical conversions. A variety of soil invertebrates, isopods, millipedes, snail, slugs and earthworms metabolize cinnamic acid and vanillin, both decomposition products and precursors of lignin, to carbon dioxide. Synthesis of humic substances is possible through the action of invertebrate synthesized peroxidases. These enzymes are involved in the polymerization of aromatic compounds associated with humification. Organisms that feed on the microbes responsible for most of the organic matter

turnover and mineralization (bacteria, fungi and actinomycetes), do not affect the total carbon metabolized, but they do affect the kinetics of decomposition: they act to maintain the dynamic nature of the nutrient cycles.

Table 2.2: Role of soil microfauna in mineralization of plant material (Paul and Clark, 1989).

1. Physical mixing of the organic matter within the soil profile
2. Inoculation of plant litter with decomposer populations
3. Adjustment of the soil physical properties
4. Physical disintegration of organic matter
5. Direct metabolism of the organic matter
6. Stimulation of decomposer populations

2.2 Role of soil microflora

The biological catalysts of soil organic matter reactions are soil enzymes originating from soil microbes. The diversity in sources of plant derived organic matter as well as the complexity of these substances result in extreme heterogeneity in the microbial reactions involved in its decomposition (Tate, 1987). These organic carbon components range from easily degradable components of the cytoplasm to the more recalcitrant components of the cell wall. Cell wall components also vary in biodegradability from the readily metabolizable celluloses and hemicelluloses to more resistant lignin. The mechanisms of lignocellulose degradation by microorganisms will be discussed in detail in sections 3, 4 and 5.

2.3 Humification processes

Humification is the biological, microbial, or chemical conversion of organic residues to humus (Tate, 1987). Humus is the relatively biodegradation resistant, predominantly dark brown to black, fraction of soil organic matter. The major sources of organic compounds are plant and animal components incorporated into soil, the

organic intermediates formed during the decomposition of these biological remains, and microbially synthesized products and biomass.

Plant chemical composition may affect the humification rate of the residues. High polyphenolic content may increase the potential for humification (Tate, 1987). Organic compounds containing more reactive groupings are also more likely to be humified than less reactive compounds. Many substances become associated with humic substances through non-covalent bonding mechanisms (hydrogen bonding, ionic bonding, Van der Waals forces), but the greatest stabilization results from formation of covalent associations (Stevenson, 1982). Polysaccharides are linked to humic substances via ester linkages, whereas proteins become associated with humic acids through peptide bonds, direct binding to aromatic rings, and linkage to quinone rings. These interactions need not be dramatic, for minor changes in the molecular structures of simple organic compounds can reduce the mineralization rate drastically.

Because of structural and chemical similarities, lignin has long been considered to be a humic acid precursor (Stevenson, 1982). The complexity of its components is amplified by the high degree of randomness of the polymer as well as the extreme variation in molecular composition of lignins isolated from various plant sources (Tate, 1987). The diversity of the enzymes necessary for its decomposition, and the high energy investment required by the microbe before it gains energy for cellular growth contribute to the long half-life of lignin in soil. The mechanisms involved in lignin biodegradation and the organisms involved will be discussed in section 4. However, many theories have been proposed to explain humic acid synthesis. The lignin theory, polyphenol theory, microbial synthesis theory and the involvement of sugars and amino acids in Maillard reactions are just a few of such attempts to explain this phenomenon (Tate, 1987; Stevenson, 1982).

New life was breathed into the proposal that lignin is the precursor of humic compounds by Shevchenko and Baily (1996). Their hypothesis assumes functionalization and restructuring but not complete depolymerization of lignin during its biotransformation into humic and fulvic acids in the environment. The biotransformation process results in the preservation of certain structural features

during the humification of dead plants. They used a genetic approach to analyze the structure, morphology and chemical reactivity of the humic compounds. The microbial synthesis theory also received support recently. Requena *et al.* (1996) indicated that the nature of the humic substances produced after incubation appeared to depend greatly on the degradation pathway carried out by the inoculants, *Trichoderma viride* and *Bacillus* spp.

2.4 Kinetics of plant organic matter decomposition

2.4.1 First-order reaction kinetics

Experimental results indicated that decomposition of plant material is reasonably well described by first-order rate kinetics (Paul and Clark, 1989). This means that the decomposition of plant residues is linearly proportional to the plant residue concentration, but the specific rate constant k is independent of the residue content. The decomposition rate described the effect of the overall reaction, while the rate constant k is a characteristic of the type of plant residue undergoing decomposition.

In first-order reactions, the rate of transformation of a substrate is proportional to the substrate concentration (Paul and Clark, 1989; Campbell, 1995).
During the following reaction

$$\text{A (substrate)} \rightarrow \text{X (product)} \qquad (1)$$

The rate of change of reactant A is

$$\frac{dA}{dt} = -kA \qquad (2)$$

The equation implies the following: the decrease of the reactant (A or the plant material to be decomposed) with time t is dependent on the rate constant k times the concurrent concentration A of the reactant. In other words, the observed rate of decomposition depends on the rate at which the particular substrate can be degraded and the available concentration of the substrate.

Integration of the last equation yields the following equation

$$A_t = A_o e^{-kt} \quad (3)$$

where A_t is the concentration of the substrate remaining at any time t. The rate constant k must be expressed per unit time. To really get an idea of the impact of the rate constant on the overall decomposition rate, one can manipulate the last equation to facilitate graphic determination of the rate constant k:

$$\ln (A_t/A_o) = -kt \quad (4)$$

A plot of this equation versus time yields a linear function with a slope of $-k$ (Figure 2.8). The equation can also be plotted in log 10 which changes the slope to $-k$ / 2.303 (Figure 2.8). In this graph, the rate constant k is independent of the substrate concentration since the slope is constant over time. To calculate the time required to transform one-half of the initial substrate, the following equations can be used:

$$\ln \frac{(A_o/2)}{A_o} = -kt_{1/2} \quad (5)$$

which is $t_{1/2} = 0.693/k$. The mean residence time (turnover time) for first-order reactions is $1/k$.

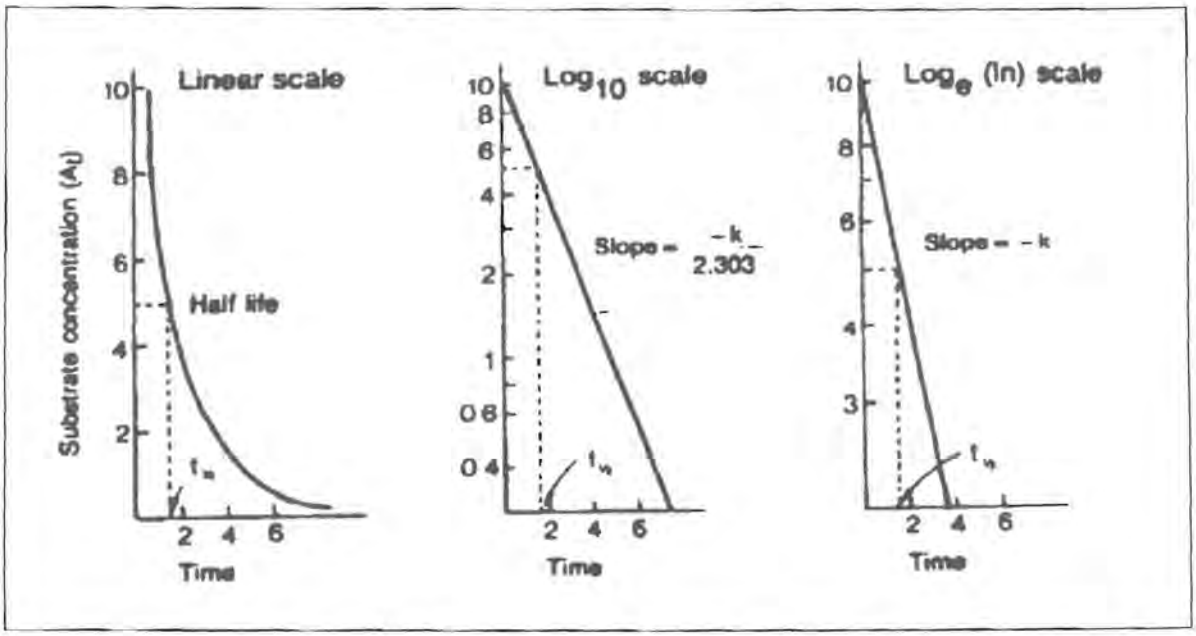

Figure 2.8: First-order function $A = 10e^{-0.5t}$, plotted on linear, $\log_{10}$ and ln scales (Paul and Clark, 1989).

2.4.2 Decomposition kinetics

The general kinetics of biomass decomposition suggests a biphasic process: a rapid catabolic period followed by a slow carbon dioxide evolution period (Figure 2.9). The initial rapid carbon dioxide evolution rate results from decomposition of readily metabolizable substrates such as proteins, amino acids, simple sugars and polysaccharides. Some carbon dioxide released during the slower degradation phase originates from polymers synthesized by the biomass itself during the initial decomposition phase (Tate, 1987). The total degradation of compost piles, as determined by the weight loss of organic matter, accounted for 64% of the organic matter applied and followed a first-order kinetic function (Bernal *et al.*, 1996).

Jenkinson and Rayner (1977) determined decomposition kinetics for succulent green plant material. Uniformly labeled ryegrass was incubated with different soils. Two-thirds of the carbon was lost as CO_2 during the initial year of incubation (Figure 2.9). The carbon remaining in the soil could be detected as non-metabolized plant constituents and newly synthesized microbial biomass and products.

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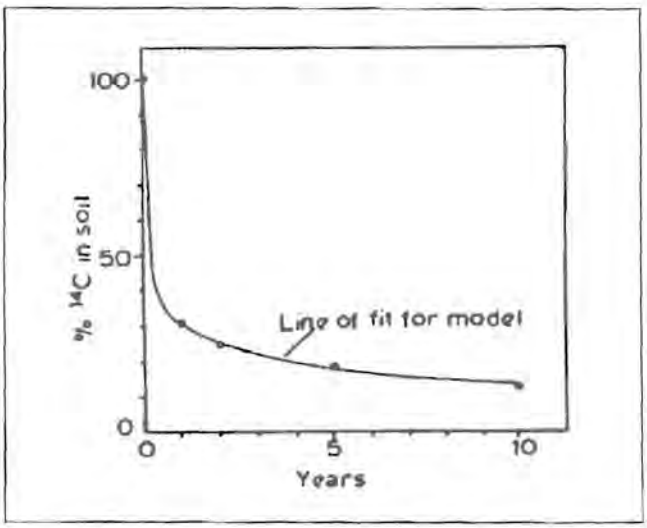

Figure 2.9: Decomposition of uniformly labeled ryegrass incubated in soil in the field (Jenkinson and Rayner, 1977).

Murayama (1984) found that a first-order kinetics model represented rice and barley straw saccharide decomposition rates under field conditions:

$$Y_t = C_1 e^{-K_1 t} + C_2 e^{-K_2 t} \qquad (6)$$

where Y_t is the residue remaining in soil at time t, k_1 and k_2 are the decomposition rate constants for the labile fraction (C_1) and the non-labile fraction (C_2). About 82% of the total saccharide content of the rice straw was in the labile fraction with a rate constant of 0.64 to 0.81. The non-labile fraction had a half-life of 9 to 59 months.

Similar decomposition kinetics is observed for highly lignified wood substrates, except the rates and extent of metabolism is reduced. The differences in decomposition rates of grasses and woods indicate the susceptibility of plant components to biodegradation varies with physical and chemical properties of the plant itself (Tate, 1987). These properties include the carbon:nitrogen ratio, lignin content and to some degree the surface area of the substrate. The mechanism of decomposition inhibition by lignin is attributed to steric hindrance of microbial and

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enzymatic interaction with the more metabolizable molecules. Plant materials with a high carbon:nitrogen ratio do not provide sufficient nitrogen for metabolism of the decomposer population under conditions of rapid microbial activity.

Research experience has shown that the decomposition rate constant *k* (describing the decomposition of plant material) is nearly always independent of the quantity added if the carbon addition does not exceed 1.5% of the soil dry mass. Higher addition rates can alter the soil characteristics and slow down decomposition (Paul and Clark, 1989).

2.4.3 Modeling plant decomposition data

Only a portion of the actual decomposition is accounted for when determining the decomposition rate by measuring CO_2 output or the amount of carbon left in the soil (Paul and Clark, 1989). The microorganisms utilize carbon compounds for biosynthesis and energy and during these processes, the carbon compounds are converted mostly to CO_2, biomass and waste products. Under aerobic conditions the production of waste products is not high. This enables us to calculate the amount of biosynthesis from the CO_2 data. This requires knowledge of yield or efficiency of substrate conversion to microbial biomass:

$$C = C_I [\, 1 + Y/(100-Y) \,] \qquad (7)$$

where C is the substrate decomposed, C_I the CO_2 evolved and Y the efficiency of the use of carbon for biosynthesis, expressed as a percentage of the total carbon utilized for production of microbial material.

Experience has shown that one makes a serious mistake when assessing decomposition rates of amendments without accounting for microbial biomass synthesis of a proportion of the utilized substrate. Therefore, the decomposition rate constant *k* must be corrected for biosynthesis to reflect true decomposition values (Paul and Clark, 1989).

Knapp *et al.* (1983a, 1983b) investigated the microbial decomposition of wheat straw. The decomposition was nitrogen limited during the initial decomposition period and

carbon limited after prolonged incubation. During the initial decomposition period when the microbes metabolised carbon-rich/nitrogen-poor substrates (such as carbohydrates) straw metabolism was stimulated by amendment with mineral nitrogen. As the readily decomposed carbon pool was exhausted and the microbial metabolic rate declined, thereby reducing the nitrogen demand, sufficient nitrogen was contained in the substrate for continued catabolism of the more resistant plant components. Because of the greater energy expenditures necessary to metabolize these substrates, the microbes at that point became carbon-limited. Therefore, amendment of the soil with carbon stimulated microbial respiration.

Decomposition curves for complex substrates, such as straw, usually yield a multislope decomposition curve (Paul and Clark, 1989). This indicates that straw consists of several components having different decomposition rate constants. Van Veen *et al.* (1984) constructed a model for the carbon and nitrogen turnover through microbial biomass in soil (Figure 2.10).

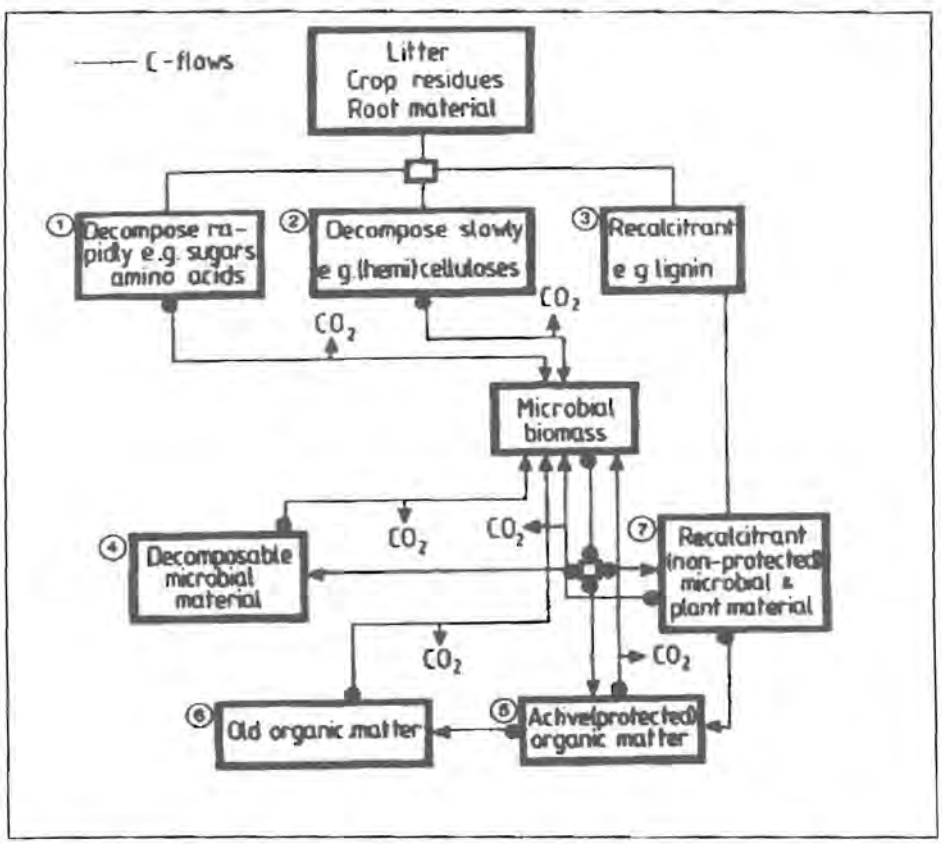

Figure 2.10 : Model describing decomposition of carbon in plant residues and the turnover of soil organic matter constituents. Numbers in circles will be discussed in Table 2.3 (Paul and Clark, 1989; Van Veen *et al.*, 1984).

Table 2.3: Pool sizes, decomposition rates, and efficiency of microbial production. These values were used to compile a carbon turnover model describing the decomposition of 1000 g carbon per gram of soil. (Paul and Clark, 1989; Van Veen *et al.*, 1984).

Pool	Residue carbon ($\mu g\ g^{-1}$ soil)	Decomposition rate k ($days^{-1}$)	Utilization efficiency (%)
Easily decomposable (1)*	150	0.2	60
Slowly decomposable (2)	650	0.08	40
Lignin (3)	200	0.01	10
Decomposable microbial products (4)*	6	0.8	40
Active protected SOM (5)	5000	3×10^{-4}	20
Old organic matter (6)	7000	8×10^{-7}	20
Recalcitrant plant and microbial products (7)*	4	0.3	25

SOM = Soil Organic Matter

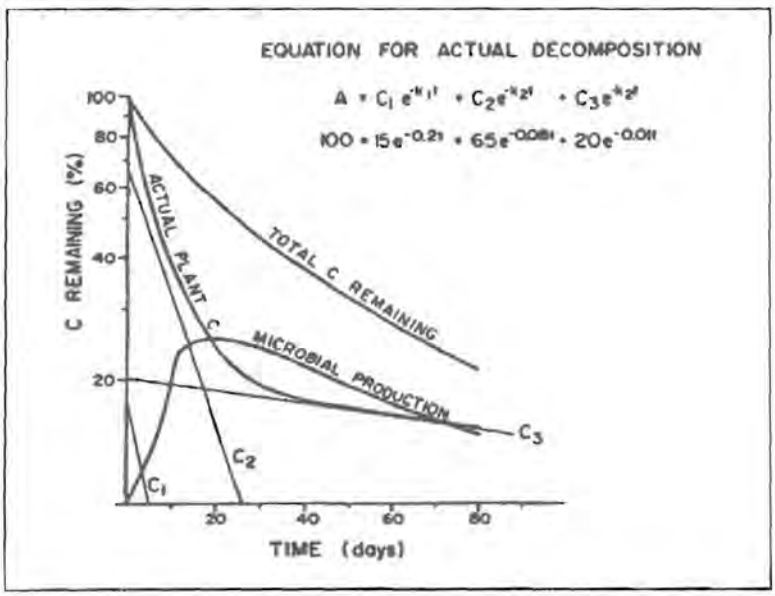

Figure 2.11: Decomposition of straw carbon (C) in the laboratory, plotted as a series of first-order reactions after correction for microbial production. The actual plant carbon remaining as proteins and solubles (C_1), cellulose and hemicellulose (C_2), and lignin (C_3) is much lower than the total carbon remaining. The equation for actual decomposition shows the initial carbon content of each component and the decomposition rate k. (Paul and Clark, 1989; Van Veen *et al.*, 1984).

The model was compiled from the following assumption: the complex substrate was considered to consist of three fractions: 1) easily decomposable sugars and amino acids, 2) slowly degradable cellulose and hemicellulose and 3) resistant lignin. However, knowledge of the decomposition rate constant k and microbial growth efficiencies were required to determine each of the flows indicated in Figure 2.10. These values are shown in Table 2.3. The model described in Figure 2.10 and Table 2.3 was based on decomposition rate constant determined under laboratory conditions and provided output curves as shown in Figure 2.11. The model output based on first-order kinetics, showed large differences between the true decomposition after correcting for microbial growth.

The kinetics of wheat straw solid state fermentation with *Trametes versicolor* and *Pleurotus ostreatus* were investigated by Valmaseda *et al.* (1991). Principle component analysis revealed that two phases could be defined during fungal solid state fermentation of wheat straw: a colonisation phase and a degradation phase (Figure 2.12).

The colonisation phase was shown by the displacement on the second axis, from the control values until this phase ended after 7 d to 10 d (Figure 2.12A). The colonisation phase was indicated by the next set of reactions, characterised by the displacement along the first axis (Figure 2.12A). This was in agreement with the biphasic decomposition of biomass as proposed by Jenkinson and Rayner (1977) (Figure 2.9).

During the initial colonisation phase, free sugars were available but were rapidly metabolised by the fungi (Figure 2.12B, bottom left sector). A few days later, xylan and arabinan were present in significant quantities (upper part of bottom left sector). Klason lignin is the insoluble material remaining after sample digestion with sulphuric acid and heat. Although this method indicates a slightly lower lignin content of the substrate, it is a good indicator of lignin loss (Deobald and Crawford, 1997).

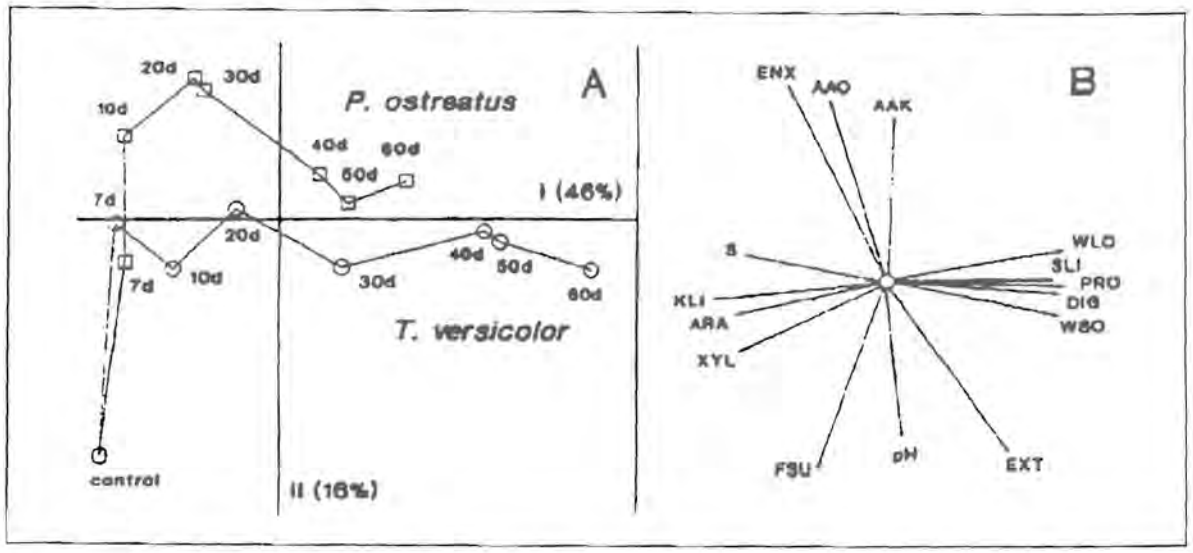

Figure 2.12: Principle component analysis (PCA) of wheat straw solid-state fermentation. The position of the samples from 0-60 d is shown in A, and the variable with the highest loading factors on the two first axis in B. Variables: AAK, Acid to (aldehyde + ketone) ratios; AAO, aryl alcohol oxidase; ARA, arabinan; DIG, digestibility; ENX, xylanase; EXT, extractives; FSU, free sugars; KLI, Klason lignin; pH; PRO, protein; S, syringyl content; SLI, soluble lignin,; WSO, water soluble; WLO, weight loss; XYL, xylan (Valmaseda *et al.*, 1991).

Therefore, the free sugar, xylan and arabinan levels were most significant when the inherent lignin levels were also most significant. Therefore, no lignin degradation has occurred during the colonisation phase. The next sector, upper left, initially indicated fungal transformation of lignin side chains. The evidence was the increase in aromatic acid yield (syringyl content, S in Figure 2.12B) from the transformed straw. No significant enzymatic activities were detected that could explain this phenomenon. Additional enzymatic activities might be present, yet they remain unknown. Xylanase and aryl alcohol oxidase levels were significant after 20 d to 30 d, especially for *P. ostreatus*. This was an indication that these inducible enzymes were active at optimal levels because sufficient substrate was available in the form of readily metabolisable hemicellulose components. After 40 d, the increase in acid to (aldehyde + ketone) ratio was significant (top right-hand sector). Side-chain modification in residual lignin was characteristic of *P. ostreatus*, preferentially degrading lignin. At this time, the lignin polymer was degraded to medium or short chain fragments, exposing the

hemicellulose and cellulose to their degrading enzymes. The final result of solid state fermentation of wheat straw with *P. ostreatus* and *T. versicolor* was indicated in the adjoining regions of the top and bottom right-hand sectors. At this stage, the wheat straw was significantly saccharified, yielding increased levels of soluble lignin (SLI), fungal protein (PRO) and digestibility (DIG). An important feature of the original substrate was significant weight loss (WLO) over 60 d and increased water solubility.

Although microflora plays a substantial part in the decomposition of plant organic matter in soil, the soil microorganisms are by far the primary decomposers. Therefore, the natural microbial population dictates natural degradation processes involving plant organic matter. However, the chemical recalcitrance of lignin is the rate-limiting factor in plant organic matter decomposition. Humification of recalcitrant plant organic matter occurs in soil and can delay the further degradation of organic polymers. Decomposition of plant organic matter under natural or laboratory conditions follows a biphasic trend: the first phase is characterized by rapid degradation of easily utilizable compounds, whereas the second phase is characterized by slower degradation rates of recalcitrant organic polymers. Correction of decomposition data for microbial biomass increases should be performed to determine the actual degradation values.

3. Cellulose and hemicellulose biodegradation

The efficient hydrolysis of cellulose requires the interaction of several enzymes: 1) endoglucanases randomly cleave intermonomer bonds; 2) exoglucanases removes monomer and dimer units from the end of the glucose chain; 3) cellobiase hydrolyzes glucose dimers (Deobald and Crawford, 1997; Tomme *et al.*, 1995). The principle of cellulose degradation is an initial loss of the crystalline structure followed by depolymerization (Figure 2.13). The glucose monosaccharides then enter energy-generating metabolic pathways.

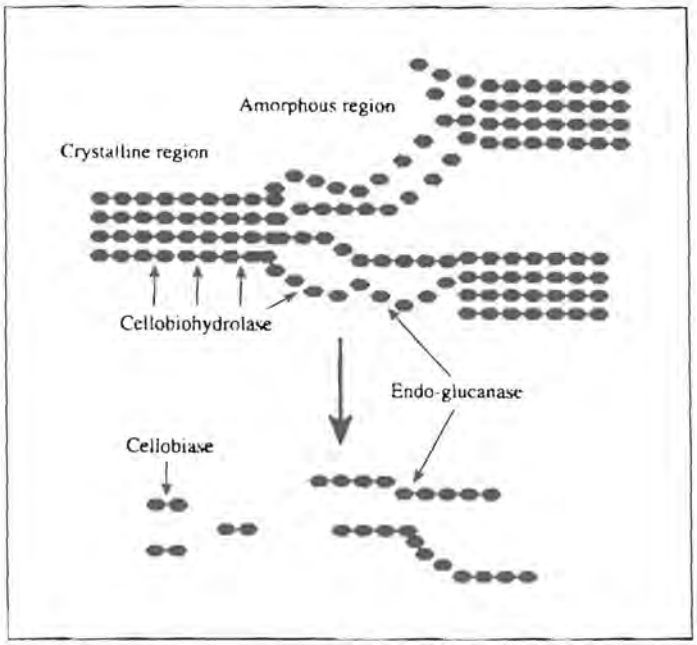

Figure 2.13: Schematic representation of the degradation of a cellulose chain (Olsen, 1995).

Three basic types of enzymes are required for the degradation of hemicellulose (Deobald and Crawford, 1997). Because of its greater complexity more enzymes are required for its complete degradation, of which xylanase is the best studied. The hemicellulose degradation products fuel energy-generating metabolic activities in microorganisms. Water-soluble LCCs often precipitate during digestion with polysaccharidases, and the residual sugars are more diverse than the bulk hemicellulose (Jeffries, 1990).

Its close association with other polymers in nature affects the degradation of cellulose and hemicellulose. Hemicellulose physically restricts the access of ligninases to lignin (Tuor *et al.*, 1995). This close association of lignin and hemicelluloses suggests that the primary attack of the wood cell wall requires degradation of the hemicellulose prior to lignin degradation: either the wood rotting fungi secrete hemicellulases or the fungal lignin degradation is preceded by hemicellulolytic activity of bacterial consortia. Hydrolyzation or depolymerization of the hemicellulose component would render lignin accessible for lignin peroxidases and Mn-dependent peroxidases (Tuor *et al.*, 1995).

After cellulose depolymerization the glucose monosaccharides enter energy generating metabolic pathways. The most common pathway for the oxidation of glucose is the Embden-Meyerhof-Parnas (EMP) pathway of glycolysis (Figure 2.14). This pathway is operational in many bacteria and fungi, but alternative glucose utilization strategies and metabolic pathways exist. The complete oxidation of one glucose molecule via the EMP pathway and the tricarboxylic acid cycle (TCA) (Figure 2.15) yields 38 adenosine triphosphate (ATP) molecules. Similar energy yielding metabolic pathways exist for the utilization of hemicellulose degradation products.

The primary goal of the biodegradation of any compound in nature by microorganisms is to obtain energy for survival. Cellulose and hemicellulose represents readily biodegradable carbon sources that can be utilized completely by natural microbial consortia.

4. Microbiological degradation of lignin

4.1 Ecology of lignocellulose degradation

Fungi and bacteria are well capable of cellulose degradation. Polysaccharide degrading enzymes from these organisms play a synergistic role in the removal of *p*-coumaric and ferulic acids from the lignocellulose matrix (Borneman *et al.*, 1990). Fungi (*Trichoderma, Chaetomium* and *Penicillium*) and bacteria (*Pseudomonas, Chromobacterium, Bacillus, Clostridium, Streptomyces* and *Cytophaga*) comprise cellulolytic enzyme systems to different extents and activities (Paul and Clark, 1989). Under laboratory conditions using sterilized substrates, lignocellulose biodegradation by white-rot fungi was characterized as a race between cellulose and lignin degradation (Reid, 1989a). Hemicellulose degradation by *Phanerochaete chrysosporium, Phlebia brevispora* and *Cyathus stercoreus* was recently studied (Chen *et al.*, 1995). *P. chrysosporium* degraded 69% of the cellulose within 28 days whilst the other fungi degraded 16% of the cellulose. Enhanced digestibility may have resulted from cleavage of lignin-carbohydrate bonds.

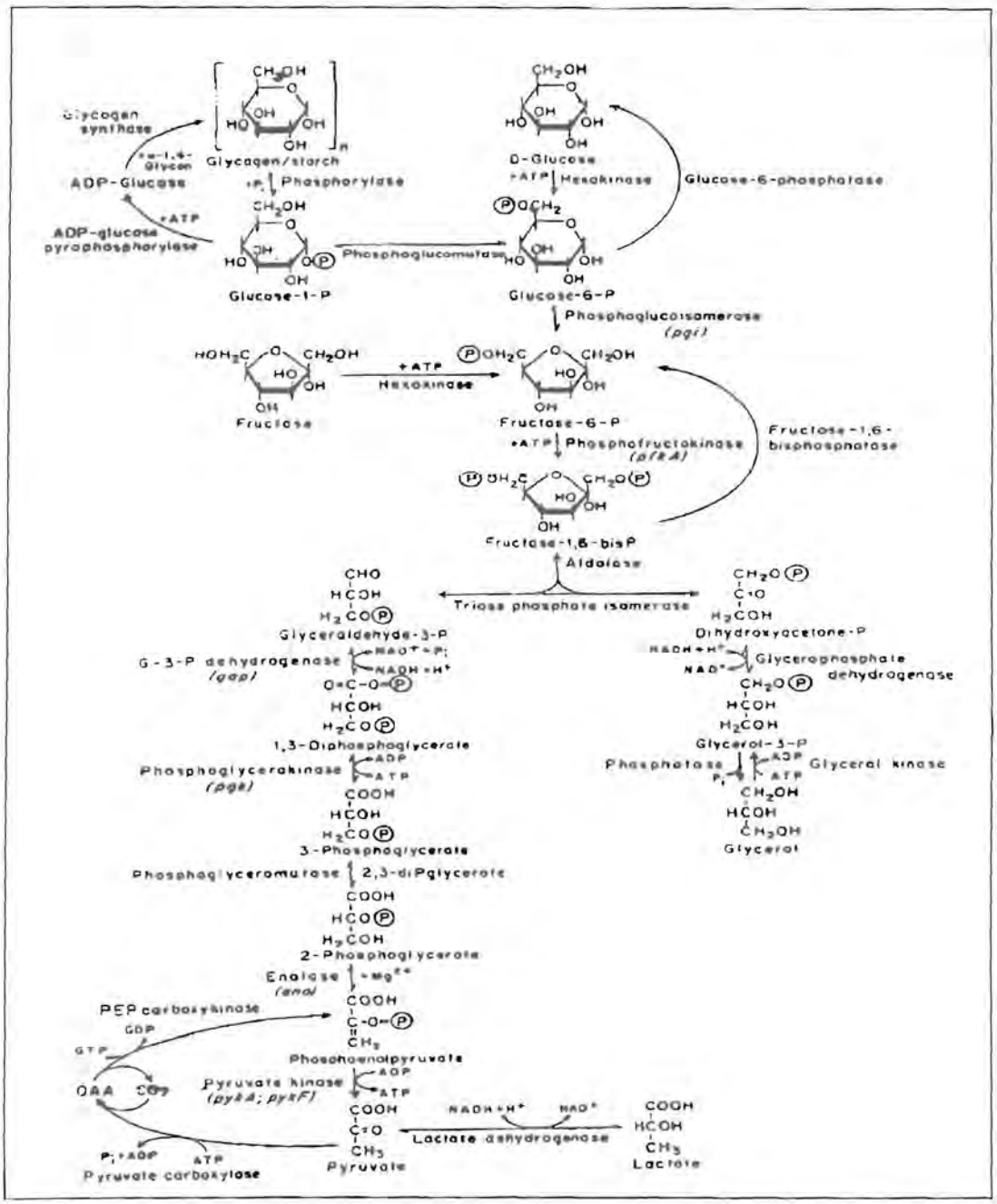

Figure 2.14: The Embden-Meyerhof-Parnas (EMP) catabolic pathway of glycolysis (Moat and Foster, 1995).

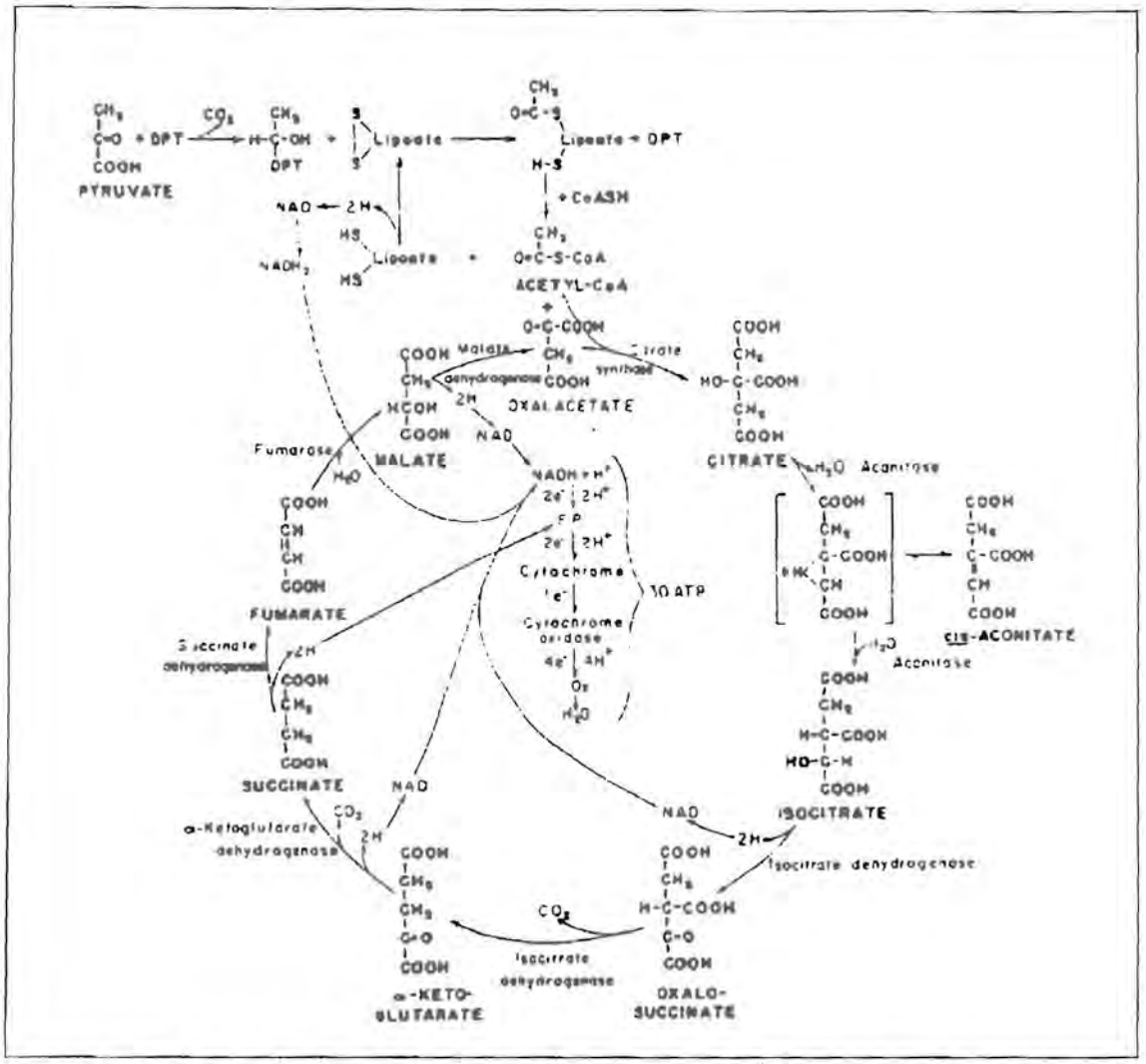

Figure 2.15: The tricarboxylic acid (TCA) cycle (also known as the Krebs cycle) and abbreviated terminal respiration system (Moat and Foster, 1995).

The race between lignin and cellulose degradation is even more extensive in nature. Fungi already present in the soil (called autochthonous fungi) quickly colonise decomposing plant materials. Most autochthonous fungi can degrade polysaccharides (Dix and Webster, 1995). Nevertheless, *Trichoderma* and *Penicillium* species caused little weight loss of plant material in monocultures. These fungi may not directly cause decomposition, but can synergistically increase rates of decay. In Europe, surface litter decays at a slower rate than in deeper layers. Basidiomycetes are more active in these deeper layers. They comprise powerful hydrolases and enzymes capable of detoxifying litter phenolics (Dix and Webster, 1995). The situation becomes more complex when fungi with restricted catabolic capabilities develop mutualistic

relationships with, and flourishing alongside species decomposing cellulose and lignin. Micro-organisms unable to degrade the lignin polymer can obtain energy from the low molecular weight intermediates released from the degradation of lignin by true white-rot fungi. A similar situation arises where competent fungi degrade cellulose.

Due to its abundance and considerable recalcitrance, the decomposition of lignin may be a rate-limiting step in the carbon cycle (Elder and Kelly, 1994). The complexity of the lignin polymer and the diversity of the subunits and linkages between the subunits predict requirements for a number of different enzymatic activities. Microorganisms exhibit different lignolytic mechanisms and capabilities. These will now be discussed in more detail.

4.2 Fungal biodegradation of lignin

4.2.1 Patterns of wood decay

Fungi can be classified according to the type of rot they cause in wood tissues. Soft rot fungi efficiently attack carbohydrates, but modify lignins only to a limited extent (examples: *Chaetomium cellulolyticum, Aspergillus niger, Trichoderma viride, Fusarium oxysporum, Penicillium* spp. etc.). Brown rot fungi exhibit preference for cellulose and hemicellulose, lignins being degraded to a limited extent (examples: *Poria placenta, Gloeophyllum trabeum, Lentinus lepidius* etc.). These fungi can cause extensive and rapid decay of cellulose early in the decay process. Brown rot fungi differ from white rot fungi with respect to the cellulolytic enzymes produced and the pattern of cellulose degradation. White rot fungi can attack any wood component to any extent (examples: *Phanerochaete chrysosporium, Pleurotus ostreatus, Ceriporiopsis subvermispora* etc.). The normal pattern of wood decay involves simultaneous attack on polysaccharides and lignin. The selectivity of polymer degradation differs between genera, species and even isolates. *Phanerochaete chrysosporium* and *Trametes versicolor* have an unselective decay pattern while *Phlebia tremellosa, Ceriporiopsis subvermispora, Pleurotus ostreatus* etc. degrade the lignin component more selectively (Chen *et al.*, 1995; Kuhad *et al.*, 1997).

4.2.2 Lignin degrading enzymes

Lignin degradation by white-rot fungi is oxidative in nature and phenol oxidases are the key enzymes (Kuhad *et al.*, 1997; Leonowicz *et al.*, 1999). Lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) oxidise the substrate by two consecutive one-electron oxidation steps with intermediate cation radical formation. Laccase has broad substrate specificity and oxidises phenols and lignin substructures with the formation of radicals. Laccase is generally considered to have a redox potential too low to allow for oxidation of non-phenolic structures. Feruloyl and *p*-coumaroyl esterases are novel enzymes capable of releasing feruloyl and *p*-coumaroyl groups from grass cell walls and their methyl esters.

Phenolic acid esterases play an important role in biodegradation of recalcitrant cell walls in grasses (Kuhad *et al.*, 1997). These enzymes accurately attack and penetrate the lignocellulose matrix without mineralization of lignin *per se*. These enzymes are responsible for selective hydrolysis of ester bonds between L-arabinosyl residues and ferulic or *p*-coumaric acids. The cinnamoyl ester hydrolase in *Piromyces equi* showed a marked preference for cleaving 1,5 ester linkages between ferulic acid and arabinose in feruloylated arabino-xylo-oligosaccharides (Fillingham *et al.*, 1999). This enzyme acted synergistically with xylanase to release more than 60% of the esterified ferulic acid from the arabinoxylan component of plant cell walls.

Anaerobic fungi (*Piromyces, Neocallimastix, and Orpinomyces*) are part of the natural microflora within the rumen. These fungi produce a wide range of active hydrolytic enzymes, notable cellulases and xylanases that provide them with the potential to degrade major structural polysaccharides (Hodrova *et al.*, 1998). Their cellulases are among the most active reported to date and solubilize both amorphous and crystalline cellulose (Wubah *et al.*, 1993). Their esterases are active against both feruloyl and *p*-coumaroyl arabinoxylans, which provides an advantage in degrading poorly biodegradable cell walls. Lignin is degraded but not metabolised. Rhizoids of vegetative thalli penetrate cell walls, and they are better able than bacteria or protozoa to attack recalcitrant tissues and weaken textural strength of plant material (Wubah *et al.*, 1993).

4.2.3 Biological pretreatment agents to be evaluated

The purpose of biological pretreatment of the Buffelsgrass will be to obtain cellulose enriched substrate with simultaneous removal of the lignin barrier. Therefore, the ideal organism will be able to accurately remove the lignin without causing severe polysaccharide loss. This was the primary criterion for selecting the fungal species to be evaluated in this study. Another very important consideration was the ability of these species to establish themselves on natural lignocellulose substrates and compete effectively with the naturally occurring microorganisms, since no substrate pasteurization facilities will be available at the AMD affected site. A third consideration was the relative ease of propagation and maintenance of these organisms in pure culture. These organisms do not have complex nutritional requirements and this makes large-scale inoculum production less complicated. Finally, much information is available on the physiology, genetics and metabolism of these organisms. *Phanerochaete chrysosporium* and *Pleurotus ostreatus* are well-studied model decomposers of wood and grass lignocellulose. *Schizophyllum commune* is frequently observed on decaying trees and is frequently included in lignocellulose degradation studies.

- ***Phanerochaete chrysosporium***

Phanerochaete chrysosporium is the model organism for studying the mechanisms involved in lignocellulose biodegradation on a chemical and molecular level. Ease of cultivation and application make this fungus a popular choice for biopulping and molecular biology projects.

Phanerochaete chrysosporium has a non-selective mode of lignocellulose degradation (Kuhad *et al.*, 1997). It degrades cellulose, hemicellulose and lignin non-specifically and causes large dry mass losses over a short period of time. This well-known lignolytic fungus strongly hydrolyses straw polysaccharides and fibres (Camarero *et al.*, 1998). Non-selective degradation of organic material caused a decrease in *in vitro* dry matter digestibility (Agosin *et al.*, 1985b; Karunanandaa *et al.*, 1992; Kerem *et al.*, 1992).

When compared to other fungi, *P. chrysosporium* caused greater dry mass loss of the original substrate: 40% on cotton straw (Platt *et al.*, 1984), 52% on sugar cane bagasse (El-Gammal *et al.*, 1998), and 55% on wheat straw within 15 d (Kerem *et al.*, 1992). However, strains of this species can differ in their severity of substrate degradation and substrate specificity (El-Gammal *et al.*, 1998).

Bacteria from polluted and agricultural soils antagonised the growth of *P. chrysosporium* on solid media. Pseudomonad isolates capable of producing phenazine derivatives were strongly inhibitory (Radtke *et al.*, 1994). A problem with the biopulping process is that poor colonisation of the freshly chipped wood chips often occurred (Wolfaardt *et al.*, 1999). It has been shown that *P. chrysosporium* can successfully biopulp wood without the need for sterilisation of the wood (Wall *et al.*, 1993). Yet the random degradation of wood components makes it unsuitable for biopulping projects.

Antagonism by soil bacteria and random degradation of lignocellulose components should be considered when using *P. chrysosporium* since this may impede the effectivity of the organism. The advantages of using this organism are ease of handling and inoculum preparation. This organism has been shown to successfully biopulp wood without the need for surface sterilisation (Wall *et al.*, 1993). Some strains might have a more specific lignocellulose degradation mechanism.

- ***Pleurotus ostreatus***

Pleurotus ostreatus has been cultivated on a wide range of lignocellulosic substrates and the end products of solid state fermentation of these substrates have been used for papermaking, upgrading of animal feed stuffs and production of edible mushrooms for human consumption (Hadar *et al.*, 1992).

Chemical analysis and electronmicroscopy revealed the potential of *Pleurotus* species for selective removal of lignin from straw; resulting in a separation of fibres (Camarero *et al.*, 1998). Laccase, MnP and aryl-alcohol oxidase was detected in *P.*

ostreatus (Pelaez *et al.*, 1995), but not all lignolytic strains exhibited laccase activity (Hadar *et al.*, 1992).

The process of substrate colonisation and utilisation by *P. ostreatus* has been well studied. It was possible to distinguish two separate phases in the degradation of wheat straw: a colonisation phase (lasting 7-10 d) and a degradation phase (Valmaseda *et al.*, 1991). The first phase involved the degradation of small, water-soluble materials and high activity of cellulase, phenol oxidases, and peroxidase. Phase two was characterised mainly by lignin degradation (Platt *et al.*, 1984). During growth of *Pleurotus* on cotton straw, both the straw in general and the lignin in particular were degraded. After 4 d of fungal growth, activity of laccase, catechol oxidase, peroxidase, and cellulase were detected. This activity declined rapidly after 8-10 d of growth. Lignin degradation began after 10 d and reached a maximum after 21 d. Kerem *et al.* (1992) observed selective lignin removal with degradation of 20% of the organic matter after 30 d of incubation.

Treatment of *P. ostreatus 'florida'* straw with cellulase released 4 times as much glucose, showing increased availability of cellulose (Platt *et al.*, 1984). Hadar *et al.* (1992) found that highest digestibility of wheat straw was obtained at 22 d of incubation with *P. ostreatus* and this value did not change much until the end of the experiment on day 42. Also, at 22 d, a substantial amount of cellulose was still left, but much was degraded by day 42. Much mineralization of organic matter was observed by day 42 (Hadar *et al.*, 1992).

The survival of *Pleurotus ostreatus* on natural substrates seems better compared to *Phanerochaete chrysosporium*. *Pleurotus ostreatus* was highly competitive with soil microbiota compared to *Ganoderma applanatum* and *Dichomitus squalens* (Lang *et al.*, 1997). Neither growth nor the production of laccase and manganese peroxidase (MnP) was markedly affected by the soil microbiota.

This fungus contains positive attributes that make it a good choice for biological delignification. *P. ostreatus* is cultivated on grass lignocellulose for the production of edible mushrooms. Studies showed that the lignin barrier was selectively removed

with much of the potentially digestible cellulose remaining intact. The ability to compete with naturally occurring micro-organisms makes it a preferred choice for biological pre-treatment of unpasteurized grass lignocellulose.

- ***Schizophyllum commune***

Schizophyllum commune is a saprophytic fungus common on decaying wood. This organism does not mineralise lignin, instead modification seems to occur (Boyle *et al.*, 1992). Known lignocellulose degrading enzyme activities include aryl-alcohol oxidase (Pelaez *et al.*, 1995), xylanase, cellulases and hemicellulases (Haltrich *et al.*, 1995; Mankel and Kothe, 1999). Peculiar features include the secretion of all the β-glucosidase activities (Willick *et al.*, 1984), cellobiose dehydrogenase with distinct and specific affinity for cellulose (Fang *et al.*, 1998) and ferulic acid esterase with novel substrate specificity (Jeffries, 1990). In several studies, *S. commune* was compared to the best-studied white-rot fungi to date (Boyle *et al.*, 1992; Platt *et al.*, 1984). In every instance, in terms of lignolytic enzyme capabilities, dry mass loss and specific degradation of lignin, the other fungi outperformed *S. commune*. Why then do researchers regularly include this organism in research projects?

If the purpose of lignocellulose treatment with white-rot fungi is to obtain a cellulose-enriched substrate, then the ideal fungus will cause accurate lignin degradation without severe polysaccharide loss. *S. commune* seems to be underpowered in terms of known enzymatic capabilities, but it is frequently observed in nature on decaying wood. Previous studies have shown that some strains can be highly effective lignin degraders (Valmaseda *et al.*, 1990).

Unique esterase enzymatic capabilities warrant the inclusion of *S. commune* in this study. This organism is a slower grower in pure culture compared to *P. chrysosporium* and *P. ostreatus*, but this enables it to compete effectively for nutrients over extended time periods. It is frequently observed on decaying plant material and therefore has some survival strategy that enables it to colonise the substrate, compete with naturally occurring micro-organisms, and effectively utilise the substrate.

4.3 Lignin biodegradation by actinomycetes

Actinomycetes are the best-studied prokaryotic lignin degraders with grass lignocellulose being more susceptible to actinomycete attack than wood (Antai and Crawford, 1981; McCarthy, 1987; McCarthy and Ball, 1991; Tate, 1987). Actinomycetes are actively involved in the natural humification process by solubilisation of the lignocellulose substrate. The lower levels of lignin degradation by actinomycetes compared to fungi may allow this group of organisms to play a significant role in humification processes in natural soils and composts (Trigo and Ball, 1994). Lignin–solubilizing actinomycetes have been isolated from the termite hindgut (Pasti *et al.*, 1990).

We did not include actinomycetes in our selection of pretreatment agents because of the lower levels of lignin degradation compared to fungi. White-rot fungi are the most capable degraders of lignin. Lignin degradation by actinomycetes is of ecological significance, but lignin degradation by white-rot fungi is of commercial importance.

4.4 Bacterial lignin degradation

Bacterial degradation of lignocellulose is very slow and takes place on surfaces with a high moisture content. Because of the lack of penetrating ability, bacteria usually invade wood cells simultaneously with fungi (Kuhad, *et al.*, 1997).

Bacteria appear to be the key organisms in lignin mineralization (Tate, 1987). Bacteria possess intracellular enzymes that cleave lignin specific linkages (Vicuna *et al.*, 1993). *Pseudomonas* spp. strains have been isolated that can cleave linkages between synthetic lignin dimers. Lignin degradation products released during fungal white rot are usually oligomeric and monomeric aromatic compounds. Bacteria can utilise these compound for they posses the enzymatic ability to cleave the alkyl-aryl ether bonds contained in these structures (White *et al.*, 1996).

Like actinomycetes, bacteria lack the powerful enzymes required to cause significant lignin degradation. They survive primarily as secondary saprophytes, living off the lignocellulose degradation products released by the enzymatic action of white-rot fungi. Bacteria are unwanted in biological delignification projects because they cause extensive deterioration of the end product, especially if a cellulose-enriched substrate is required.

5. Biodegradation of other plant components

5.1 Protein biodegradation

Proteins, peptides and amino acids are readily metabolised in soil (Kuzyakov, 1997). Soil micro-organisms rapidly degrade amino acids in the free state. The build-up of amino acids in soil can be attributed to their being a component of a stable biomass in their interaction with other organic matter and with soil clays, and their incorporation into aggregates (Paul and Clark, 1989).

Aerobic and facultative aerobic bacteria are equally capable of oxidising proteins and amino acids. These organisms are able to utilise amino acids as their sole source or carbon and energy (Doelle, 1975). Microbial proteinases and peptidases hydrolyse peptide bonds of proteins and peptides. Enzymes such as trypsin produce proteosomes by partial hydrolysis. Microbial enzymes, such as pronase and subtilisin, carry out terminal amino acid chain removal (Paul and Clark, 1989). Amino acid oxidases, which are flavoproteins with a redoxpotential of –0.004 V, are mainly involved in the initial oxidation step. The amino acid is oxidised to an imino acid, followed by a hydrolysation to the corresponding keto acid (Doelle, 1975). L-amino acids will undergo transamination reactions first, before further degradation takes place. These oxidation reactions are unique for the different types of amino acids and may differ between species or genera. The end products are however simple compounds (like succinyl-CoA, acetyl-CoA, acetoacetate, propionyl-CoA etc.) that can be oxidised to CO_2 and H_2O in any of the oxidative metabolic pathways.

Therefore, free proteins represent easily utilisable substrates for most micro-organisms. Proteins released from decaying plant material might become inaccessible once interactions with humic compounds occur.

5.2 Lipid biodegradation

Triglycerides, the core structure of membrane phospholipids, are enzymatically degraded by lipases to yield fatty acids and glycerol (Moat and Foster, 1995). The glycerol is oxidised to glycerol-3-phosphate, which is then metabolised via the triose phosphate pathway of the EMP system. The free fatty acid is converted to acyl-CoA (Figure 2.16). The next sequence of oxidation reactions is repeated until the fatty acid has been reduced to acetyl-S-CoA fragments. These fragments are metabolised under normal circumstances by the Krebs or glyoxylate cycles.

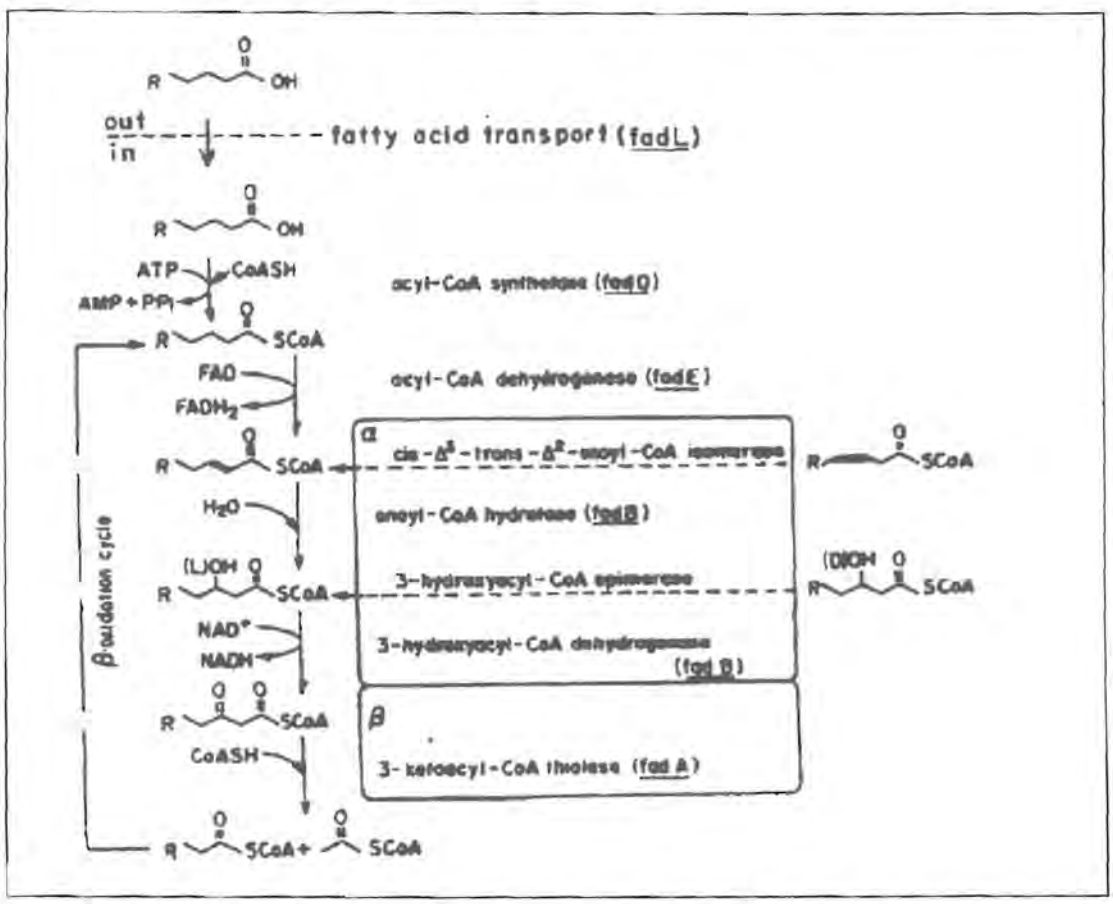

Figure 2.16: General catabolic pathway for the degradation of fatty acids by micro-organisms (Moat and Foster, 1995).

Most organic phosphor compounds like phospholipids cannot be taken into the living cell, therefore the hydrophilic carbon chain must be degraded to liberate the phosphate ion. Wild-type *Escherichia coli* can utilise long-chain fatty acids, such as oleate (C_{18}:1, but not medium-chain fatty acids, such as decanoate (C_{10}), as a sole source of carbon and energy (Moat and Foster, 1995). *Neurospora crassa* also possesses an inducible α-oxidation system. *Candida tropicalis* contains the enzymes for α-oxidation in peroxisomes. These organelles have a novel long-chain acyl-CoA synthetase whose product is exclusively used for α-oxidation and not for lipid synthesis (Moat and Foster, 1995).

Therefore, lipids can also serve as a source of energy for most micro-organisms. Lipids and related compounds are readily utilised once they become available. Also, interactions with humic substances can prevent the complete degradation of these compounds.

5.3 Nucleic acid degradation

Nucleic acids are readily degraded by soil microflora and it is the only cell constituent that does not accumulate in the soil organic matter (Paul and Clark, 1989; Kuzyakov, 1997). Degradation products that have been identified in soil are cytosine, adenine, guanine, uracil, hypoxanthine and xanthine. The salvage pathways by which micro-organisms can utilise these compounds may vary considerably between species and genera (Stanier *et al.*, 1986). Of the total organic phosphorous in soil, only 1% can be identified as nucleic acids or their derivatives.

6. Inhibition of lignocellulose degradation

6.1 Physical barriers

Physical barriers prevent biological catalysts (micro-organisms or secreted polymer degrading enzymes) access to the substrate. The waxes and cuticle of an intact epidermis prevent bacterial access to the interior of leaves and stems (Wilson and

Mertens, 1995). The accessibility of enzymes to wood and fibres is limited due to factors such as adsorption to surface areas, low fibre porosity, and low median pore size of fibres (Tomme *et al.*, 1995).

The limits to ruminant digestion of plant cell walls have been studied. According to Wilson and Mertens (1995) degradation of the middle lamella-primary wall region is not only prevented by the chemical nature (lignin concentration) but also by physical structure and cell architecture. Bacterial digestion of fibre cells can progress only from the interior (lumen) surface because their middle lamella-primary wall region is consistently found to be indigestible (Figure 2.17). Fungi may also play an important synergistic role in the ruminal digestion of forages by physically disrupting the lignified stem tissue (Varga and Kolver, 1997). This allows the ruminal microbes greater access to the plant stem tissue and the digestible portions of the plant.

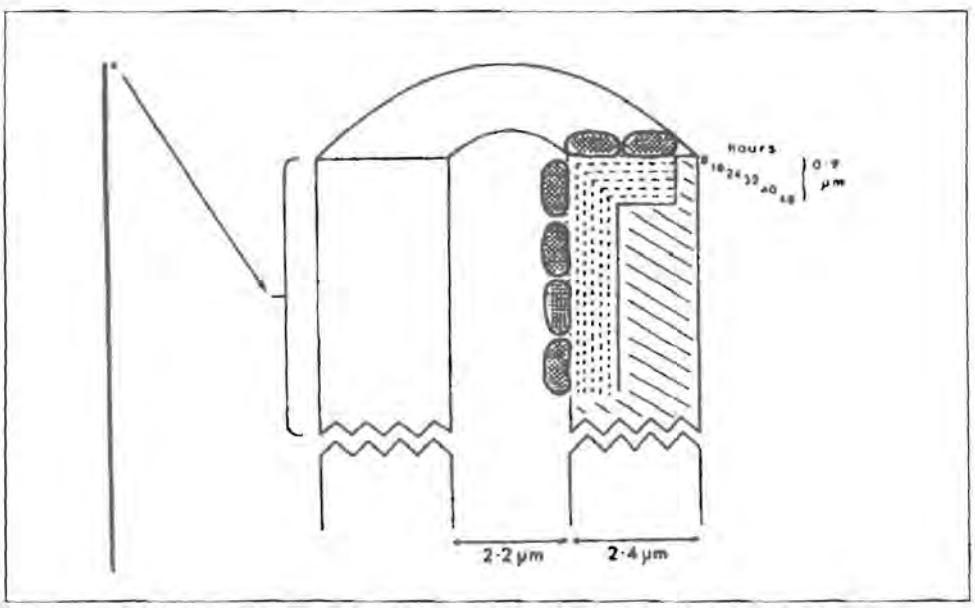

Figure 2.17: Enlargement of sclerenchyma fiber at the broken end showing progressive digestion fronts adjacent to bacteria (stippled) (Wilson and Mertens, 1995).

The wall surface area to cell wall volume ratio can be used to predict digestibility of thick-walled cells (Wilson and Mertens, 1995). Ignoring the lignin component of plant cells, digestive loss of sclerenchyma and other thick walled cells (like parenchyma

cells) are predicted to be slow because of the very small surface area for bacterial colonization relative to the amount of cell wall to be digested. Considering Buffelsgrass, Wilman and Moghaddam (1998) estimated that at least 2/3 of the thickness of the thick cell walls of stems can be degraded during the time the walls are likely to remain in the rumen. Despite the higher concentrations of lignin in the stems than in the leaf blades or sheaths, the thick walls in the stems were at least as digestible as those in the leaves (Wilman and Moghaddam, 1998).

Physical barriers to lignocellulose degradation remain a difficult problem to solve. Too severe particle size reduction might influence the intended process adversely. In the rumen scenario, the physical barrier is a problem only because of the low retention times. In other words, the microorganisms do not have sufficient time to establish themselves on the substrate before the rumen contents leave the rumen.

6.2 Chemical recalcitrance

The molecular organisation of the different components of the plant fibre cell walls (cellulose, hemicellulose, lignin) also limits the accessibility of microorganisms and their enzymes to wood and its fibre components (Kuhad *et al.*, 1997). Therefore, mechanical, chemical, biological or thermal pre-treatment improves the accessibility of lignocellulosics to degradative enzymes by removing lignin and hemicelluloses as well as by partially disrupting lignified tissue structure (Kuhad *et al.*, 1997; Reid, 1995; Tomme *et al.*, 1995).

LCCs retard digestion of cellulose and hemicellulose by ruminants (Jeffries, 1990; Varga and Kolver, 1997). Low digestibility of total hemicellulose in immature plants low in lignin was attributed to the inability of the ruminant to adequately digest branched xylans. In mature forages, the major limiting factor of hemicellulose digestion was greater amounts of lignin (Bittner, 1983).

Crystalline cellulose is highly resistant to microbial attack and enzymatic hydrolysis, whereas amorphous cellulose is degraded at a much faster rate (Kuhad *et al.*, 1997; Tomme *et al.*, 1995). As its crystallinity increases, cellulose becomes increasingly resistant to hydrolysis. Native cellulose contains both crystalline and amorphous regions.

Lignification is widely accepted as the barrier preventing complete degradation of cellulose but attempts to shift this paradigm recur. Lignification reduced the degradability of plant cell walls by fungal hydrolases, but degradability was not affected by lignin composition (Grabber *et al.*, 1997). *Arabidopsis* mutants unable to deposit syringyl-lignin were studied and revealed that lignin composition does not alter cell wall degradability or that the mutation had no other effects on the cell walls of the mutants (Jung *et al.*, 1999).

Pure cultures of microorganisms cannot effectively degrade a complex polymer like lignocellulose. Microbial consortia, comprising an array of hydrolases with different substrate specificities, will synergistically cause more extensive degradation of any complex polymer. This is especially true for natural microbial consortia and will be discussed in section 8.

6.3 Interaction of lignocellulose degradation products with humic substances

In a study by Verma *et al.* (1975) 71-95% of ^{14}C-labeled proteins, peptides and amino acids were oxidised to ^{14}C-labeled CO_2 after 4 to 12 weeks of incubation. Metabolism of the amino acids could be reduced by a variety of associations with soil humic acids, but the greatest reduction was found when the substrates were covalently combined with the humic acids. Reduction in degradation rates of up to 90% was observed.

Aminosugars are stabilised into humic acid type polymers through nucleophilic addition of the amino group to the aromatic nuclei of quinones (Bondietti *et al.*, 1972). After 12-week incubation period, 15-23% of the ^{14}C-labeled glucosamine

covalently linked to a humic acid polymer was released as ^{14}C-labeled CO_2. The control incubation (without the humic acid polymer linkage) released 70% of the labelled glucosamine as ^{14}C-labeled CO_2.

Verma and Martin (1976) demonstrated that decomposition of microbial cellular components was affected by humification. Cytoplasmic and cell wall fractions complexed with model phenolase polymers showed a three-fold decrease in decomposition rates than the control fractions.

Therefore, humification processes in soil will prevent the degradation of any organic compounds. If the decomposition of grass lignocellulose by natural consortia reach a stage where humification processes are well underway, the complete digestion of any organic compounds will become more unlikely.

7. Toxic effect of lignocellulose degradation

7.1 Intrinsic substrate toxicity

Toxic substances contained within plant cells might be released during subsequent biodegradation or autolysis. Naturally occurring wood extractives are potent inhibitors of white rot fungi used for biopulping (Wolfaardt *et al.*, 1999). Buffelsgrass is widely used for animal feed and no examples of intrinsic toxicity are known. South African Buffelsgrass was recently screened for compounds of medical interest but no toxins were detected (Lindsey *et al.*, 1999).

7.2 Lignin-carbohydrate complexes

In addition to the physical barrier effect (paragraph 6.1), LCCs constitute a biochemical barrier, sterically hindering cellulases and hemicellulases (Cornu *et al.*, 1994). Also, reduction in microbial adhesion might occur, but this effect is not significant (Cornu *et al.*, 1994). Treatment of barley straw cell walls with cellulase

released compounds containing *p*-coumaroyl and feruloyl groups bound to carbohydrates (Mueller-Harvey and Hartley, 1986). Also, high molecular weight lignin-polysaccharide complexes are released during rumen digestion (Wilson and Mertens, 1995). These large polymers diffuse away from the site of digestion at a very slow rate and their concentration can reach levels that are toxic to microflora. Since lignin preparations do not inhibit *in vitro* fermentation, the inhibition caused by phenolics is due, directly or indirectly, as a result of linkages between phenolics and carbohydrates (Cornu *et al.*, 1994). However, in terms of the rumen, the toxic effect of these molecules *in vivo* is insignificant due to dilution and rapid detoxification by the microflora (Wilson and Mertens, 1995). The toxic potential of LCCs is released when lignin monomers are cleaved from the complex. The toxic effects of LCCs in microenvironments are unknown. Hot water treatment of straw gave solutions with carbohydrates and phenolics. Most of the sugars extracted by hot water treatment were bound either to polysaccharides or to phenolics in the lignin (Vered *et al.*, 1981). The implication is that phenolic compounds can indirectly accumulate to toxic levels within microenvironments at the sites of lignocellulose degradation.

7.3 Lignin monomers and phenolic degradation intermediates

Evidence suggests that potential toxicity of phenol, lignin degradation intermediates and lignin building blocks is neutralised within complex ecosystems via co-operative metabolic actions of consortia. Free phenolic acids and aldehydes (*p*- and *o*-hydroxyphenyl acetic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillic acid, aniline, syringic acid, *p*-coumaric acid, syringaldehyde, and ferulic acid) were detected in wheat straw extracts (Galletti *et al.*, 1990; Milstein *et al.*, 1981; Vered *et al.*, 1981). Low molecular weight fragments containing methoxyl groups are released during exposure of lignin to peroxidases (Leonowicz *et al.*, 1999). These degradation products are introduced to a variety of natural and man-made environments.

In the rumen, limited antimicrobial effect by these degradation products occurs due to rapid detoxification (Cornu *et al.*, 1994). Phenolic acids are usually produced in subtoxic amounts (except in microenvironments) and may be transformed to

phenylpropanoic acid, which is a growth factor. The physical cell wall architecture has instead been identified as the primary barrier to cell wall degradation in the rumen (paragraph 6.1).

The degradation of lignin by white rot fungi in particular releases intermediates that may enter anaerobic environments. Lignin degradation is very slow under anaerobic conditions and lead to the accumulation of humus to form peat, organic soil matter, lignite and coal (Heider and Fuchs, 1997). Although polymeric lignin remains stable in anaerobic environments for long periods of time, bacterial consortia actively metabolise smaller degradation products (Egland *et al.*, 1997). Because of its chemical stability, the anaerobic reduction and cleavage of aromatic rings by microbial enzymes is a significant biochemical feat (Elder and Kelly, 1994). A reductive benzoate pathway is the central conduit for the anaerobic biodegradation of aromatic pollutants and lignin monomers (Egland *et al.*, 1997).

It is now accepted that the microorganisms inhabiting anaerobic environments make a significant contribution to the overall turnover of aromatic compounds (Elder and Kelly, 1994). Bacterial strains and consortia have been isolated for their ability to degrade, under anaerobic conditions, homocyclic monoaromatic compounds, such as phenolic compounds, methylbenzenes and aminobenzenes (Villemur, 1995). Anaerobic degradation of phthalic acid isomers was dependent on syntrophic consortia consisting of fermentative bacteria and methanogens (Kleerebezem *et al.*, 1999).

Lignin degradation products influence sulphate reducing bacteria in a variety of ways. The metabolic activities of both sulphate reducing bacteria and methanogens were required for complete anaerobic degradation of phenol in swine manure (Boopathy, 1997). Toluene and benzene metabolism was coupled to sulphate reduction and ceased when sulphate was depleted or when sulphate reducing bacteria were inhibited (Beller *et al.*, 1992; Lovley *et al.*, 1995). Some sulphate reducers are able to completely oxidise benzene to carbon dioxide without the production of extracellular intermediates (Lovley *et al.*, 1995).

Compounds released from lignocellulose by the action of microorganisms might have potential toxicity effects, but natural consortia are effective in detoxifying these degradation compounds either by direct metabolism or inactivation.

7.4 Other degradation products

Anaerobic environments often have low pH and high concentrations of organic acids (Russell and Diez-Gonzalez, 1998). The toxicity of organic acids toward bacteria at low pH is explained by inhibition of intracellular metabolism. Antibiotics can disturb the production of biogas during anaerobic digestion (Sanz *et al.*, 1996). Therefore, indirect toxicity can be caused by specific members of natural microbial consortia due to stimulation of a metabolic activity by lignocellulose degradation products.

8. Role of natural consortia in lignocellulose biodegradation

8.1 Rumen

The ruminant provides nutrients that support the growth of rumen microorganisms. In return for the provision of this excellent habitat, rumen microbes provide activities and products that are essential for the animal. The most important of these are microbial cellulases. Cellulose is the most important source of carbon and energy in the animal's diet, but the animal itself does not produce cellulose hydrolyzing enzymes (Czerkowski, 1986). The microorganisms (bacteria, fungi and protozoa) utilise the carbohydrates and other plant carbohydrates as their sources of carbon and energy, they produce large amounts of acetic, propionic and butyric acids which the animal can then use as its sources of carbon and energy (Figure 2.18) (Colberg, 1988).

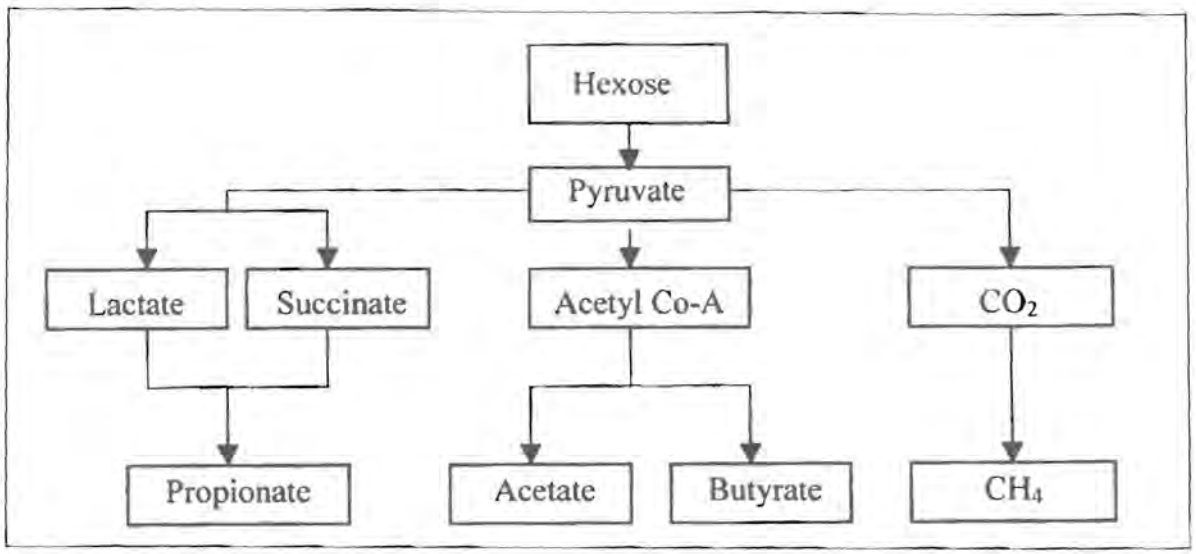

Figure 2.18: A simplified scheme depicting the degradation of glucose in the rumen to organic acids and methane gas (Czerkowski, 1986).

Cellulolytic, hemicellulolytic and proteolytic activities of rumen fungi were observed *in vitro* in the degradation of plant components (Fonty *et al*, 1990). Five genera of fungi have been described for the rumen ecosystem: *Neocallimastix, Caecomyces, Piromyces, Orpinomyces* and *Ruminomyces* (Leschine, 1995).

Genetic engineering was applied to improve the lignocellulose digestion ability of the anaerobic bacteria to contribute to the acceleration of ruminant growth and production (Ohmiya, 1990). The role of protozoa in fibre digestion is also well known (Jouany and Ushida, 1990). Bacteria are the best studied of the rumen inhabitants (Colberg, 1988; Leschine, 1995). Ruminococci are the most numerous but anaerobic, cellulolytic bacteria indigenous to the rumen include *Bacteroides succinogenes, Butyrivibrio fibrisolvens*, and *Eubacterium cellulosolvens*. These organisms comprise cellulases which enable them to degrade the digestible fraction of the plant fibre. However, because these organisms do not secrete their cellulases, they need to attach to the substrate before degradation can commence. This can be a disadvantage in a very dynamic environment.

The rumen is a model system for evaluating the effect of retention time and fibre accessibility on the ability of micro-organisms to degrade lignocellulose. The end

products of the rumen fermentation are organic acids, which are then utilised by the animal. The biological pre-treatment of lignocellulose under conditions simulating the rumen might give high yields of organic acids. These organic acids can be utilised as a carbon source by the sulphate reducing bacteria. However, significant amounts of the plant material is converted to methane gas and lost to the atmosphere.

8.2 Termite hindgut

The carbon and energy nutrition of lower termites is centered on wood polysaccharides. These compounds constitute 70% of the dry mass of wood and 99% is degraded in the gut of this insect. Most of the degradation occurs in the hindgut, a region often referred to as an anaerobic fermentation chamber. The termite gut resembles the rumen of sheep and cattle: it is characterised by high concentrations of volatile fatty acids, the presence of fermenting bacteria and protozoa, and the occurrence of typical anaerobic activities such as methanogenesis and acetogenesis (Brune, 1998).

The hindgut microflora consist of a heterogeneous bacteria population and unique genera and species of flagellate protozoa. These protozoa appear to be the dominant players in hindgut fermentation (Odelson and Breznak, 1983; Yamin, 1981) whereas bacteria are of secondary importance in this activity. The presence of these protozoa is crucial to the survival of lower termites. True cellulolytic bacteria appear to be quantitatively insignificant in this activity (Odelson and Breznak, 1983). A recent publication provided direct evidence that lignin-biodegradation occurs in the hindgut of *Nasutitermes takasagoensis*. The bacterium *Burkholderia cepacia* was implicated in lignin degradation because it was isolated form an enrichment containing vanillic acid as sole carbon source (Kato *et al.*, 1998).

The crucial question is whether low molecular weight carbon sources in the form of volatile fatty acids are produced? Odelson and Breznak (1983) indicated that fermentative bacteria produce such carbon sources, of which acetate was the main product (Figure 2.19). Termite hindgut fermentation can be viewed essentially as a homoacetic fermentation of cellulose (Odelson and Breznak, 1983; Breznak and

Switzer, 1986). The fermentation begins with the hydrolysis of cellulose and the fermentation of the glucosyle units to 2 acetate + 2 CO_2 + 4 H_2 by anaerobic cellulolytic protozoa. Anaerobic acetogenic bacteria form a third acetate molecule and then consume the CO_2 and H_2. These three acetates are then taken up from the hindgut and oxidised aerobically by termite tissue cells to 6 CO_2 and 6 H_2O. Methane and hydrogen are formed during this fermentation but the amounts produced per glucose are negligible. Breznak and Switzer (1986) determined that CO_2 reduction to acetate, rather than CH_4, represents the main electron sink reaction of the hindgut fermentation and can provide the insects with a significant fraction of their principle oxidisable energy source, acetate. Also, HPLC analysis revealed that the $^{14}CO_2$ fixed by *R. flavipes* gut homogenates was present in compounds that coeluted with acetate and formate. Only trace amounts of $^{14}CO_2$ were associated with propionate, butyrate, and lactate-succinate (Breznak and Switzer, 1986).

Several researchers also addressed the question of how lignin is degraded in the termite hindgut. Lignin degradation is the rate-limiting step in lignocellulose degradation because of the recalcitrant nature of the lignin monomers. Pasti *et al.* (1990) confirmed the existence of lignin-solubilising actinomycetes in the termite hindgut. A screening procedure based on $^{14}CO_2$ evolution from [^{14}C-lignin] lignocellulose combined with measurement of acid-precipitable polymeric lignin (APPL) yield was the most effective in identifying lignin-solubilising strains. Five strains caused 40.11% to 49.64 % lignocellulose weight loss in solid state fermentation after 4 weeks of incubation at 37°C (Pasti *et al.*, 1990). Higher termites do not contain any protozoan endosymbionts as described for the lower termites (Yamin, 1981). Therefore, the ability of hindgut flora of higher termites to degrade lignin monomers and related aromatic compounds were investigated (Kuhnigk *et al.*, 1996). Under anaerobic conditions, no aromatic compound was degraded, only modifications of the side chains occurred. Their main observation was that in the anaerobic hindgut the breakdown of aromatic ring systems required oxygen, which was supplied via the aerated paunch epithelium. This highlights some unanswered questions: 1) Why are the majority of the isolates from the hindgut obligatory aerobic, aerotolerant or facultative? 2) How does lignin degradation take place since degradation of lignin and humic compounds cannot take place in the absence of

oxygen? 3) From a physiochemical viewpoint, how can such a small ecosystem maintain its anoxic status within an aerobic environment? (Brune, 1998).

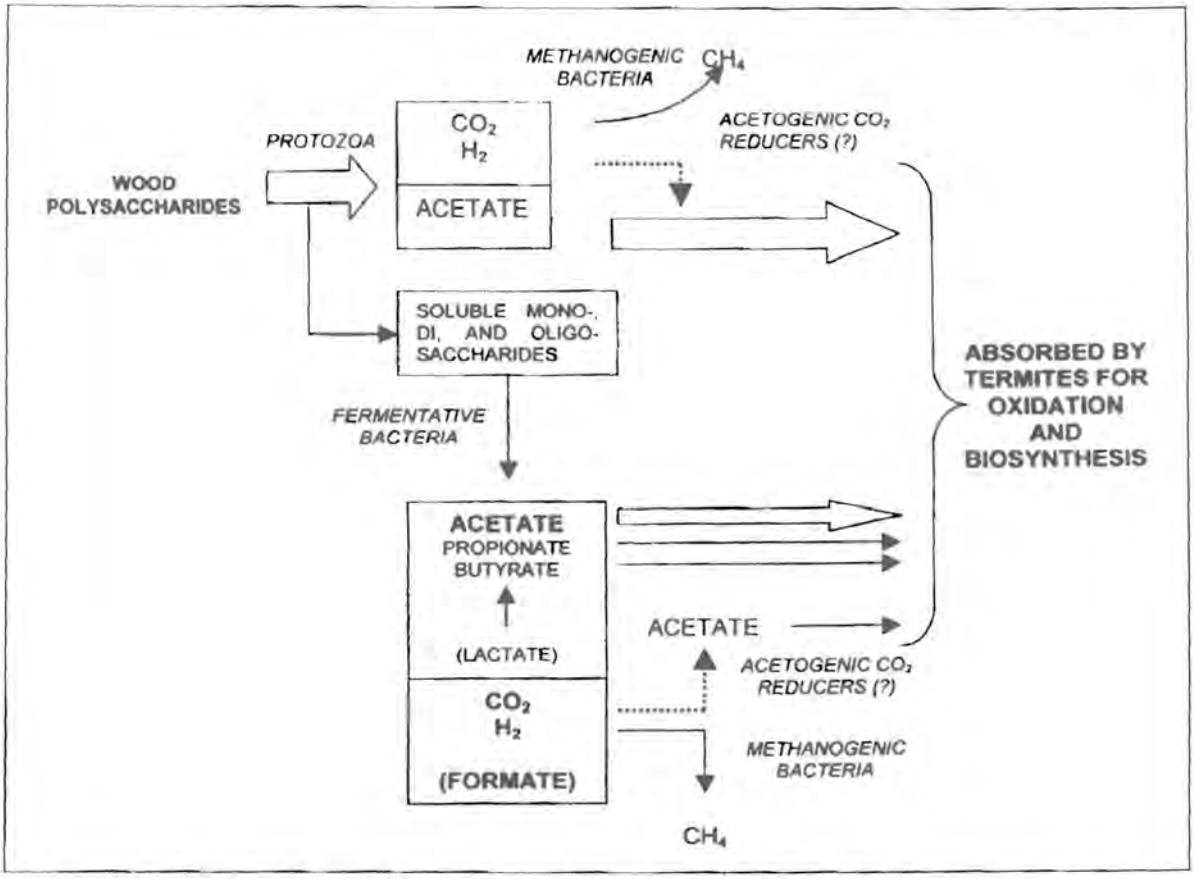

Figure 2.19: Proposed working model for symbiotic wood polysaccharide dissimilation in *R. flavipes*. Thickness of arrows represents approximate relative contribution of the respective reactions to the overall dissimilatory pathway (Odelson and Breznak, 1983).

The discovery of micro-aerotolerant sulphate reducing bacteria in the termite hindgut added to the confusion. The ability of the sulphate-reducing anaerobe *Desulfovibrio desulfuricans* to modify lignins (synthetic lignins like Kraft lignin and lignosulfonate), were demonstrated (Ziomek and Williams, 1989). Interestingly, the biologically modified lignin had an increased capacity for binding heavy metals like lead and mercury. A novel species, *Desulfovibrio termitidis* sp. nov., was isolated from the hindgut of a termite (Trinkerl *et al.*, 1990). Finally, a feasible model for the role of sulphate reducing bacteria in the termite gut was proposed and recent publications confirmed this hypothesis (Figure 2.20; Brune, 1998; Kuhnigk *et al.*, 1996).

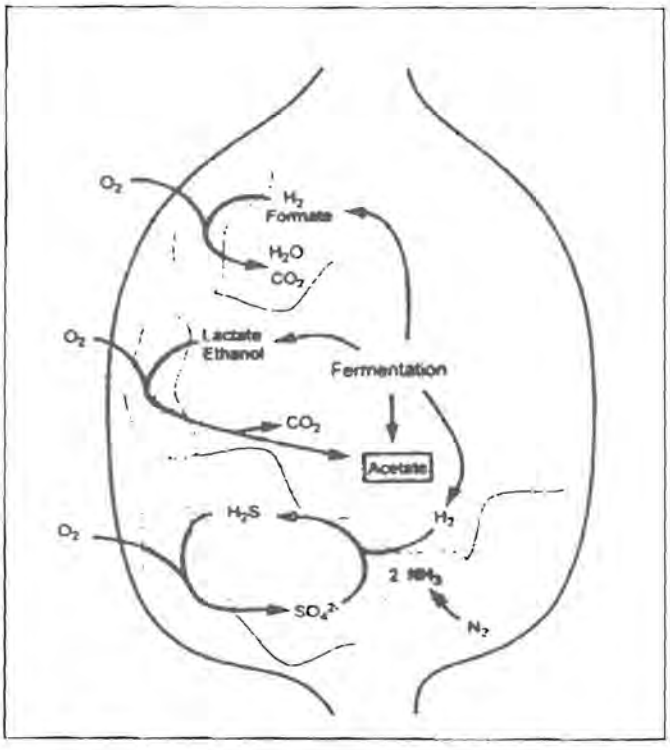

Figure 2.20: Suggested roles of sulphate reducing bacteria in the termite gut (Kuhnigk *et al.*, 1996).

Typical anaerobic metabolic activities within the termite hindgut are acetogenesis and methanogenesis. Yet most bacteria isolated from the termite hindgut are either obligately aerobic, aerotolerant or facultative. However, the respiratory activity of the hindgut microbiota maintains steep oxygen gradients within the gut periphery (Figure 2.21). Hydrogen accumulates to high concentrations at the gut center and is consumed by methanogens and sulphate reducing bacteria to form acetate from CO_2 (Breznak and Switzer, 1986). Very steep oxygen and hydrogen partial pressure gradients exist within the termite gut (Figure 2.21). Thus, oxygen is present in the termite gut and the role of oxygen in lignin degradation is less debated, for it is required for the mineralization of lignin monomeric aromatic compounds. Molecular oxygen is required for aromatic ring cleavage (Kuhnigk *et al.*, 1996), but in the light of the latest findings, aromatic ring cleavage can occur in the steep oxygen gradients that are maintained within the termite gut (Brune, 1998). The breakdown product of aromatic ring cleavage can then be anaerobically metabolised by the resident microbiota. The efficient and constant removal of oxygen is a determinant of the metabolic activities of the gut microflora.

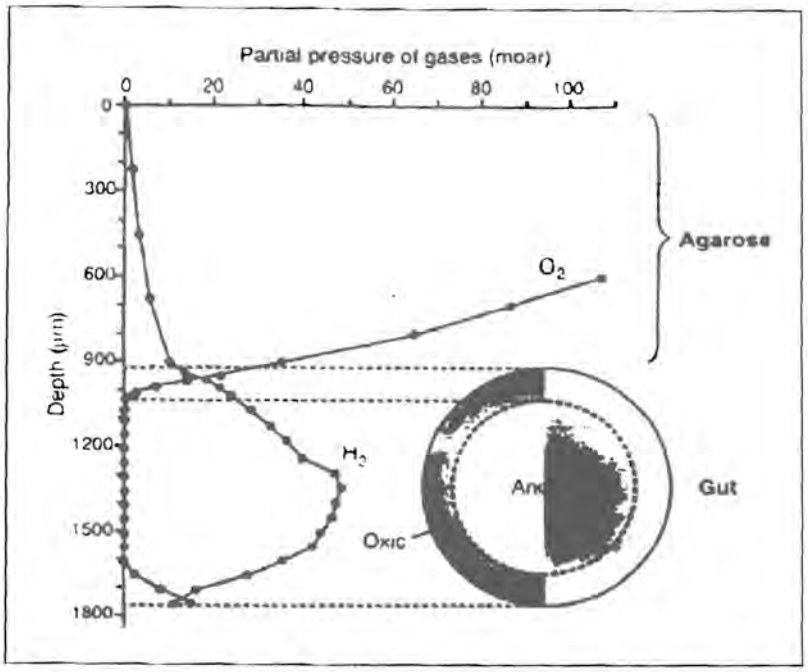

Figure 2.21: Radial profiles of oxygen and hydrogen partial pressure in the agarose-embedded hindgut of the woodfeeding termite *R. flavipes* (Brune, 1998).

8.3 Anaerobic aquatic sediments

Lignocellulose-containing detritus is a significant source of particulate organic carbon in aquatic ecosystems. Both waterlogged sediments and anoxic conditions characterise these environments. Such conditions facilitate slow but significant turnover of native cellulose present in the lignocellulose complex. The degradation of lignocellulose in waterlogged aquatic sediments proceeds by a different route compared to classic soil models. A range of organic compounds is present in anaerobic sediments (Table 2.4).

The anaerobic degradation of polymeric lignin has long been disputed but publications that proved the contrary have emerged. It is now accepted that polymeric lignin and lignin monomers are slowly degraded to CO_2 and CH_4 in anoxic sediments at environmentally significant rates (Colberg, 1988). Complex chemical and biological interactions occur in anaerobic aquatic sediments and exogenously added plant or animal organic matter fuel these interactions (Figure 2.22).

Table 2.4: Organic compounds present in wet sediments (Stevenson, 1982).

Class	Comments
Fermentation products	Incomplete oxidation leads to production of CH_4, organic acids, amines, mercaptans, aldehydes and ketones.
Modified or partially modified remains of plants	In addition to slightly altered lignins, carotenoids, sterols, porphyrins or chlorophyll origin are preserved.
Synthetic organic chemicals	Many man-made chemicals decompose slowly if at all under anaerobic conditions.
Carcinogenic compounds	Synthesis of methylmercury, dimethylarsine, dimethylselenide, and nitrosamines of various types.

Most cellulose is degraded aerobically, but 10-15% is degraded anaerobically. A community of physiologically diverse micro-organisms is responsible for the anaerobic degradation of cellulose (Leschine, 1995). Several mesophilic cellulolytic anaerobes have been isolated from soils, sediments and composts. Most of the isolates are species of the genus *Clostridium* (*C. papyrosolvens, C. lentocellum, C. cellulofermentans*). Other organisms include *Bacteroides cellulosolvens* and *Acetovibrio cellulolyticus*.

High moisture content, causing oxygen depletion and the accumulation of acid substances, is sometimes particularly favourable for the accumulation of humus. In such waterlogged aquatic systems, undegraded or extremely slowly degraded lignocellulose accumulates to form peat.

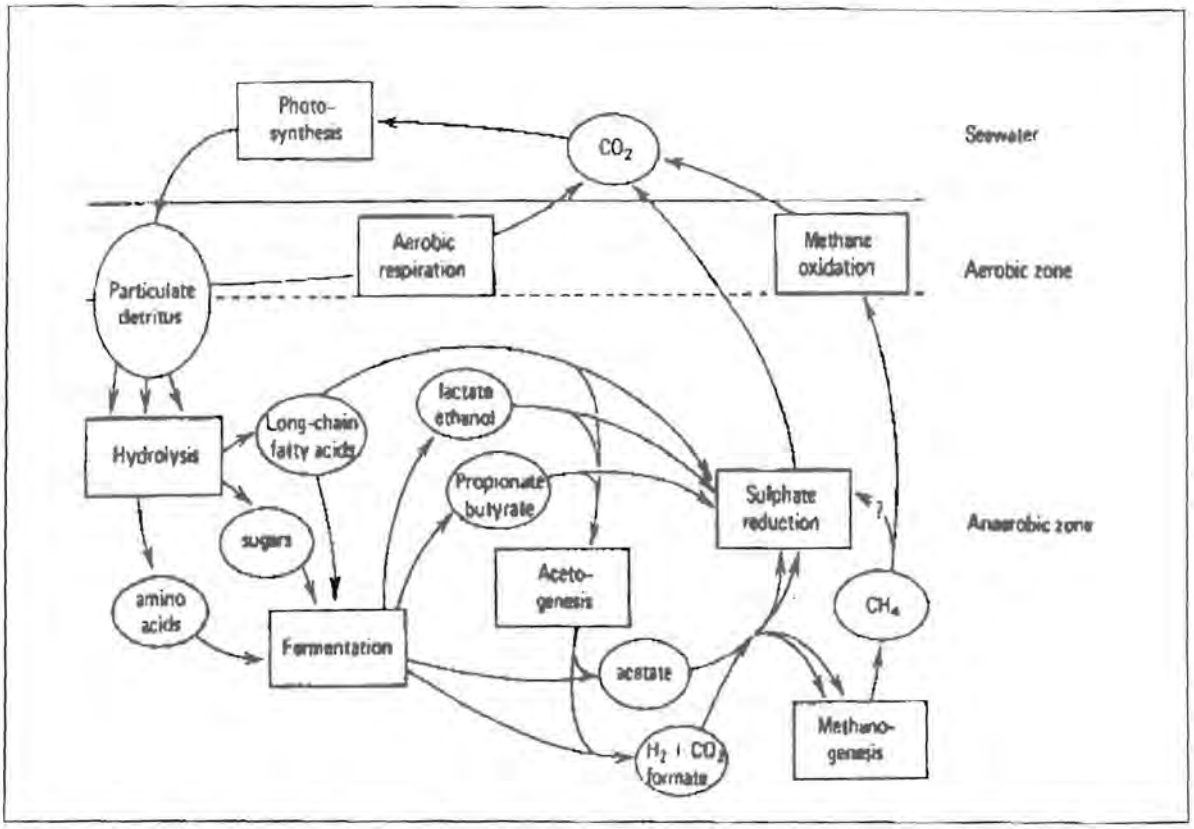

Figure 2.22: Degradation and cycling of organic matter in sediments (Stanier *et al.*, 1986).

The cooperative action of natural microbial consortia is undoubtedly responsible for the natural degradation of lignocellulose. Our understanding of lignocellulose degradation by mixed populations will greatly enhance our ability to imitate such systems in order to initiate a successful scale-up lignocellulose biodegradation project. All the environments discussed here have some common features. All are anaerobic environments where acetogenesis, sulphate reduction and methanogenesis can occur. These environments are home to numerous species of known or poorly characterised microbes that comprise highly diverse range of metabolic activities and capabilities. Finally, these environments are dynamic and pose serious stress on the inhabitants in terms of nutrient, oxygen and pH gradients, and high levels of species interactions (competition, predation etc.). Of these environments, the hindgut of the termite is the most effective in lignocellulose degradation and possesses interesting features to ensure efficient lignocellulose utilization. Conditions similar to those encountered in

anaerobic sediments should be avoided because it leads to the immobilization of all organic plant material for very long periods of time.

9. Analysis of lignocellulose biodegradation

Several methods exist by which one can determine the lignin content of a lignocellulosic sample. These methods differ in their complexity, the information they give and their applicability to certain types of materials. A major concern regarding all current lignin determination methods is the resultant change or modification of the lignin structure upon isolation or characterisation. Many authors have voiced their dissatisfaction with current analytical methods but reliable alternatives are scarce and costly (Kerem *et al.*, 1992; Lewis and Yamamoto, 1990).

9.1 Acid-insoluble lignin

The gravimetric analysis of 72% H_2SO_4 insoluble lignin (also known as Klason lignin after its inventor) is a generally adopted method for the lignin determination. In this procedure the strong acid hydrolyses polysaccharides and the hydrolysate can be used for carbohydrate determinations. The remaining hydrolysis-resistant fraction is weighed as lignin and is expressed as a percentage of the dry mass.

The method works well for conifer woods, which contain predominantly guaiacyl lignins; less well for angiosperm woods which contain a fair proportion of partially acid-soluble syringyl lignins, and least well for lignins of herbaceous plants including those of monocotyledonous species (Selvendran *et al.*, 1989).

Although this procedure is ubiquitous in many lignocellulose related publications, it is fundamentally limited in its interpretation (Table 2.5). The lignin residue obtained by this procedure is not identical with protolignin (native, intact or natural lignin), because the lignin is damaged and chemically modified during acid treatment. Determining lignin in this manner does not take into account the lignin that has been

dissolved in the sulphuric acid. Lewis and Yamamoto (1990) dismissed all claims that "wet" chemistry techniques, like the Klason procedure, proves that lignin is present in plant residues. Accordingly, the Klason procedure only gives a measure of the residual plant material remaining after acid digestion.

Despite the critique, this procedure remains a very simple and relatively reliable method for estimating lignin content. This procedure and modifications thereof can be found in most publications on lignocellulose degradation (Agosin *et al.*, 1985b; Antai and Crawford, 1981; Arora and Garg, 1992; Blanchette, 1984; El-Gammal *et al.*, 1998; Hernadez-Coronado *et al.*, 1997; Ramos *et al.*, 1993; Valmaseda *et al.*, 1990; Valmaseda *et al.*, 1991).

Table 2.5: Major advantages and disadvantages of the 72% H_2SO_4 or Klason procedure.

Advantages	Disadvantages
1. Widely used by wood chemists for its simplicity and reliability. 2. Good reproducibility.	1. Proteins, tannins and suberins may analyse as Klason lignin. 2. Not directly applicable to grass-like samples that contain syringyl residues that is partially solubilised. 3. Older variants of the method slightly underestimate lignin content.

Other less used methods are the kappa method, thioglycolic acid method, potassium permanganate method, acetyl bromide method (Kirk and Obst, 1988). More complex analytical methods rely on the use of cellulase hydrolysis of structural carbohydrates and extraction with dioxane-water solvent (Jung and Himmelsbach, 1989).

9.2 Detergent method for forage fiber analysis

The detergent method is preferred for analysis of cereals, forages and feed ingredients that often have high contents of protein and other non-lignocellulosic components (Kirk and Obst, 1988). Samples are pre-treated with acid detergent to remove protein,

hemicelluloses and other components from the cellulose and lignin. Lignin is determined as acid-insoluble lignin (9.1). Insoluble fibre in food represents the cross-linked matrix of the plant cell wall and is measured as neutral-detergent fibre (NDF), which includes the cellulose, hemicellulose and lignin as the major components. Acid detergent fibre (ADF) gives an estimate of cellulose plus lignin. These two components may be estimated separately in the ADF fraction by removal of lignin or cellulose. Hemicellulose is estimated as the difference between NDF and ADF. These techniques are frequently encountered in literature on lignocellulose degradation (Agosin *et al.*, 1985b; Karunanandaa *et al.*, 1992; Kerem *et al.*, 1992; Müller and Trösch, 1986).

9.3 Carbohydrate analysis

Structural carbohydrates (cellulose and hemicellulose) are usually not quantified in lignocellulose biodegradation studies because these molecules are chemically complex. However, methods exist that can give an estimation of the carbohydrate content. In most cases, the Klason lignin is prepared by digesting the sample with strong acid. The acid hydrolyses the carbohydrates, leaving the acid resistant fraction. The hydrolysed fraction is used for sugar determinations by the appropriate methods.

The method of Somogyi (and modifications thereof) (Nelson, 1944; Shaffer and Somogyi, 1932; Somogyi, 1951) is very reliable and is frequently used (Antai and Crawford, 1981; El-Gammal *et al.*, 1998; Seelenfruend, *et al.*,1990). The procedure is however laborious. Polysaccharide content can also be determined by gas chromatography (Camarero *et al.*, 1998) and high performance liquid chromatography (HPLC) (Dale *et al.*, 1996).

9.4 Spectroscopy

Infrared (IR) spectroscopy has been used extensively to analyse the structure of lignin. IR peaks are assigned to functional groups and chemical linkages based on observations using model compounds. Nuclear magnetic resonance (NMR) spectroscopy has been used for the analysis of lignin structure and progress in the

technique enabled the analysis of native and decayed lignins as well as degradation products of lignin. These non-degradative methods have been used to characterise the reaction products obtained from analytical procedures (Hernandez-Coronado *et al.*, 1997; Akin *et al.*, 1995; Trigo and Ball, 1994; Valmaseda *et al.*, 1990) to monitoring chemical and biological pulping conditions (Guadalix *et al.*, 1997).

9.5 Chromatography

Chromatographic procedures are by far the most frequently used technique to characterise lignocellulose degradation products. High performance liquid chromatography (HPLC) provides a rapid and convenient method to estimate the molecular weight distribution of lignin degradation products (Mason, 1988; Müller and Trösch, 1986; Ramos *et al.*, 1993; Seelenfreund *et al.*, 1990; Trigo and Ball, 1994). HPLC can also be used for identifying individual sugar components (Blanchette, 1984). Gas chromatography (GC) was used for determining polysaccharide content (Camarero *et al.*, 1998) and identification of low-molecular mass compounds (Trigo and Ball, 1994).

9.6 Microscopy

- **Light microscopy**

Differential staining of polysaccharides and lignin can be utilised for light microscopy techniques. Histological studies recently revealed different degradation patterns of lignified tissues by *Pleurotus eryngii* and *Phanerochaete chrysosporium* (Camarero *et al.*, 1998). Scanning electron microscopy (SEM) subsequently confirmed these observations.

- **Ultraviolet (UV) microscopy**

The UV microscopic method makes it possible to estimate the lignin content of various regions of wood cell wall both qualitatively and quantitatively. UV

microscopic studies of wood and pulp are carried out on ultra-thin cross sections (Akin *et al.*, 1995). Measurements of absorbance caused by aromatic structures are made directly on a microscope equipped with a microscope photometer. It is also possible to differentiate distribution of syringyl and guaiacyl units based on their absorption maxima.

- **Electron microscopy**

Scanning electron microscopy (SEM) was used to examine degradation of wheat straw (Camarero *et al.*, 1998) and Bermuda grass stems (Akin *et al.*, 1995). SEM was also used as part of an approach to screen white rot fungi for preferential lignin degradation (Blanchette, 1984). Transmission electron microscopy (TEM) of parenchyma cell walls from untreated and pre-treated materials showed overall loss of electron denseness, removal of middle lamella and disruption of other cell wall components (Akin *et al.*, 1993; Akin *et al.*, 1995). Ultrastructural analysis of wood decay using TEM was applied for the selection of white rot fungi for biopulping (Blanchette *et al.*, 1988).

9.7 Digestibility

When the purpose of delignification is to increase the accessibility of polysaccharides to enzymatic hydrolysis, either *in vitro* or in the rumen, digestibility measurements show the success of the process (Reid, 1989b). Rumen digestibility can be estimated by suspending the samples in a porous nylon bag inside the rumen of a fistulated animal, or by incubating the sample in a test tube with an inoculum taken from the rumen of a fistulated animal (*In vitro* rumen digestibility, IVRD or IVD). These techniques measure the susceptibility of the material to digestion by rumen micro-organisms. The digestible dry matter in lignocellulose is the sum of the material that is already soluble and the material that can be solubilised by enzymatic hydrolysis. This potentially hydrolysable fraction is the total insoluble carbohydrate minus the part that is protected from enzymatic attack by lignification.

The method developed by Tilley and Terry (1963) is based on the concept of incubating the lignocellulose material with rumen fluid. The gas production by the rumen bacteria is monitored during 70 h fermentation. Using the method of Tilley and Terry (1963), Agosin *et al.* (1985b) investigated the structural changes in wheat straw components during decay by lignin-degrading white-rot fungi. The amount of lignin removed correlated well with *in vitro* dry matter digestibility (IVDMD) improvement. Highest improvement of *in vitro* digestibility was seen at the beginning of a fungal fermentation by Agosin *et al.* (1985b), indicating that partial delignification is necessary for achieving maximal increases in digestibility. Akin *et al.* (1993) made an in-depth study on fungal delignification and found that microbial delignification with white-rot fungi improves forage digestibility.

Other digestibility-like assays convey the same information. The efficiency of cellulase to hydrolyse lignocellulose is inversely related to the lignin content (Dale *et al.*, 1996; Müller and Trösch, 1986).

9.8 Fungal biomass estimations on decomposing lignocellulose

Obtaining representative and reproducible data on fungal biomass growing on a complex substrate such as lignocellulose remains a daunting task. Since lignocellulose substrates contain proteins and some fungi secrete proteases into the substrate, degradation of proteins in the substrate frustrates attempts to measure production of fungal protein. The degree of straw colonisation by white rot fungi can be inferred from chitin (Valmaseda *et al.*, 1990) or protein content (Valmaseda *et al.*, 1991). Ergosterol is an index molecule present in most eumycotic fungi and is absent from vascular plants (Gessner and Newell, 1997). Monitoring fungal biomass is not frequently encountered in literature but it becomes an important parameter when considering scale-up of solid-state fermentation bioreactors.

9.9 Monitoring lignin degradation using radioisotope-labeled lignins

Mineralisation of lignin to carbon dioxide is currently the most sensitive and accurate procedure for testing lignolytic activity of an organism (Deobald and Crawford, 1997). The lignin degrading ability of a microorganism is commonly evaluated by measuring $^{14}CO_2$ evolution from labeled lignin preparations (Agosin *et al.*, 1985a). Experiments are carried out in flasks and require special equipment for trapping of $^{14}CO_2$ (Deobald and Crawford, 1997). The carbon dioxide trapped in NaOH solution is determined by carbonate precipitation with $BaCl_2$ and titration of the residual NaOH with HCl, using thymol blue as indicator (Reid, 1989a). Specially synthesised radioisotope-labeled lignins and the disposal of radioactive waste products make this method very expensive and time consuming.

9.10 Enzymatic activities and the release of enzyme end-products

Valmaseda *et al.* (1990; 1991) utilised the activity of several enzymes and the release of reaction products to infer lignocellulose degradation kinetics (Table 2.6). They monitored carbohydrate hydrolysis products (glucose, xylose, arabinose) and lignin alteration products (*p*-hydroxyphenyl, guaiacyl and syringyl units; cinnamic acids; sinaptic acid; *p*-hydroxybenzoic acid and aliphatic compounds like azeleic acid).

The monitoring of enzymatic activity is an indirect way to measure the changes of the substrate over time. This approach can be used in concert with chemical analysis to get a better picture of actual substrate changes. However, this approach is based on the knowledge of existing enzymes that have been identified and characterised. Unknown enzymatic activities may be present in other fungi, therefore standard enzymatic assays can not be used to infer biodegradation potential or fungal biomass.

Table 2.6: Significance of enzymatic activities monitored to characterise lignocellulose degradation (Valmaseda *et al.*, 1990; Valmaseda *et al.*, 1991).

Enzyme activities estimated	Significance
1. Exocellulase	Cleaves cellobiose from non-reducing ends of glucose chains.
2. Endocellulase	Attacks randomly within glucose chain.
3. β-glucosidase	Hydrolyses cellobiose and dextrins to glucose
4. Xylanase	Endo- and exoxylanases release oligosaccharides and xylose residues from the hemicellulose polymer respectively.
5. Laccase	Oxidises phenolic substances to phenoxy radicals.
6. Lignin peroxidase (ligninase)	Oxidative cleavage of C-C backbone, oxidation and hydroxylation of benzylic methylene groups, oxidation of phenols and benzyl alcohols etc. H_2O_2 is essential for ligninase activity.
7. Aryl-alcohol oxidase	High levels are produced by *Pleurotus ostreatus*. H_2O_2 is produced as part of enzyme activity.
8. Mn(II)-dependent peroxidase	Oxidises Mn^{2+} to Mn^{3+} and in turn oxidises phenolic substances to phenoxy radicals.

10. Industrial exploitation of lignocellulose biodegradation

10.1 Solid state fermentation technology

Solid-state fermentation (SSF) technology has the potential to improve the biodegradability of lignocellulose. Man has used this technology for thousands of years to manufacture food, feed and chemicals. Solid-state fermentation offers the advantages of a robust technology that outperforms conventional fermentation technologies with respect to simplicity, cost effectiveness and maintenance requirements. These advantages make solid-state fermentation processes an attractive option when designing a passive AMD treatment system.

10.1.1 Unique features of SSF

Solid-substrate fermentations are distinguished from submerged cultures by the fact that microbial growth and product formation occur on the surfaces of solid substances

(Mudgett, 1986). Solid substrates may be viewed as gas-liquid-solid mixtures in which an aqueous phase is intimately associated with solid surfaces in various states of sorption and is in contact with a gas phase continuous with the external gas environment.

SSF is in many ways a unique process. The following points listed by Mudgett (1986) are a summary of the general characteristics of solid-state fermentations.

- Traditional solid-state fermentations (fermented foods) may involve mixed cultures of indigenous microbial flora, seed inocula, or both.
- Solid substances provide selective environments for a large number of filamentous fungi and a few bacteria that grow in mycelial form.
- Natural solid substances (for example lignocellulose materials) provide mixed carbon energy sources and a complex source of nutrients that may or may not be complete with respect to nutritional requirements of organisms to be cultured.
- Traditional substances (for example grain hulls etc.) contain some small carbon compounds. The bulk of the dry mass is in high molecular weight polymers (starch, cellulose, lignin, protein etc.), which requires enzymatic hydrolysis for assimilation in growth associated primary metabolism.
- Hydrolytic enzymes for the assimilation of high molecular weight compounds by mycelial organisms generally employed in the solid state are extracellular and may be surface bound or free.
- Mixtures of high- and low-molecular-weight carbon compounds lead to complex patterns of induction, repression and inhibition in the regulation of microbial metabolism.
- Apical growth of mycelia on solid surfaces may permit primary and secondary metabolism to occur simultaneously in different parts of the mycelia.
- Microbial growth and product formation occur at or near solid surfaces in a liquid phase which interfaces with both the gas phase and the external gas environment.
- The gas-liquid interface provides a boundary for O_2-CO_2 exchange and heat transfer at high liquid surface-to-volume ratios.

- Fungi commonly employed in solid-state fermentations are obligate aerobes and need to obtain oxygen from the gas phase under relatively stagnant conditions for gas transfer.
- Growth-associated enzyme synthesis or product formation in some SSF is extremely shear sensitive.

10.1.2 Advantages and disadvantages of SSF

Several authors have listed the advantages and disadvantages of solid-state fermentation as compared to shake-flask or submerged incubations (Aidoo *et al.*, 1982; Cannel and Moo-Young, 1980; Hesseltine, 1972; Mudgett, 1986).

- **Advantages**

- Some solid substances require only the addition of water, whilst other may require other nutrients.
- Fermentation vessels may be small relative to fermentation yield, because small volumes of water are used and the substrate is concentrated.
- Spore inoculum excludes the use of seed tanks.
- Low moisture reduces the problem of contamination.
- Conditions are similar to those in natural habitats.
- Culture agitation, if applied, inhibits sporulation and prevents laboratory contamination.
- Aeration is facilitated by inter particle spaces.
- Product yields are usually higher and is reproducible.
- Fermented solids may be extracted immediately by direct addition of solvents.
- Products may be incorporated directly into animal feeds.
- Provides low shear environments for shear sensitive fungi.

- **Disadvantages**

- The types of organisms that can be applied in SSF are limited to fungi, yeasts, and some bacteria and streptomycetes.

- Fermentations requiring continuous agitation or rotation may involve high power requirements.
- Addition of water in early fermentation stages may increase risk of bacterial contamination.
- Spore inoculum may be large and aseptic conditions must be maintained throughout propagation and inoculation into the fermentation vessel.
- Heat becomes a major problem when large quantities of moist substrates are used.
- Monitoring and control of the fermentation is a problem, especially moisture and pH.
- The major problem encountered in studying SSF is the difficulty in the estimation of mycelial biomass.
- Some agricultural substrates may require pre-treatment like abrasion or cracking.
- Considerable development work may be required for successful scale-up.

10.1.3 Ecophysical requirements for biological delignification

Due to the previously described uniqueness of the SSF process, a unique set of parameters determines whether the process will be successful or not. These parameters must either be monitored during the process or considered when designing a SSF bioreactor.

- **The selection of the delignifying agent**

See section 4

- **Aeration**

Oxygen concentration is not a critical parameter in SSF, but the uniform distribution of air throughout the substrate is (Kerem *et al.*, 1992). However, different species have wide-ranging atmosphere requirements. Uniform distribution of air through the substrate will become necessary when considering scale-up of the experiment.

- **Agitation**

Shear sensitivity is attributed to disruption of mycelial-substrate contact, particularly for organisms with mycelial-bound enzymes for the hydrolysis of solid polymers (Mudgett, 1986). Forced aeration will be necessary for scale-up experiments because physical agitation will interrupt colonisation of the substrate by the fungus.

- **Heat transfer**

Metabolic heat produced by the biomass can influence the kinetics of SSF significantly. In packed bed reactors, overheating is a major problem causing moisture loss and inhibition of the micro-organisms (Marsh *et al.*, 1998). Heat transfer in SSF is therefore closely linked to aeration (Cannel and Moo-Young, 1980). The temperature within the substrate matrix increases due to the respiration of the microbes. Therefore, forced aeration is necessary to prevent overheating of the bioreactor with subsequent destruction of the inoculum.

- **Incubation time**

Biological delignification is a race between degradation of lignin and degradation of carbohydrate by the fungus. As the lignin is removed and the polysaccharides become progressively more accessible to enzymatic hydrolysis, the rate of carbohydrate hydrolysis tends to increase. Therefore, an optimum incubation period exists after which digestible polysaccharide yield decreases although lignin degradation continues. In straw, there is typically an initial decrease in digestibility as the easily digested components are metabolized during colonization, before lignin degradation begins and digestibility increases (Reid, 1989b). Maximum digestibility typically requires 3-4 weeks in straw (Zadrazil and Brunnert, 1981) and 6-8 weeks for wood chips or shavings (Reid, 1989b). Therefore, the optimum window wherein the desired product is obtained should be determined experimentally.

- **Inoculum density**

It is generally necessary to optimise inoculum density in solid-state fermentations (Mudgett, 1986). Too low a density may give insufficient biomass and permit growth of undesirable micro-organisms; too high densities may produce too much biomass and deplete the substrate of nutrient necessary for product formation. Reid (1989a) found that inoculum levels as low as 2% were adequate for initiating sufficient fungal activity on wood chips. Inoculum production will require aseptic conditions to prevent contamination by unwanted microorganisms. Fungi that sporulate easily in pure culture are desired because it simplifies inoculum manufacture, transport and the actual inoculation procedure. A fungus that establishes an effective colonisation of the substrate with the minimum inoculum is preferred. Once again, this should be determined in laboratory experiments.

- **Mass transfer**

Interparticle and intraparticle mass transfer are important in SSF, and it is usually advantageous to physically alter the nature of the substrate (by steaming, abrasion and sorting) to reduce these mass transfer resistances. Interparticle mass transfer is most important for oxygen transfer (Cannel and Moo-Young, 1980). The void fraction is the volume fraction that is occupied by air within the substrate mass. It is a function of the moisture content and the nature of the substrate. If the void fraction is high enough, continuous mixing and aeration is not required as the voids contain enough oxygen to support cell growth. Periodic mixing is required to expel CO_2 and resupply the voids with oxygen. A sufficiently porous substrate allows the diffusion of enzymes into the substrate where degradation can take place (Cannel and Moo-Young, 1980). The water-soluble fragments released by that degradation must also diffuse out of the solid matrix through the same pores. Since the growing microbe requires water-soluble substrates, the action of these secreted enzymes is a key step. Large resistance to the intraparticle mass transfer of enzymes and substrate fragments can cause this to be a rate-limiting step. Therefore, a small pore radius increases the resistance to intraparticle mass transfer with increasing particle diameter. If the pore radius is large,

then intraparticle mass transfer is not likely to be limiting. This explains why particle size reduction should not be too severe.

- **Moisture content**

The optimum moisture content for each microbe-substrate system is based on the desired product and the conditions for cultivation (Mudgett, 1986). The optimum water content for degradation of straw by white-rot fungi was near 3 g water per g straw (Lee, 1997). According to Zadrazil and Brunnert (1981), optimum SSF of straw was obtained at medium water content of 75 ml / 25 g substrate. The weight ratios of water to substrate are usually between 1:1 and 10:1 (Reid, 1989b). The secret to successful solid-state fermentations is having the substrate moist enough for fungal growth, but not so moist that bacterial growth is promoted. Too much water will block interparticle spaces and increase heat transfer resistance.

- **Particle size reduction**

There may be a lower limit in particle size at which heat transfer or gas exchange becomes rate limiting and an upper limit at which nutrient transfer becomes limiting (Mudgett, 1986). Reid (1989a) found that lignin loss and digestibility increases were comparable in the wood shavings and smaller particles, but significantly less in the wood chips. The cost of size reduction will in the end determine which particle size is best for large-scale solid-state fermentations. The largest particle size required for a successful solid-state fermentation is the preferred option.

- **pH**

The bulk pH of the liquid phase may also be considerably different from local pH levels at solid surfaces near which major biological activity occurs (Mudgett, 1986). Not only is it almost impractical to monitor pH of the SSF, but more so for controlling or adjusting it. Laboratory studies should give an indication of the bulk pH and

whether that pH is acceptable or not. Adjustments should be made accordingly (for example chemical manipulation of pH or change of substrate).

- **Sterilisation**

Sterilisation is another expensive part of substrate preparation and should be just intense enough to prevent the growth of harmful contaminating microbes and permit the desired fungus to establish itself (Reid, 1989a). Sterilisation of aspen wood was essential for selective delignification by *Phlebia tremellosa* (Reid, 1989a). Autoclaving not only removed contaminants but also might have influenced the susceptibility of the substrate to enzymatic attack (Figure 2.23). *Phanerochaete chrysosporium* was used to successfully biopulp wood without the need for sterilisation of the wood (Wall *et al.*, 1993). Selective conditions for fungal growth may also be obtained by moistening the substrate with buffered or unbuffered medium at low pH and by using high densities of spore inoculum (Mudgett, 1986). Therefore, sterilisation of the substrate will be necessary if the inoculated fungus is a weak competitor.

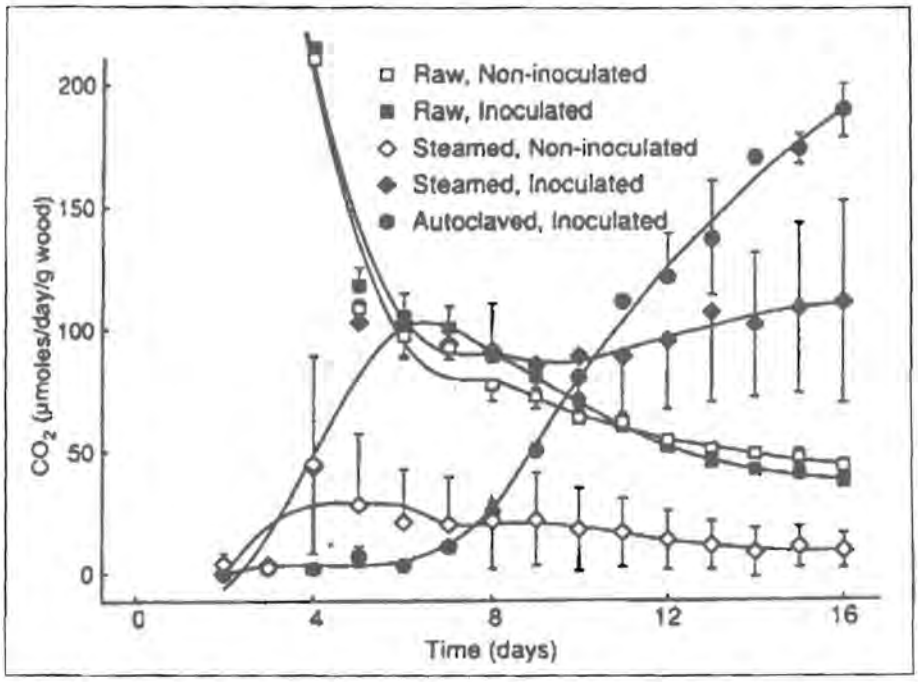

Figure 2.23: Effect of sterilisation and inoculation with *P. tremellosa* on CO_2 evolution from early stages of solid-state fermentation of aspen wood (Reid, 1989a).

- **Nutrient supplementation**

It may be beneficial to provide supplemented media to initiate biomass production, induce enzyme synthesis, provide balanced growth conditions or prolong secondary metabolite production (Mudgett, 1986). Some lignin-degrading fungi require a supplemental carbon source for growth and further lignin degradation. Carbohydrate supplements may have small beneficial effects, but the benefits are not large enough to make them of practical interest (Lee, 1997; Reid, 1989b). Lignin degradation by many white-rot fungi is suppressed by nitrogen. In addition, nitrogen added to lignocellulose substrates generally accelerates degradation and consumption of carbohydrates (Reid, 1989b). However, lignocellulose is a nutrient rich polymer and the fungus degrades a portion of the readily degradable compounds or the structural polysaccharides to satisfy its energy requirements.

10.1.4 Kinetics of SSF

Useful kinetic models for fungal growth in the solid state are not easy to obtain because of the complex nutritional nature of the substrate and the difficulty of estimating rates of biomass formation in the presence of the substrate (Mudgett, 1986). Lack of kinetic and design data on various fermentations and the fermenters further complicate matters, especially for industrial applications. Such models are instrumental for scale-up of pilot plants and are essential for determining the engineering feasibility of the project. Accurate predictions regarding the SSF process can be made from good mathematical models, provided that the correct parameters are monitored (as indicated by the model). Despite the tendency to protect industrial secrets, mathematical models describing aspects of SSF are published more frequently.

Two kinds of mathematical modelling efforts can be identified for SSF: 1) those dealing with microscopic surface growth and 2) those dealing with macroscopic variables (Mariano *et al.*, 1995). Valmaseda *et al.* (1991) has used an extensive biochemical and physiological approach to describe the kinetics of lignocellulose

biodegradation by two white-rot fungi. However, models dealing with both microscopic and macroscopic observations are required.

The importance of adequate heat transfer has led to the development of mathematical models describing two-dimensional heat transfer (Sangsurasak and Mitchell, 1995; Sangsurasak and Mitchell, 1998) and the effect of temperature on SSF kinetics (Smits *et al.*, 1998). The model of Sangsurasak and Mitchell (1995) combines the physical processes of heat transfer with microbial growth kinetics, to predict the temperature and the resulting growth at any location with a packed bed-reactor. The temperatures reached in the packed-bed column is most sensitive to parameters which affect the peak heat load, including the substrate packing density, the maximum specific growth rate and the maximum biomass concentration (Sangsurasak and Mitchell, 1998). Their model suggests that evaporation can remove as much as 78% of the heat from the bed during times of peak heat generation.

Most of the models described here were not developed for SSF of lignocellulose. However, the principles are valuable and can be modified for our purposes. The modelling of plant litter degradation in soil has been reviewed in paragraph 2.4.3. By understanding these recent developments in mathematical modelling of SSF, the models described for lignocellulose biodegradation in soil can be used to make accurate predictions and increase the feasibility of our analytical approach. Clever mathematical modelling combined with practical and cost saving engineering will overcome most of the disadvantages associated with solid state fermentations like overheating, aeration and moisture content.

10.1.5 SSF bioreactor configurations

- *Koji reactors*

The rice koji process provides an interesting and useful model for aerobic SSF (Mudgett, 1986). Washed, filtered air is circulated through the grain bed, humidified by a spray shower, and heated to control the temperature and humidity of the fermenting solids (Figure 2.24).

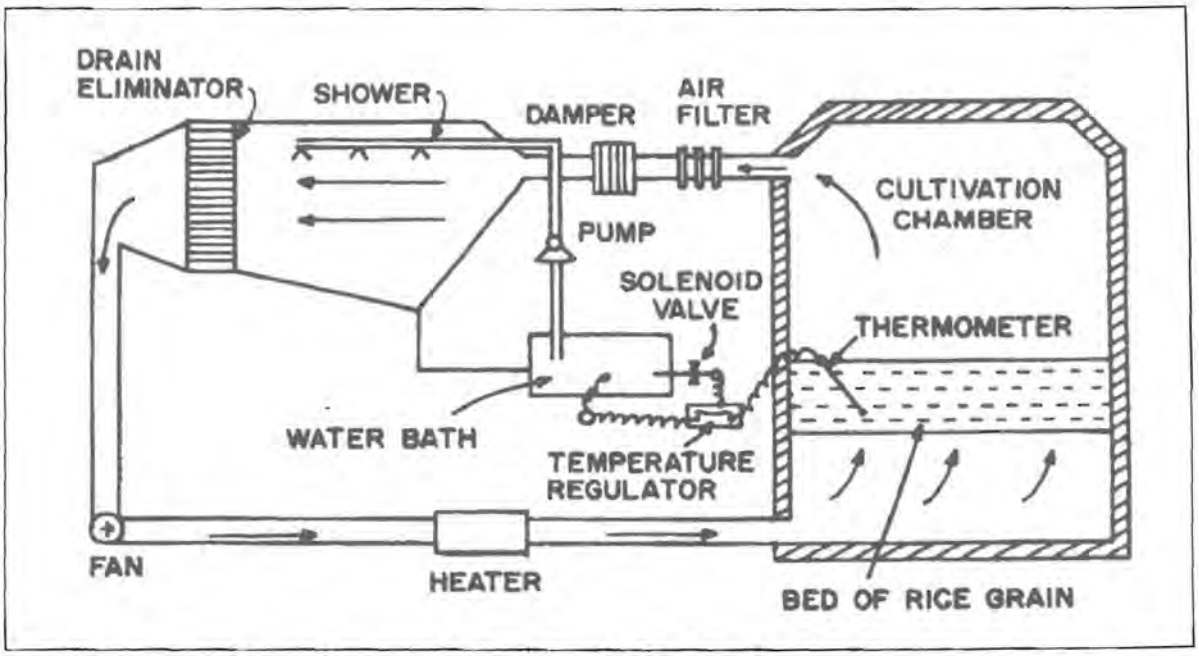

Figure 2.24: Schematic representation of a koji-making apparatus. (Mudgett, 1986).

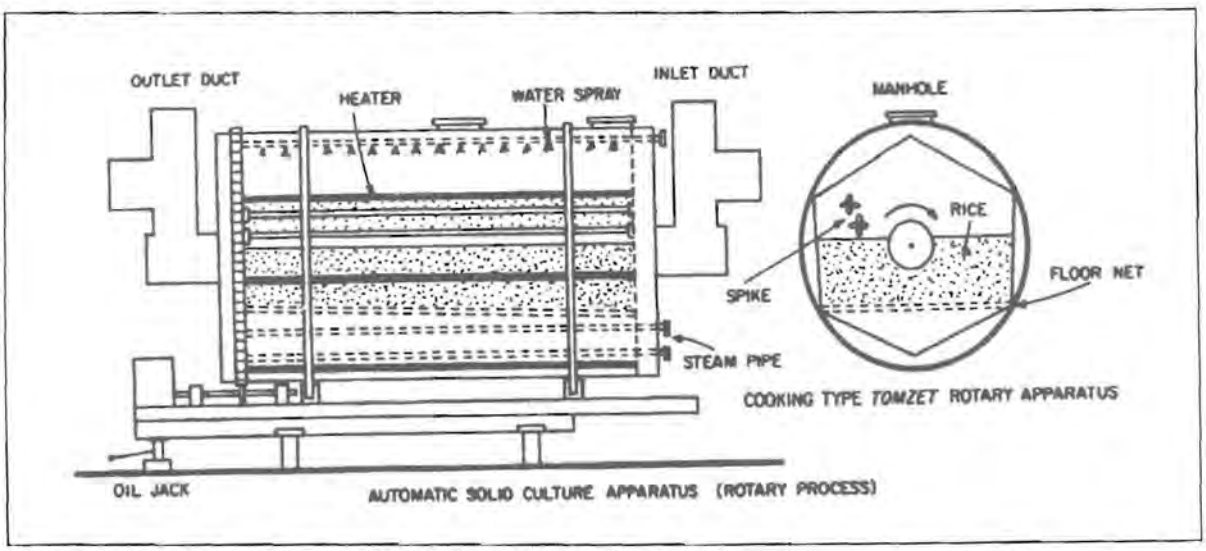

Figure 2.25: Rotary drum-type koji-making apparatus. Unit operations like washing, cooking, inoculation, water spraying, cooling, air circulation, filling and exhausting can be done in this apparatus (Mudgett, 1986).

Many koji-making apparatus have been developed but the principles of operation are similar. In the rotary drum systems, mixing and temperature control is accomplished by rotating the drum while blowing in conditioned air (Figure 2.25) (Cannel and Moo-Young, 1980).

Aidoo *et al.* (1982) described the pot method previously described by Hao, Fulmer and Underkofler. According the original authors, this method was superior to the rotating drum. The equipment required less space and was less complicated. The mould mycelium was not disturbed during growth and uniform aeration was obtained, resulting in good growth. The method consisted of packing 750 g moist wheat bran into a 3-quart (approximately 3408 ml) aluminium pot with holes at the bottom. Air was passed through the mass after inoculation.

- *Bench-scale reactors*

Levonen-Munoz and Bone (1985) studied the effect of different gas environments on bench-scale solid-state fermentations of oat straw by white-rot fungi. Polycarbonate jars (4.5 l volume; 23 x 16 cm) were used as the solid-state fermenters. The jars could be closed with a lid equipped with several ventilation holes. Gas was introduced to the fermenters through stainless-steel aeration coils situated in the bottom. The coils had small holes at regular intervals. Samples of the gas composition were taken with a syringe through a stainless-steel port situated in the middle of the straw bed.

- *Stirred tank reactors*

The cultivation of *P. chrysosporium* in stirred tank reactors has not been successful to date. Earlier attempts achieved low-level production of lignin peroxidases, but scale-up to a 300 l reactor failed (Janshekar and Fiechter, 1988). This supports the statement that SSF is the method of choice for biological delignification (Reid, 1989b). Although the stirred tank reactor described here functions on the submerged fermentation principle, its failure to facilitate the growth of the fungus emphasises the advantages of SSF.

- *Chip pile-based systems*

A modified chip pile-based system was described for biopulping of wood chips (Figure 2.26) (Wall *et al.*, 1993). The modifications include temperature control and aeration by forced or free convection, chip turning to decrease pile heterogeneity, and sprinklers or forced convection of humidified air to supply moisture. The advantage of this system is reduced cost as compared to packed bed reactors; the disadvantage is reduced process control. A recent publication emphasised the economic feasibility of this system, however scale-up experiments are still being run (Scott *et al.*, 1998).

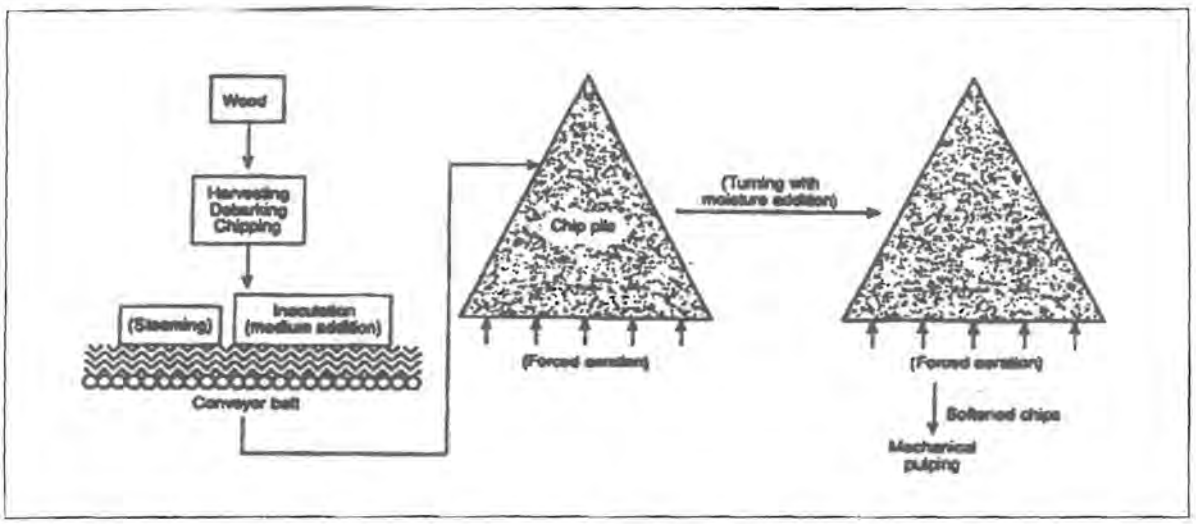

Figure 2.26: Chip pile-based system process flowsheet, () = optional (Wall *et al.*, 1993).

- *Packed bed reactors*

Wall *et al.* (1993) evaluated the scale-up considerations for two biopulping reactor types: a chip pile-based system (Figure 2.26) and a packed bed reactor (Figure 2.27). Packed bed reactors allow better control of process conditions such as aeration rate, gas concentration, moisture delivery and exposure to contaminants than chip pile-based systems. These reactors require greater capital expenditure and higher operating costs. Also, the packed bed reactors are unlikely to be economical unless the two-week treatment is reduced to 2-3 d. A simple and practical design is also a major

requisite. Steaming the wood chips at atmospheric pressure can perform reduction of the contamination load in a packed bed reactor.

Packed-bed SSF reactors with forced aeration have performance superior to that of tray-type (shallow or deep static) fermentations but were also found to have regions of non-uniform protein production (Silva and Yang, 1998).

Lu *et al.* (1998) utilised a multi-layer packed-bed reactor for citric acid production by *Aspergillus niger* (Figure 2.28).

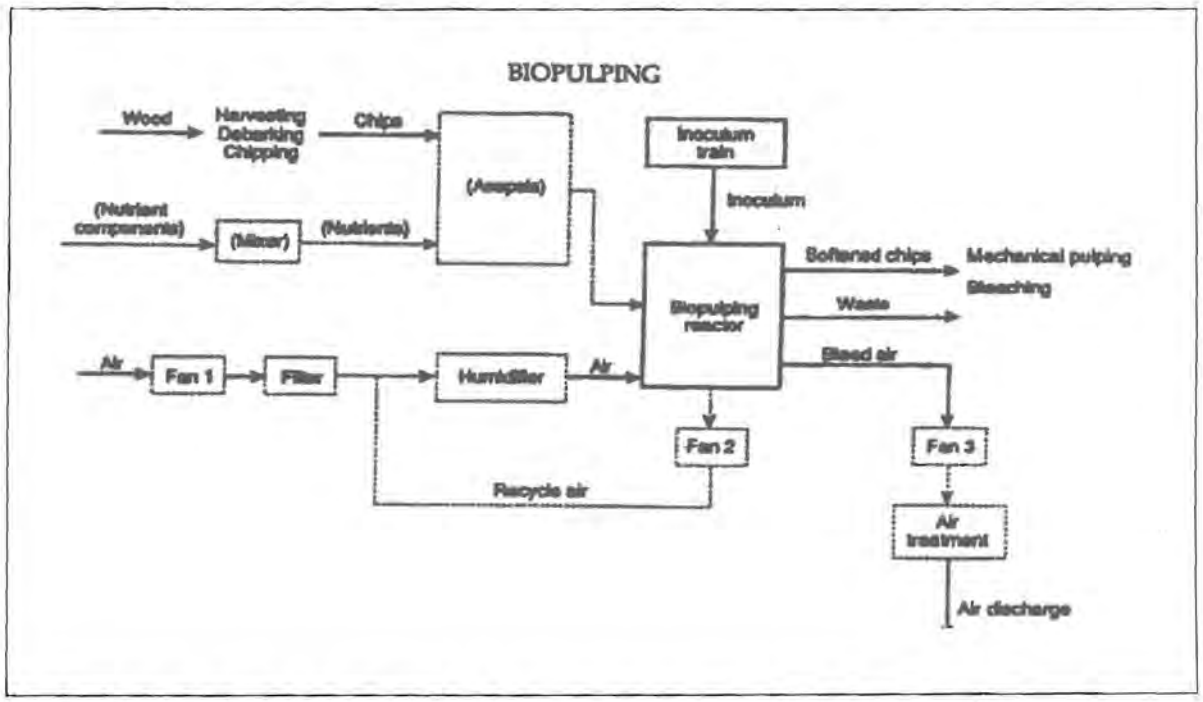

Figure 2.27: Packed bed reactor process flowsheet, () optional (Wall *et al.*, 1993).

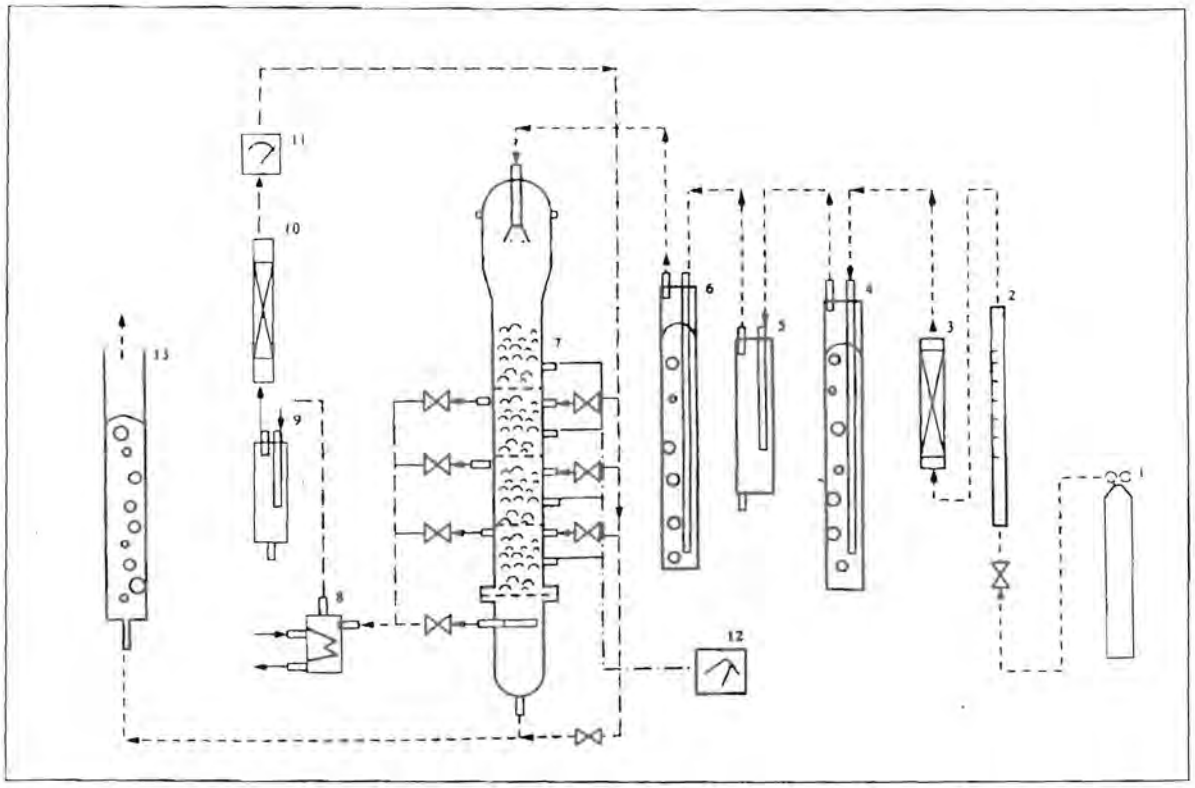

Figure 2.28: Schematic diagram of the multi-layer packed-bed reactor system: (1) compressed air cylinder, (2) rotameter, (3) filter, (4) NaOH solution, (5) water trap, (6) humidifier, (7) reactor, (8) condenser, (9) water trap, (10) silica gel column, (11) carbon dioxide analyser (12), temperature recorder, (13) collector. Air entered the reactor from the top and passed successively through layers 1, 2, 3 and 4 (Lu *et al.*,1998).

- *Spouted-bed bioreactors*

The first report on a gas-solid spouted-bed reactor described its application in the production of amylase from rice in SSF conditions (Figure 2.29) (Silva and Yang, 1998). The spouted bed reactor was developed to overcome many of the problems inherent to large-scale SSF systems, including mass- and heat-transfer limitations in the conventional tray reactors and solids-handling difficulties seen in packed-bed reactors. The spouted bioreactor with intermittent spouting with air achieved high production levels in both total protein and enzymes (α-amylase, β-amylase and glucoamylase). Continual spouting was found to be detrimental to the SSF, because of shear or impact damage to fungal mycelia during spouting.

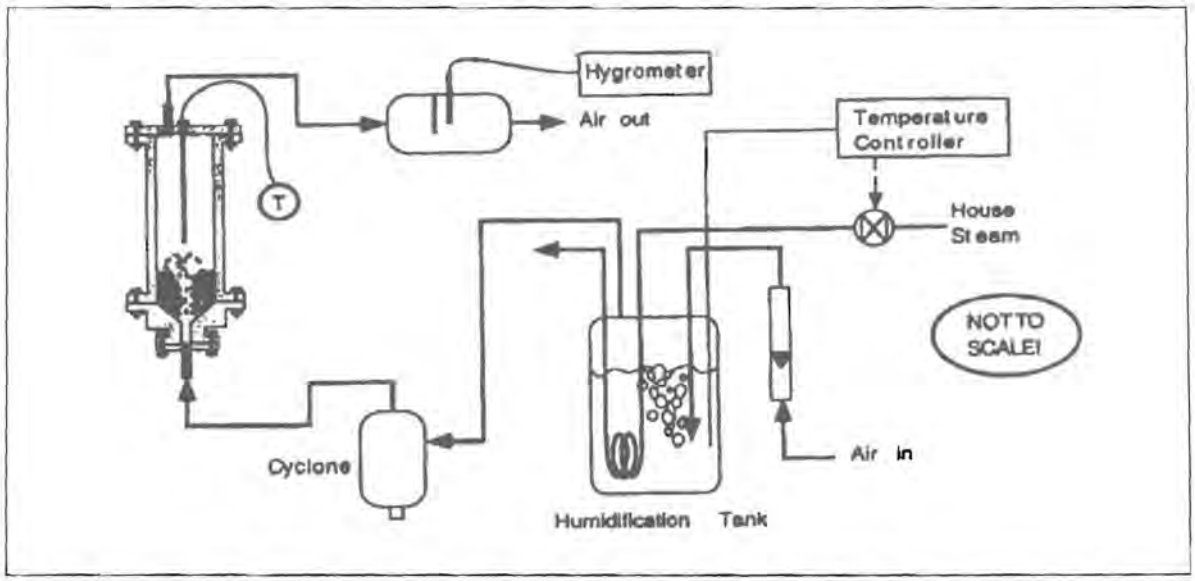

Figure 2.29: Schematic diagram of the spouted-bed bioreactor (Silva and Yang, 1998).

- *The INRA-DIJON reactors*

The Institut de la Recherche Agronomique (INRA) in Dijon, France developed a range of reactors for solid-state fermentations (Durand *et al.*, 1993). An important component of these reactors are a specially designed device for regulating temperature and water content of a solid medium. This device aims to overcome heat and mass transfer restrictions commonly associated with SSF and can be used for all their reactor types. The lab scale reactors are 2 l wide-mouthed jars sealed by a cap, and supplied with thermostated air. These lab scale reactors are used for strain and medium screening, optimisation of culture conditions, physiology studies and the development of analytical procedures. The prepilot reactors are used for studying physical parameters (air channelling, medium compaction, heat and oxygen transfers etc.) (Figure 2.30).

The pilot reactor was used for studying scaling up (effect of agitation, shape of agitation device etc.) and the downstream processes (Figure 2.31). Agitation is accomplished by means of three screws mounted on a conveyor that moves them back

and forth across the reactor. Humidified air is forced through the substrate bed from beneath. Probes are strategically placed to monitor key variables. The process control is automated to better regulate key parameters.

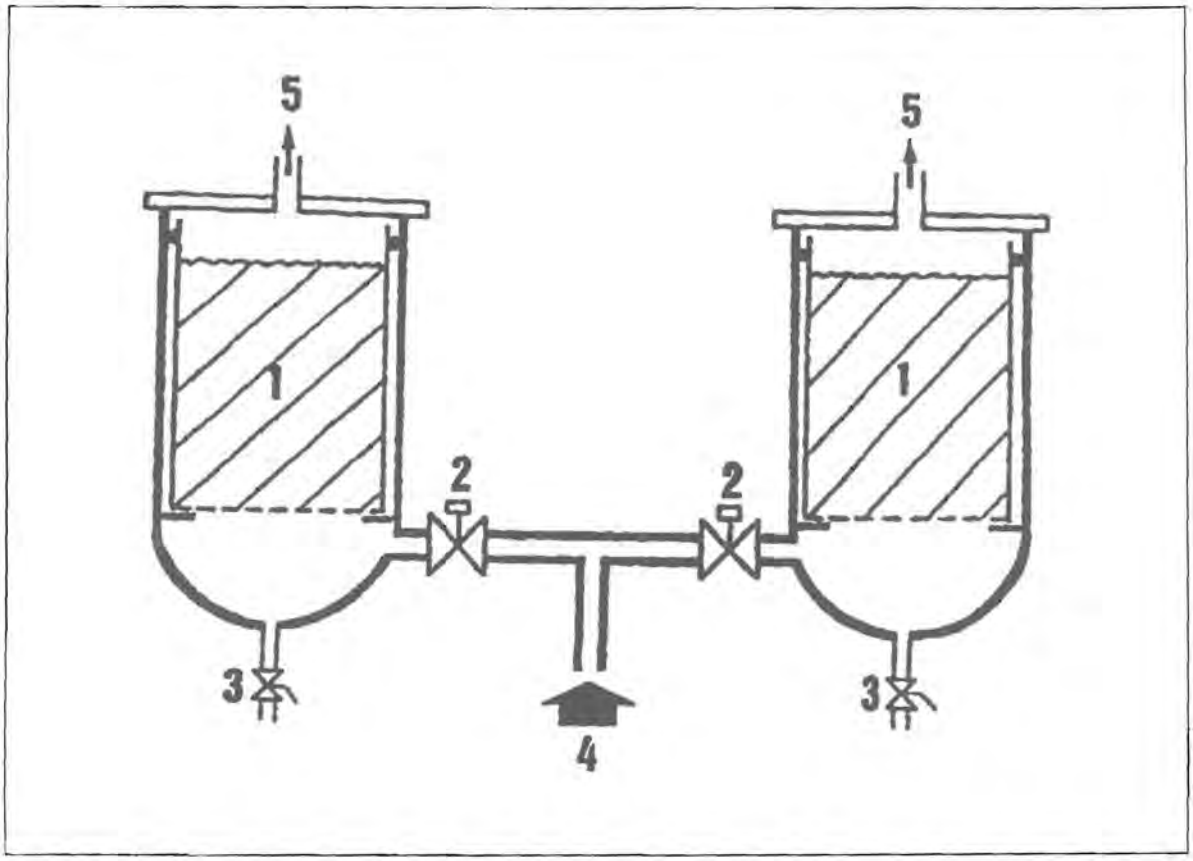

Figure 2.30: Schematic diagram of the INRA-DIJON prepilot reactors: (1) baskets with a perforated bottom containing medium up to 30 cm height, (2) valves for air flow adjustment, (3) valves for cleaning, (4) thermostated air coming from special device, (5) air out to gas analysers (Durand *et al.*, 1993).

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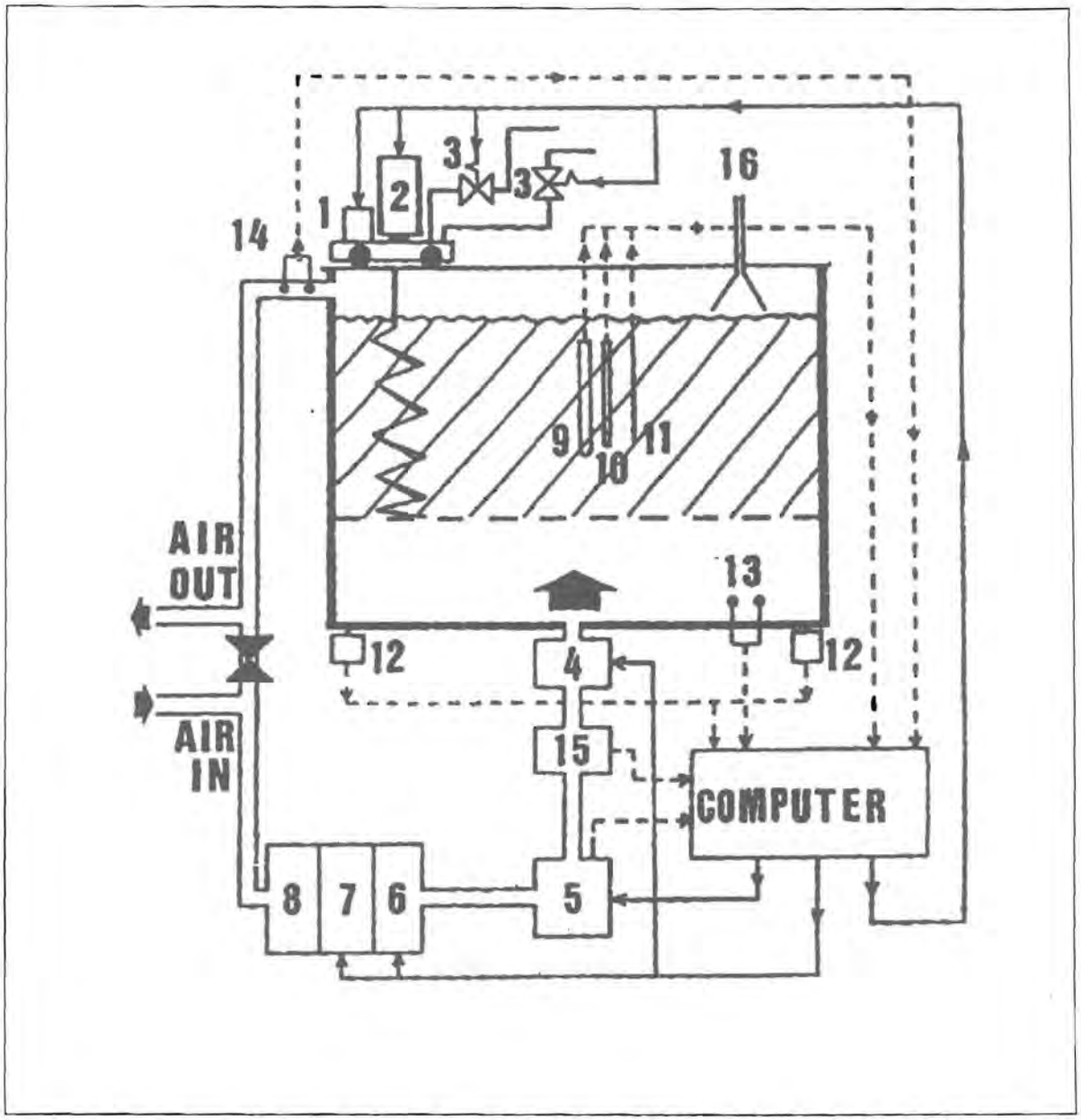

Figure 2.31: Schematic diagram of the INRA-DIJON pilot plant: (1) carriage motor, (2) screw motor, (3) electric valves, (4) humidifier, (5) fan, (6) heater, (7) cooler, (8) filter, (9) oxygen probe, (10) pH probe, (11) temperature probe, (12) weight gauges, (13) relative humidity probe, (14) temperature probe, (15) flowmeter, (16) to gas analysers (Durand *et al.*, 1993).

- ***Other reactor types***

The pneumatic and mechanically agitated reactors described by Bonnarme *et al.* (1993) are ideal for producing fungal biomass and secondary metabolites like lignin and manganese peroxidases (Figure 2.32). The cultures are grown in a synthetic medium containing glycerol and nitrogen. However, these reactor configurations are not similar to the classic SSF systems. Entirely different parameters must be monitored and the conditions inside the reactors are very dynamic. This makes these types of reactors more difficult to construct and maintain than SSF reactors, although better process control is obtained.

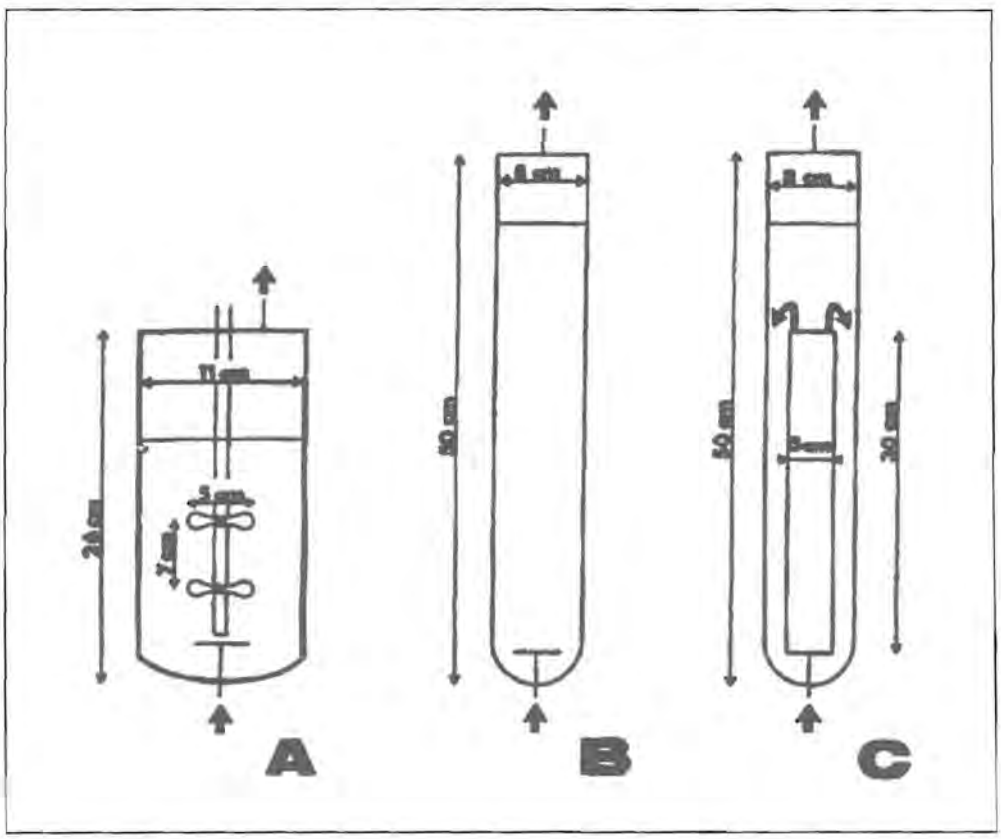

Figure 2.32: Agitated vessels used for lignin and manganese peroxidases production by *Phanerochaete chrysosporium*. A) stirred tank reactor (STR); B) bubble column reactor (BCR) and C) airlift reactor (ALR) (Bonnarme *et al.*, 1993).

Laukevics *et al.* (1984) compared the efficiency of SSF with submerged fermentation and alternative lignocellulose conversion processes. Six different reactor configurations were evaluated and will be discussed briefly (Figure 2.33). The SSF had lower overall efficiency but higher product concentration per reaction volume than the other conversion schemes.

- *1.5 m^3 Mixed-layer pilot fermenter*

The mixed-layer pilot fermenter is provided with an internal diffuser type heat exchanger (circular chamber) in addition to an outside jacket for more efficient heat removal (Figure 2.33, a). Mixing is provided by an Auger drive that moves the substrate upwards and spreads it over the diffuser to synchronously rotating paddles. The mixer is operated periodically at 5-10 rpm for 1 min at 2-5 min intervals to avoid fragmentation of mycelia.

- *Hollow shaft horizontal fermenter*

The hollow shaft horizontal fermenter is the improved version of the horizontal paddle fermenter (Figure 2.33, b and c). Heat removal is facilitated through the circulation of cold water through the hollow shaft. It has interchangeable blades to suit the nature of the substrate, and has a reversible drive to spread the substrate more evenly.

- *SF-SSF fermenter*

SSF may be conducted in the upper cylindrical apparatus while submerged fermentation (SF) may be conducted in the bottom part (Figure 2.33, 1d and 2d). The apparatus is suitable for combined mixed fermentations, permitting percolation and recirculation of nutrients. Air is bubbled through the liquid and led under the perforated plate or through the solid substrate by a special diffuser.

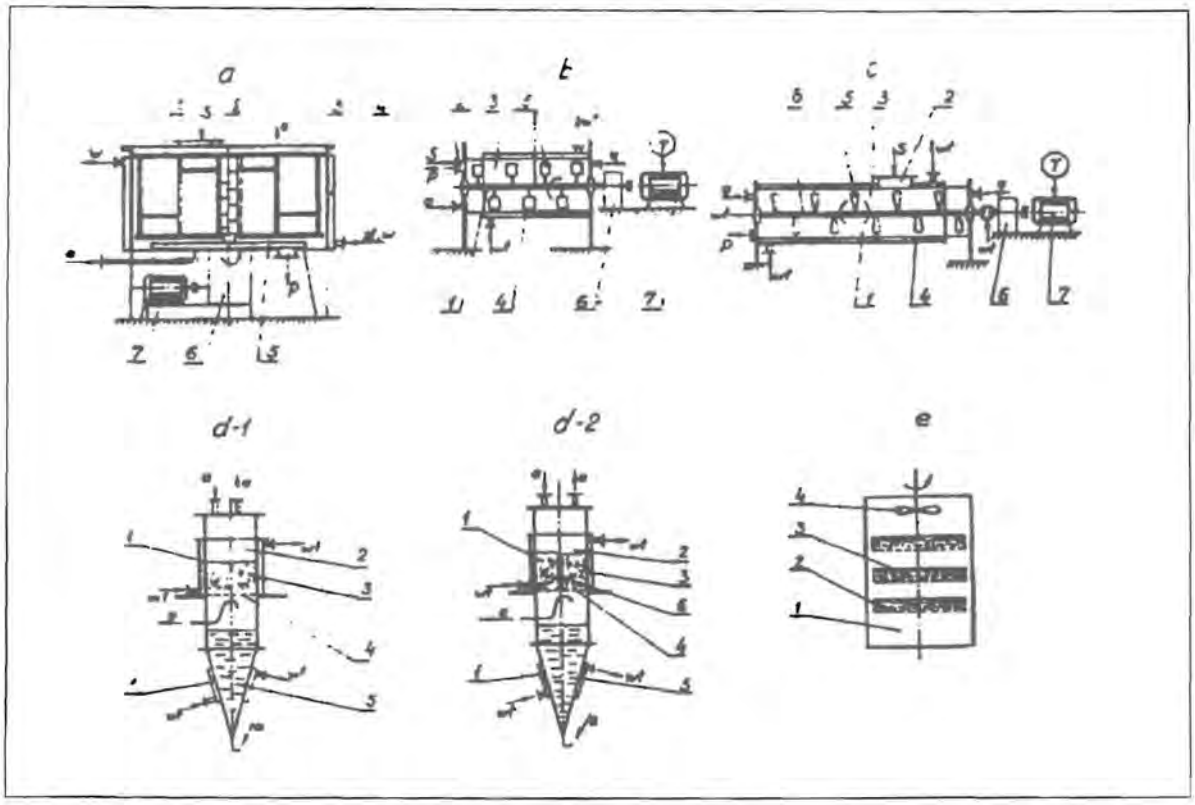

Figure 2.33: Fermentation equipment used for comparing SSF with submerged fermentation. **(a) Mixed-layer pilot fermenter**: (1) substrate chamber, (2) loading port, (3) circulation-type diffusion heat exchanger, (4) jacket, (5) rotating platform with Auger drive and paddles, (6) shaft, (7) motor. **(b) Horizontal paddle fermenter**: (1) shaft, (2) loading port, (3) substrate chamber, (4) jacket, (5) paddles, (6) gearbox, (7) motor. **(c) Horizontal hollow shaft fermenter**: (1) body of apparatus, (2) loading port, (3) substrate chamber, (4) jacket, (5) mixer with interchangeable blades, (6) gear box, (7) reversible motor, (8) hollow shaft. **(d1) Submerged fermentation – SSF bioreactor**: (1), jacket, (2) body of apparatus, (3) substrate, (4) perforated tray, (5) submerged cultivation vessel. **(d2) Submerged fermentation – SSF bioreactor with special air diffuser**: (1) jacket, (2) body of apparatus, (3) substrate, (4) perforated tray, (5) submerged cultivation vessel, (6) air diffuser. **(e) Stationary trays in climatic chamber**: (1) chamber, (2) trays, (3) substrate, (4) fan. The symbols are: **(S)** substrate, **(w)** water, **(P)** product, **(a)** air, **(st)** steam, **(wt)** water by temperature of fermentation, **(T)** timer. (Laukevics *et al.*,1984).

10.1.6 SSF bioreactor design considerations

Key design considerations that should be included in any scale-up design are techniques of inoculation; sampling; transfer systems; sterilisation of equipment, air and fermenting medium; monitoring, and measurement and control of various parameters (Aidoo *et al.*, 1982). Waal *et al.* (1993) listed the key design decisions as

- Choice of organism,
- Degree of asepsis,
- Size and type of inoculum,
- Physical conditions such as temperature, aeration and moisture content, and
- Type and amount of added nutrients if such supplementation is required.

According to Durand *et al.* (1993) the choice of reactor design and the elaboration of an efficient parameter control system are very important. As interest in developing SSF technology increased over the past years, so did the complexity of the variables involved. Classical key variables that should be monitored are cell growth, substrate consumption and product formation (Mariano *et al.*, 1995). Other variables identified by the authors are bulk and inlet air temperatures, water availability, water activity, and void space availability. These variables have strong effects on the physiological and biochemical activities of the organisms used, and consequently, the global effectiveness of the system. The bioengineering aspects of SSF have recently been summarised: "the various substrates used in SSF will have different physical and heat transfer properties, while microbes used in SSF will vary widely in growth characteristics such as maximum specific growth rate, maximum biomass density, and the sensitivity of the specific growth rate to increases in temperature." (Sangsurasak and Mitchell, 1998).

The type of measurements will affect the incorporation of sampling ports into the reactor design. The monitoring of gas evolution (O_2, CO_2, N_2 and H_2S) will require the instalment of appropriate gas analysers at various levels within the solid matrix. The monitoring of the temperature (solid matrix and headspace) and relative humidity of the incoming air will require the presence of adequate measuring devices. It is the

responsibility of the investigators to decide which variables should be monitored in order to give clear answers to the questions at hand. Sampling should be done aseptically, and samples (physical, chemical or biological) should be representative. Biological delignification is sufficiently slow that it would be practical to take samples of the fermenting substrate for off-line analysis (Reid, 1989b). This introduces us to the analytical approach used to accurately describe the physical, chemical and biological parameters and their respective interactions. A major problem in SSF bioreactor operation is the inability to get true biomass estimations. The analytical approach should be based on simplicity and reproducibility, without sacrificing reliability.

Solid-state fermentation technology is robust enough for it to be implemented at the AMD affected mine site without large financial investment. However, the larger the substrate mass to be pre-treated, the greater the need for process monitoring (biological activity, uniform colonisation etc.) and control (aeration, heat transfer etc.). Substrate inoculation will be a very sensitive part of the operation and must conform to rigorous quality standards.

10.2 Silage manufacturing

Silage is the material produced by the controlled fermentation of a crop of high moisture content. Lactic acid bacteria ferment the naturally occurring sugars in the crop to a mixture of acids, predominantly lactic acid. The nutritional value of the silage produced depends firstly upon the species and stage of growth of the harvested crop, and secondly upon the changes resulting from the activities of plant enzymes and microorganisms during the harvesting and storage period (McDonald *et al.*, 1995; Shaver and Batajoo, 1995).

10.2.1 Role of plant enzymes in silage manufacture

Chemical changes occur in the plant immediately after harvesting. Plant respiration will continue as long as oxygen and substrate is available. Soluble carbohydrates are lost during the respiration process. Proteolysis after harvesting may cause the protein yield to be reduced by 50% within a few days. Proteolysis declines during ensilaging as the pH decreases.

10.2.2 Role of microorganisms in silage manufacture

Lactic acid bacteria are normally present on crops in small numbers. When the crop is ensilaged, their numbers increase rapidly, fermenting the water-soluble carbohydrates to organic acids.

Clostridia are present on crops in the form of spores and will only grow under strictly anaerobic conditions. Saccharolytic clostridia ferment lactic acid and water-soluble carbohydrates to butyric acid resulting in an increase of the pH. Proteolytic clostridia ferment mainly amino acids to a variety of products (mainly acetic and butyric acids, amines and ammonia).

Enterobacteria are present in low numbers on crops and being facultative anaerobes, they compete with the lactic acid bacteria for the water-soluble carbohydrates. The fermentation products include acetic acid, ethanol and hydrogen. Proteolytic activity of these organisms led to increased levels of ammonia.

Yeasts and fungi play an important role in the deterioration of silage when it is exposed to air. The majority of these species are strict aerobes and occurs on the surfaces of the plant material. The growth of filamentous fungi on the plant material is undesirable because of mycotoxin production that might negatively affect the desired silage microflora and the animals fed on the silage. Species of *Candida*, *Saccharomyces* and *Torulopsis* are the dominant yeast genera associated with silage.

Aspergillus, *Fusarium* and *Penicillium* species are known for their ability to produce toxins and are frequently isolated from deteriorated silages.

10.2.3 Loss of nutrients during silage manufacture

With crops cut and ensiled the same day, nutrient losses are negligible and even over a 24 h wilting period, losses of dry matter of not more than 1-2% may be expected. Longer wilting periods cause considerable losses of nutrients with water-soluble carbohydrates and proteins the nutrients most affected.

Oxidation loss results from the action of plant and microbial enzymes on substrates such as sugars in the presence of oxygen, leading to the formation of CO_2 and water.

Overall dry matter and energy losses due the activities of lactic acid bacteria are low. In clostridial and enterobacterial fermentations, because of the evolution of CO_2, hydrogen and ammonia, nutrient losses will be much higher than in lactic acid bacterial fermentations.

In most silage silos, free drainage occurs and the liquid or effluent carries with it soluble nutrients. The amount of effluent produced depends largely upon the initial moisture content of the crop.

Silage is a natural fermentation of forage lignocellulose by naturally occurring bacteria. Silage has an improved nutritional value in terms of organic acids the animal can utilize. These organic acids produced during silage making can serve as a carbon source for sulphate reducing bacteria. Therefore, silage is an anaerobic process that can be used as an alternative carbon source pretreatment method. However, unlike silage, previous studies have shown that hay was the best carbon source for sustaining sulphate removal from artificial AMD.

10.3 Biopulping

Lignin becomes problematic to postharvest, cellulose-based wood processing, because it must be separated from cellulose at enormous energy, chemical and environmental expense. Biopulping is a solid-state fermentation process in which wood chips are treated with white-rot fungi to improve the pulping process. Repression of lignin biosynthesis resulting in cellulose accumulation and healthy growth of transgenic trees has been achieved (Hu *et al.*, 1999).

Biological pulping has the potential to improve the quality of pulp, properties of paper and to reduce energy costs and environmental impact relative to traditional pulping operations (Breen and Singleton, 1999; Scott *et al.*, 1998). Several problems still plague this concept: Scale-up to industrial process requirements presents challenges that are difficult to simulate in laboratory tests. Inoculation, aeration and heat dissipation are key parameters for maintaining fungal activity. Hence, problems regarding the management of these parameters to ensure adequate colonisation and metabolic activity are complex. Also, poor colonisation of wood chips has been attributed to competition with naturally occurring microorganisms or to inhibition by wood components (Wolfaardt *et al.*, 1999). Breen and Singleton (1999) summarised biopulping research as follows: "Overcoming these challenges will determine, in a large part, if biopulping becomes a reality."

The biopulping project was an ambitious move by man to overcome increasing resistance against conventional chemical pulping operations. However, this endeavour appears to be unsuccessful and this casts a shadow on future commercial applications of biological delignification by white-rot fungi. The natural process of lignocellulose decay is more complex that initially anticipated. The major problem remains the choice of organism. Wood chips can be completely sterilised, but with large expenses. Secondary contamination by airborne microorganisms must then be prevented and this incurs additional costs. An organism capable of establishing itself in a hostile environment with no additional nutrient and atmosphere requirements will solve most of the problems. The existence of such an organism remains unproved.

11. References

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CHAPTER 3

BIOLOGICAL PRETREATMENT OF *Cenchrus ciliaris* cv. Molopo (BUFFELSGRASS)

ABSTRACT

The biological treatment of acid mine drainage using sulphate reducing bacteria is an alternative to chemical treatment. However, substrate availability normally becomes the limiting factor for sustaining sulphate reduction. Biological pretreatment of complex carbon sources by white rot fungi was investigated to enhance the biodegradability of the lignocellulose substrate. The effect of substrate particle size was evaluated for natural and steam pasteurized *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) which was inoculated with *Phanerochaete chrysosporium, Pleurotus ostreatus,* and *Schizophyllum commune*. Uninoculated controls were run in parallel. *In vitro* dry matter digestibility was used to measure the effectiveness of the fungal delignification processes. In all treatments, the initial digestibility was high (64.8%), rapidly decreasing after 1 to 2 weeks. None of the treatments increased the digestibility of the natural substrate. Only *P. ostreatus* was capable of improving the initial digestibility of steam pasteurized grass by 7% over 6 weeks. Substrate particle size influenced substrate degradation in terms of fungal community structures, but did not influence the overall extent of degradation by the inoculated fungi and the naturally occurring microflora. The pH of all natural grass treatments increased rapidly to an alkaline pH in excess of 9. It is known that alkaline treatment can increase forage digestibility, however, in this study the alkaline pH decreased the substrate digestibility by leaching potentially digestible compounds. This was confirmed by the brown leachaté that was produced over the experimental period in all treatments. The leachate that was produced could be a direct result of the breakdown of lignocellulose, yielding potentially readily utilisable carbon.

1. INTRODUCTION

Agricultural wastes and lignocellulose are potential inexpensive and available sources of fermentable carbon compounds. During the development of a mixed aerobic-anaerobic microbial treatment process for acid-mine drainage using straw as substrate, substrate availability became the limiting factor in terms of sulphate reduction (Béchard *et al.*, 1994). The authors assumed that the biodegradation of the straw would provide organic carbon necessary to sustain the treatment process. However, the long-term stability of their bioreactors could not be maintained and supplementation with urea and sucrose was required. Carbon was the primary limiting factor in the treatment process.

Plant biomass is made up of predominantly cellulose, hemicellulose and lignin. Up to 50% of the lignocellulose biomass in nature consists of cellulose and the potential for producing fermentable sugars from the cellulose biomass is undergoing intense investigation. However, cellulose fibers are embedded into a lignin matrix, which protects them from enzymatic hydrolysis (Leonowicz *et al.*, 1999; Tomme *et al.*, 1995). Lignin makes up about 20-30% of wood and up to 15% of grasses, and is found in cell walls in a complex with cellulose and hemicellulose polysaccharides (Jeffries, 1990; Mueller-Harvey and Hartley, 1986). In this natural composite material, the cellulose fibrils provide tensile strength, and the hemicellulose and lignin provide cross-linking, binding the structure together (Jeffries, 1990; Tomme *et al.*, 1995).

The biological process for converting the lignocellulose to fermentable carbon compounds requires delignification to liberate cellulose and hemicellulose from their complex with lignin, and depolymerization of the carbohydrate polymers to produce free sugars or short sugar chains (Lee, 1997). These compounds can then be fermented to organic acids by microbial consortia occurring in either aerobic or anaerobic environments, which in turn will provide a carbon source for sulphate reducing bacteria. Water-soluble carbohydrates and phenolics are released from grass lignocellulose during hot water treatment (Milstein *et al.*, 1981; Vered *et al.*, 1981). Such a leachate can contain a mixture of growth promoting substances, antimicrobial compounds and fermentable substrates.

If the purpose of biological delignification is to obtain a cellulose enriched substrate that can be further fermented under anaerobic conditions to low molecular weight organic acids, then the mechanism of delignification should be very specific. White rot fungi are the only microorganisms capable of degrading lignin completely (Reid, 1995). Different species of these fungi exhibit different degradative specificities towards cellulose, hemicellulose and lignin (Kuhad *et al.*, 1997). Many species with accurate lignin degradation capabilities have been described in literature (Lee, 1997; Reid, 1989). An additional requirement should be that the polysaccharide component of lignocellulose must be left untouched. *Pleurotus ostreatus* is one fungus that meets these requirements (Camarero *et al.*, 1998; Hadar *et al.*, 1992; Platt *et al.*, 1984). Therefore, white rot fungi can potentially be used for the biological pretreatment of lignocellulose biomass to make the cellulose and hemicellulose components more accessible for further use in a passive AMD treatment.

Biological pretreatment of complex carbon sources must be accomplished on site in order to meet the requirements for a passive AMD treatment system. This implies that the substrate cannot be sterilized and that the pretreatment agents should be able to compete effectively with the natural microflora present on the substrate. Survival of *P. ostreatus* in soil has been demonstrated, but not for *Phanerochaete chrysosporium* (Lang *et al.*, 1997; Radtke *et al.*, 1994). This implies that potential pretreatment agents should be screened for their ability to survive on the natural substrate without inhibition of its delignification capabilities. *Schizophyllum commune* is frequently observed on decaying trees and mechanisms for ensuring efficient colonization and degradation must exist within this fungus. *Phanerochaete chrysosporium* is the model organism used for studying lignocellulose biodegradation. Ease of handling and rapid growth makes it a favourite choice for lignocellulose biodegradation studies.

The objective of this study was to investigate the biological pretreatment of *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) with *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Schizophyllum commune*. The fungal community structure of the decaying grass was also studied.

2. MATERIALS AND METHODS

2.1 Fungal strains

Pleurotus ostreatus was obtained from the Department of Botany basidiomycete culture collection, University of Pretoria. *Phanerochaete chrysosporium* strain WR 230 was obtained from the Council for Scientific and Industrial Research (CSIR) fungi culture collection. *Schizophyllum commune* was isolated from decayed plant material found in a suburban garden. All strains were maintained on Malt Extract Agar (MEA) at 25°C.

2.2 Substrate preparation

Buffelsgrass (*Cenchrus ciliaris* cv. Molopo) was obtained from a farm near Pienaarsrivier, 60 km north of Pretoria. A hammer mill was used to prepare two particle sizes: fine cut (1-2 cm pieces) and medium cut (5-10 cm pieces). Uncut grass (longer than 15 cm) was used as the third particle size.

2.3 Preparation of fungal inocula and experimental setup

2.3.1 Preparation of pre-inoculum

Agar squares (6 mm x 6 mm) from 10 d old *Pleurotus ostreatus* and *Schizophyllum commune* pure cultures were inoculated aseptically into Malt Extract (ME) broth (supplemented with 250 mg/l chloramphenicol) and incubated on a rotary shaker (130 rpm) at 28°C. Growth was detected visually after 24 to 72 h. The broths were aseptically homogenised for 20 s with a high-speed homogeniser (Ultra-Turrax supplied by Janke & Kunkel Ika-werk).

50 g grass was placed in a 2 l Erlenmeyer flask, moistened with 150 ml of distilled water (dH_2O) and autoclaved twice at 121°C for 30 min. The autoclaved grass was inoculated separately with 5 ml of the liquid inoculum prepared for *P. ostreatus* and *S. commune* and 20 or more agar squares (6 mm x 6 mm) from 10 d old *P.*

chrysosporium pure cultures. The inoculated grass was incubated at room temperature for 7 d or until sufficient fungal colonization was visible.

2.3.2 Preparation of large-scale inoculum

Medium cut grass was soaked in tap water for 18 h and steamed for 4 h. 288 g steamed grass was aseptically inoculated with 12 g of the fungal pre-inocula and packaged into plastic tubing reactors (20 cm x 50 cm x 50 µm) (Figure 3.1). Ventilation holes were made 5 cm apart. The experimental reactors were incubated at 20°C – 25°C until sufficient fungal growth was visible.

2.4 Substrate inoculation

280 g natural grass were moistened with 500 ml of tap water and inoculated with 20 g (wet weight; 5 g dry mass) of inoculum. 740 g steamed grass was inoculated with 60 g (wet weight) of the inoculum. The inoculated substrate was placed in plastic tubing reactors (20 cm x 50 cm x 50 i m) sealed and ventilation holes were made 20 cm apart. Completed reactors were incubated at 20°C-25°C. Reactors were constructed in triplicate for all substrate treatments and particle sizes. Uninoculated controls were run in parallel.

Figure 3.1: Completed plastic tubing reactor constructed for the biological pretreatment of Buffelsgrass.

2.5 Sampling

Series containing triplicate reactor sets were harvested on a weekly basis for six weeks (Figure 3.2). One reactor per set was stored at 4°C in case additional analyses were required. The entire contents of the second reactor was dried and a composite sample milled (hereinafter referred to as "Dry sample"). The remaining reactor was used for wet chemistry analyses (hereinafter referred to as "Chem sample"). Composite samples from this reactor were used for drying, microbiological analysis, pH and digestibility experiments.

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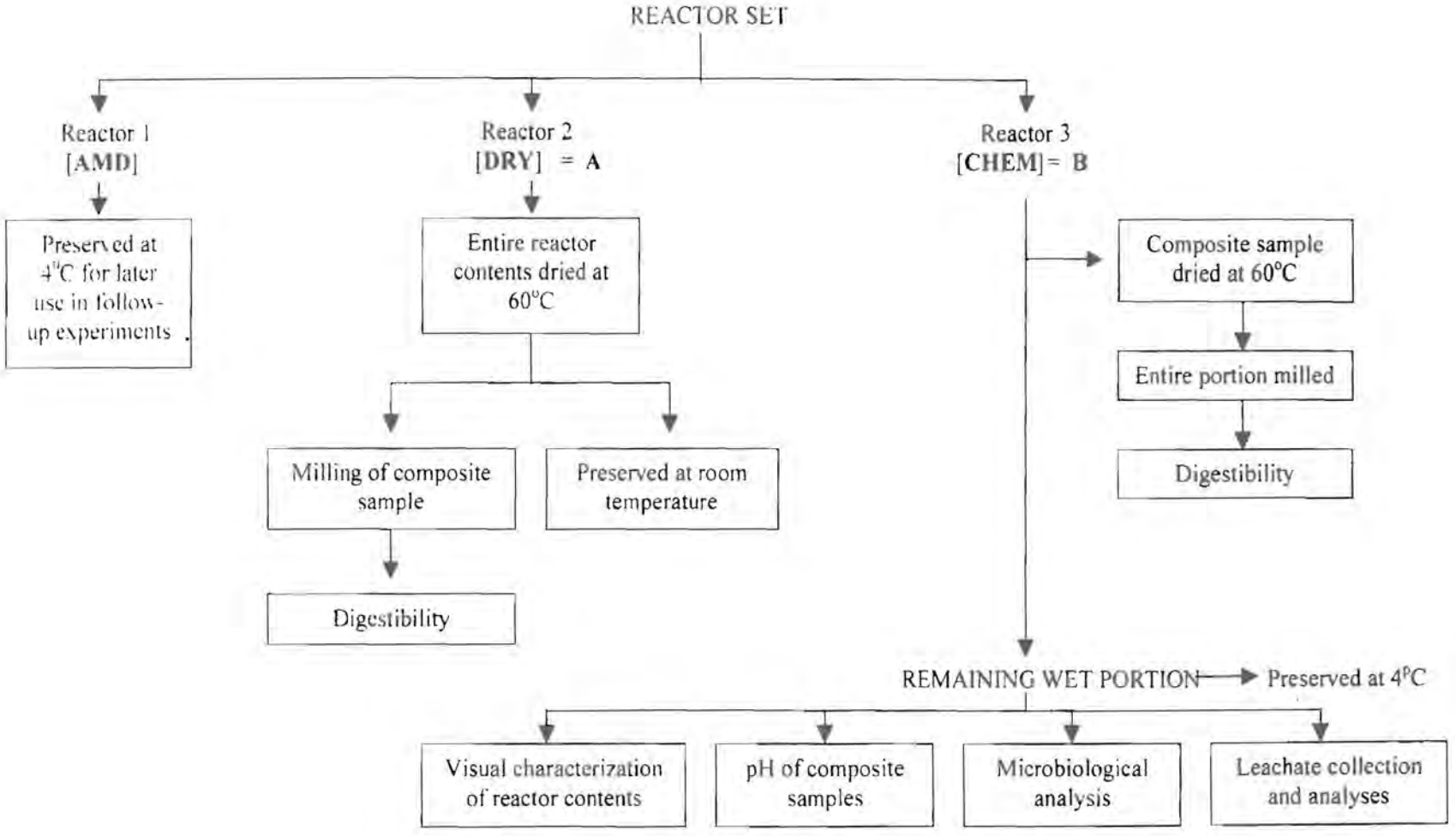

Figure 3.2: Allocation of analyses to individual reactors per reactor set.

2.6 Physical and chemical analyses

2.6.1 Dry mass loss

Dry mass loss was estimated after drying at 60°C for 48 h and expressed as the percentage of the starting material dry mass. Composite samples were milled in a Willey mill to pass a 1 mm sieve.

2.6.2 pH

5 g of wet composite sample was mixed with 200 ml of distilled water and kept overnight at 4 °C. The pH for duplicate (fine and medium grass) or triplicate (uncut grass) samples was determined the following day using a Beckman ϕ 34 pH meter.

2.6.3 *In vitro* dry matter digestibility of experimental reactor contents

A modified two-stage Tilley & Terry (1963) procedure was used for measuring the *in vitro* dry matter digestibility (IVDMD) of the dried samples. Dry matter (DM) determinations were made on separate samples at 100°C. The percentage dry matter (% DM) was used to correct the initial sample mass to dry matter.

Phase 1

Fresh artificial saliva solution was prepared using $NaHCO_3$ (9.80 g/l), KCl (0.57 g/l), NaCl (0.47 g/l), $MgSO_4.7H_2O$ (0.12 g/l), anhydrous $CaCl_2$ (0.04 g/l), and anhydrous Na_2HPO_4 (3.71 g/l). The solution was thoroughly saturated with CO_2 at 39°C. Duplicate subsamples (0.2 g) from the Dry and Chem samples were weighed into glass centrifuge tubes. Alfalfa of known *in vivo* digestibility (75-77%) was included as a positive control. Rumen contents were obtained from a fistulated sheep maintained on lucerne. Rumen liquor was filtered through 4-layer cheesecloth and mixed with the artificial saliva in a 1:3 ratio. This mixture was continuously gassed with CO_2. To each tube 2 ml of 8.68 g/l urea solution and 20 ml of the artificial saliva/rumen solution were added. The space above the liquid in each tube was thoroughly flushed with CO_2. "Blank" tubes contained no sample and were filled with the urea and

artificial saliva/rumen mixtures only. The tubes were sealed with a rubber stopper fitted with a Bunsen gas release valve. The tubes were then incubated at 39°C for 48 h in a shaking water bath.

Phase 2

A fresh solution of 4 g pepsin in 1 l of 0.1 M HCl was prepared in a 1 l glass beaker. The rubber stoppers were removed and gently rinsed with water to remove solid particles adhered to the sides of the stopper and the tube. The tubes were centrifuged at 2500 rpm for 15 min. The supernatant was carefully decanted and discarded. 20 ml of the pepsin/HCl-solution was added to each tube. The tubes were sealed with the rubber stoppers and incubated at 39°C for 48 h in shaking water bath. The tubes were then centrifuged at 2500 rpm for 15 min. The supernatant was gently decanted and 20 ml warm tap water was added. The tubes were again centrifuged at 2500 rpm for 15 min. The supernatant was gently decanted and the tubes were dried at 100°C for 18 h, cooled in a dessicator and weighed. The IVDMD of the residue was calculated as follows:

$$\text{IVDMD} = 100 - \text{Undigested material}$$

$$= 100 - \frac{[(\text{Final sample} - \text{Blank}) - \text{Tube mass}}{\text{Initial sample x \% DM}}$$

Digestibility data was corrected for dry mass loss as follows:

$$\text{Digestibility (corrected)} = \frac{\text{Digestible mass + Dry mass lost}}{\text{Starting Dry mass}}$$

2.7 Microbiological analysis

1 g sample of plant material was taken from sites in the reactor representing visually dominant fungi, diluted in 9 ml of distilled water and vortexed for 15 s. Dilution series were prepared and plated on Potato Dextrose Agar (PDA) (Biolab) plates. All steamed grass samples were plated on normal PDA plates (pH ± 5.6) while the remaining samples were plated on modified PDA plates (pH adjusted to 9.3 prior to autoclaving)

to simulate the pH of the decaying plant material. Chloramphenicol (250 mg/l) was added to the microbiological media. Fungal and yeast colonies were selected and isolated for identification. Water agar (12 g agar/l distilled water) was used for identification of *Fusarium* species. Light microscopy was used to identify the fungal species and scanning electron microscopy (SEM) was used to study substrate decomposition.

3. RESULTS

3.1 Physical and chemical analyses

- **Fine natural grass**

Dry mass

The dry mass in the control experiment decreased from 100% to 71.3% over the experimental period. This decrease occurred gradually at a consistent rate over the experimental period (Figure 3.3). The dry mass of the natural grass inoculated with *S. commune* decreased from 100% to 80.3% (19.7%) over the experimental period. Dry mass loss occurred rapidly during the first week (13.0%) and then slowly for the rest of the experimental period (Figure 3.3). The dry mass of the natural grass inoculated with *P. ostreatus* decreased from 100% to 76.9% (23.1%) over the experimental period. Dry mass loss occurred rapidly during the first week (14.6%) and remained constant for the rest of the experimental period (Figure 3.3). The dry mass of the natural grass inoculated with *P. chrysosporium* decreased from 100% to 74.7% (25.3%) over the experimental period. Dry mass loss occurred rapidly within the first week (17.0%) and remained constant for the rest of the experimental period (Figure 3.3).

Digestibility

The digestibility of the control experiment grass decreased gradually at a consistent rate from 59.166% to 34.016% (25.150%) over the experimental period (Figure 3.3).

The adjusted digestibility decreased gradually from 59.2% to 45.4% over 5 weeks after which it again increased to 52.9%. The digestibility of the grass inoculated with *S. commune* decreased from 59.166% to 27.025% (32.141%) over the experimental period (Figure 3.3). A more rapid decrease was observed for the first 2 weeks (by 30.522%) and then remained constant for the rest of the experimental period. Adjusted digestibility followed the same trend, stabilizing after 2 weeks at 41.4%. The digestibility of the grass inoculated with *P. ostreatus* decreased from 59.166% to 22.068% (37.098%) over the experimental period (Figure 3.3). A more rapid digestibility decrease was observed within the first week (by 24.523%) and decreased slowly for the rest of the experimental period. The adjusted digestibility decreased rapidly within 1 week and then remained constant for the rest of the experimental period. The digestibility of the grass inoculated with *P. chrysosporium* decreased from 59.166% to 26.031% (33.135%) over the experimental period (Figure 3.3). A more rapid decrease in digestibility was observed within the first week (21.339%) and then remained constant for the rest of the experimental period.

pH

The pH in the control experiment increased from 6.00 to 10.06 over the 6 week experimental period. A rapid increase to pH 9.95 was observed within the first week. The pH of the grass inoculated with *S. commune* increased from 6.00 to 9.95 over the 6 week experimental period. A rapid increase to pH 9.88 was observed within the first week. The pH of the grass inoculated with *P. ostreatus* increased from 6.00 to 10.09 over the 6 week experimental period. The pH increased within the first 2 weeks to 8.02. The pH of the grass inoculated with *P. chrysosporium* increased from 6.00to 10.05 over the 6 week experimental period. A rapid increase to pH 9.81 was observed within the first week.

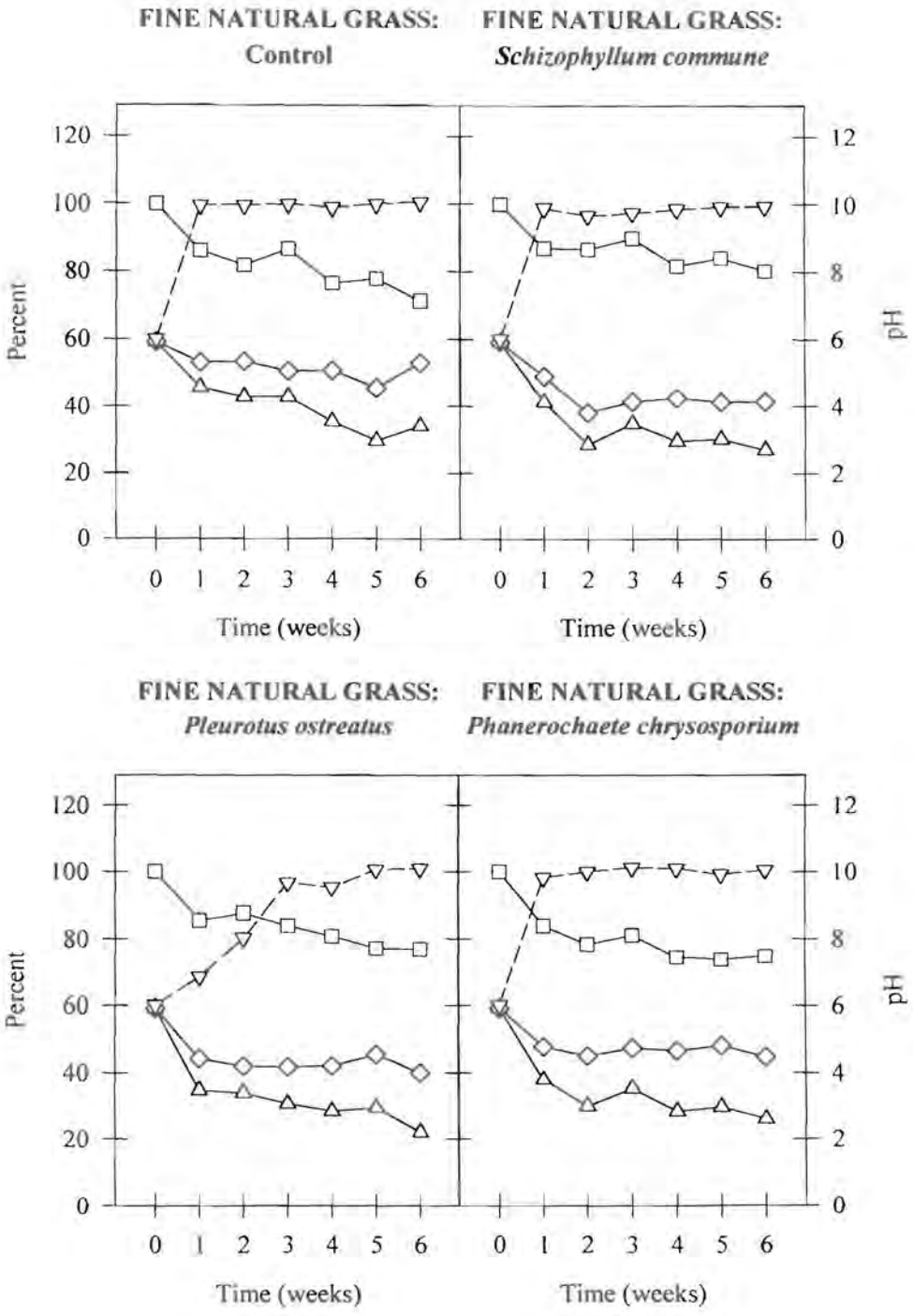

Figure 3.3: Influence of different treatments on solid-state fermentation of fine natural grass as described by dry mass loss (□), corrected digestibility (◇), digestibility of the remaining residue (△), and pH (▽).

- **Medium natural grass**

Dry mass

Dry mass in the control experiment decreased gradually from 100% to 71.7% (28.3%) over the experimental period (Figure 3.4). Dry mass in the grass inoculated with *S. commune* decreased gradually from 100% to 69.3% (30.7%) over the experimental period (Figure 3.4). Dry mass in the grass inoculated with *P. ostreatus* decreased gradually from 100% to 68.1% (31.9%) over the experimental period (Figure 3.4). Dry mass in the grass inoculated with *P. chrysosporium* decreased gradually from 100% to 68.5% (31.5%) over the experimental period (Figure 3.4).

Digestibility

The digestibility of uninoculated medium-cut grass decreased gradually from 59.166% to 24.752% (34.414%) over the experimental period (Figure 3.4). The adjusted digestibility decreased very slightly to 46.1% over the experimental period. The digestibility of medium-cut grass inoculated with *S. commune* decreased from 59.166% to 16.594% (42.572%) over the experimental period (Figure 3.4). A more rapid decrease was observed within the first week (36.009%) and then remained constant. The adjusted digestibility decreased over the first three weeks but increased to 42.5% over the experimental period. The digestibility of medium-cut grass inoculated with *P. ostreatus* decreased from 59.166% to 17.383% (41.783%) over the experimental period (Figure 3.4). A more rapid decrease was observed within the first week (33.625%) and then remained constant. The adjusted digestibility also decreased within the first week but increased to 43.7% over the experimental period. The digestibility of medium-cut grass inoculated with *P. chrysosporium* decreased from 59.166% to 23.226% (35.940%) over the experimental period (Figure 3.4). A more rapid decrease was observed within the first 2 weeks (29.748%) and then remained constant. The adjusted digestibility decreased within the first 2 weeks but increased to 47.4% over the experimental period.

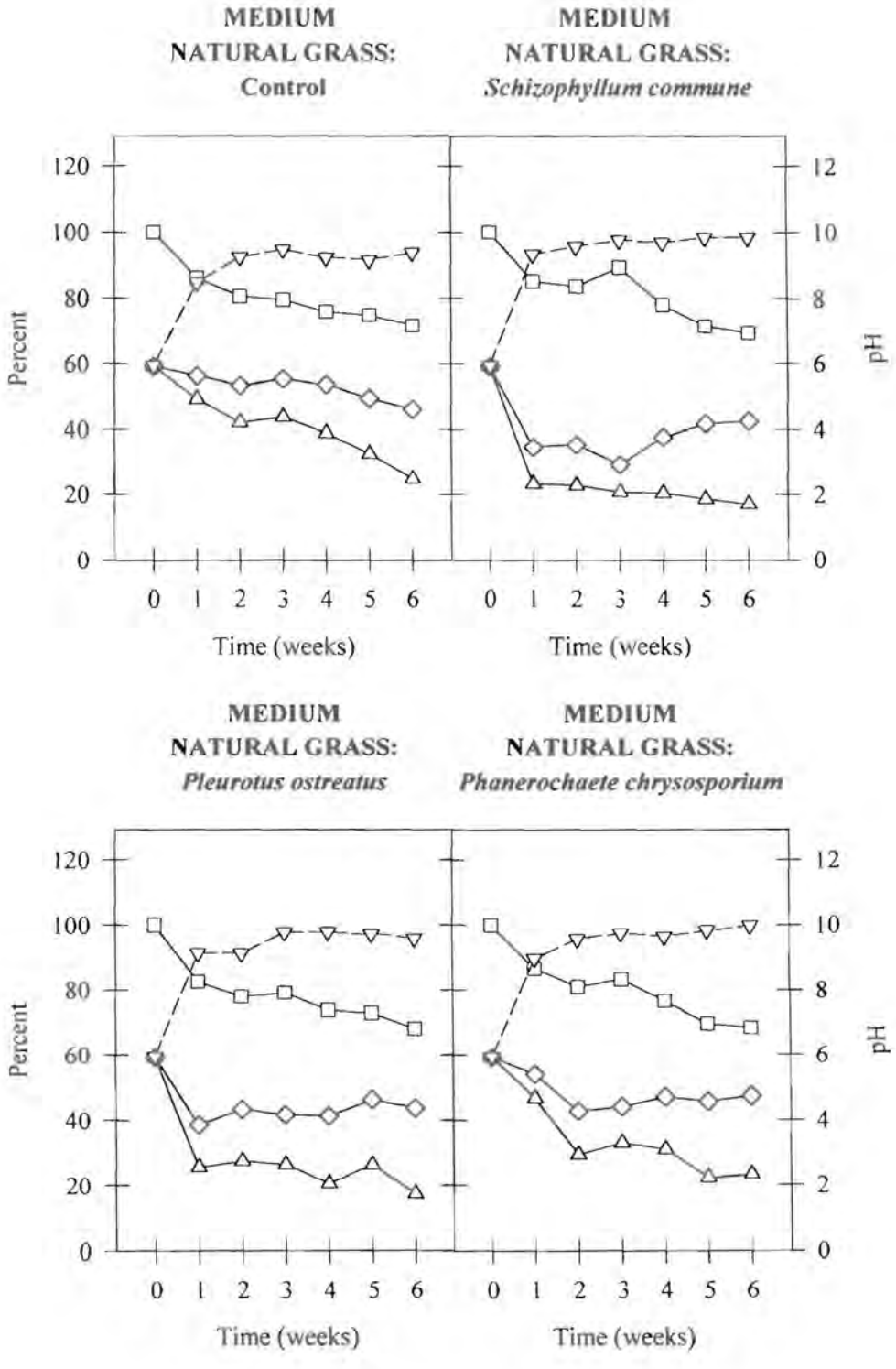

Figure 3.4: Influence of different treatments on solid-state fermentation of medium natural grass as described by dry mass loss (□), corrected digestibility (◊), digestibility of the remaining residue (△), and pH (▽).

pH

The pH of the uninoculated medium-cut grass increased rapidly from 5.94 to 9.37 over the experimental period. A rapid increase to pH 8.47 was observed within the first week. The pH of the medium-cut grass inoculated with *S. commune* increased from 5.94 to 9.84 over the experimental period. A rapid increase to pH 9.32 was observed within the first week. The pH of the medium-cut grass inoculated with *P. ostreatus* increased from 5.94 to 9.58 over the experimental period. A rapid increase to pH 9.13 was observed within the first week. The pH of medium-cut grass inoculated with *P. chrysosporium* increased from 5.94 to 9.98 over the experimental period. A rapid increase to pH 8.96 was observed within the first week.

- **Uncut natural grass**

Dry mass

Dry mass in the control experiment for uncut grass decreased from 100% to 97.6% (2.4%) over the experimental period (Figure 3.5). An increase in dry mass was observed after the first 2 weeks. Dry mass in uncut grass inoculated with *S. commune* remained unchanged over the six week experimental period (Figure 3.5). Periodic dry mass increases and decreases were observed. After the first 3 weeks the dry mass increased to 105.7% (5.7%) and decreased to 88.4% (11.6%) after the fifth week of the experimental period. Dry mass in uncut grass inoculated with *P. ostreatus* decreased from 100% to 97.3% (2.7%) over the experimental period (Figure 3.5). The dry mass increased after the first 2 weeks to 131.7% (31.7%) and decreased gradually to 97.3% over the experimental period. Dry mass in uncut grass inoculated with *P. chrysosporium* remained unchanged at 99.1% over the experimental period (Figure 3.5). The dry mass increased to 115.9% (15.9%) and 109.5% (9.5%) respectively after the first and fourth weeks of the experimental period.

Digestibility

The adjusted digestibility of uncut grass decreased from 64.8% to 40.7% (24.1%) over the incubation period (Figure 3.5). The residue digestibility followed the same trend. Digestibility decreased gradually over the first 4 weeks to 33.9% (30.9%) after which it again increased to 40.7% (24.1%). The adjusted digestibility of uncut grass inoculated with *S. commune* decreased from 64.8% to 38.3% (26.5%) after the experimental period (Figure 3.5). A more rapid decrease in digestibility was observed within the first 2 weeks and then remained constant. The residue digestibility followed the same trend. The adjusted digestibility of uncut grass inoculated with *P. ostreatus* decreased from 64.8% to 34.7% (30.1%) over the experimental period (Figure 3.5). A more rapid decrease in digestibility to 19.5% (45.3%) was observed within the first 2 weeks after which it again increased. The residue digestibility followed the same trend. The adjusted digestibility of uncut grass inoculated with *P. chrysosporium* decreased from 64.8% to 26.3% (38.5%) over the experimental period (Figure 3.5). The digestibility initially decreased to 34.9% (29.9%) within the first 2 weeks after which it again increased to 55.8%. The digestibility again decreased for the remainder of the experimental period.

pH

The pH of the uncut grass control experiment increased from 6.04 to 10.03 over the experimental period (Figure 3.5). The pH increased within the first 2 weeks to 9.12. The pH of uncut grass inoculated with *S. commune* increased from 6.04 to 9.87 over the experimental period (Figure 3.5). The pH increased within the first week to pH 9.40. The pH of uncut grass inoculated with *P. ostreatus* increased from 6.04 to 10.08 over the experimental period (Figure 3.5). The pH increased within the first week to 9.51. The pH of uncut grass inoculated with *P. chrysosporium* increased from 6.04 to 9.89 over the experimental period (Figure 3.5). The pH increased within the first week to 9.61.

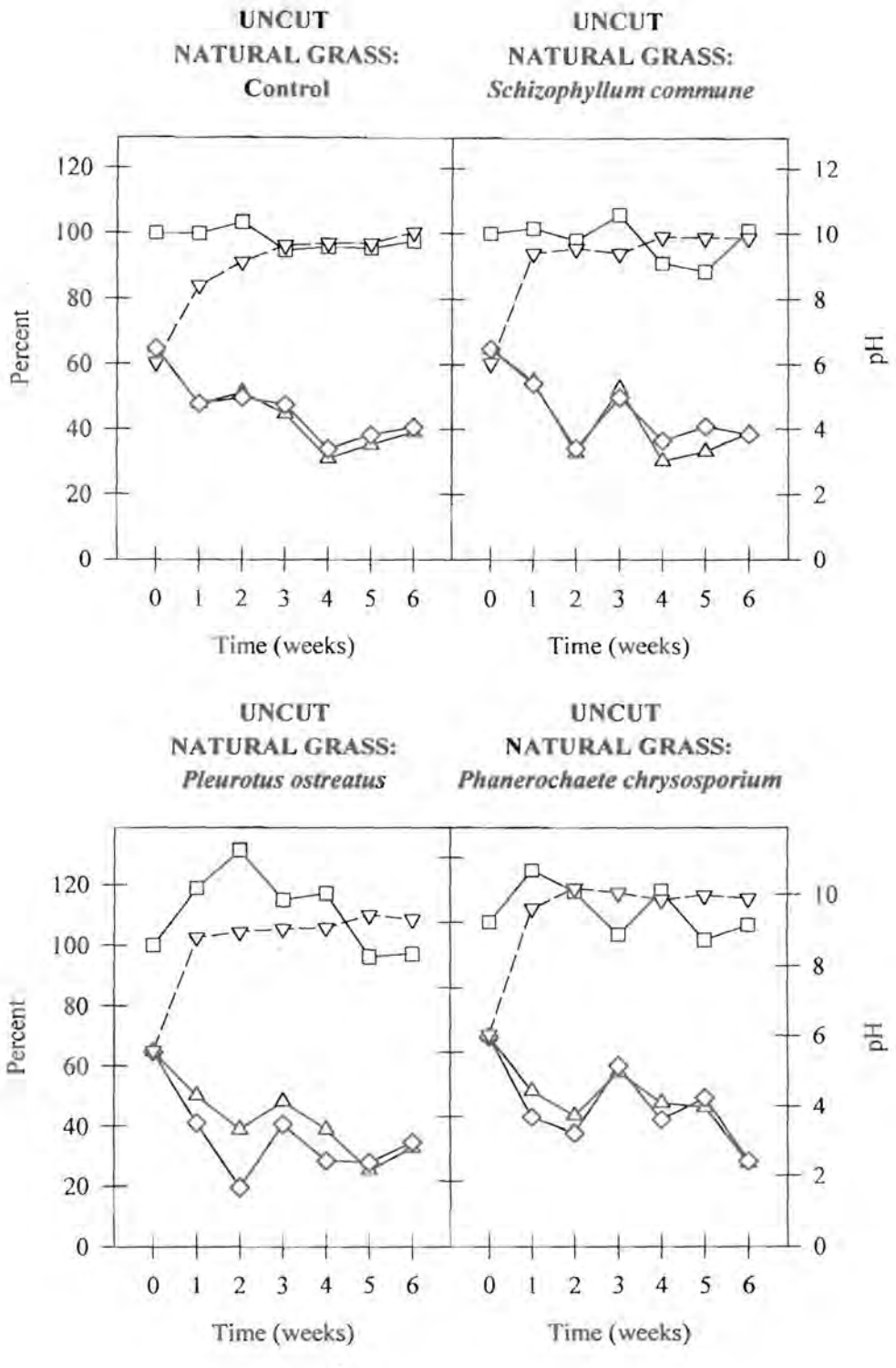

Figure 3.5: Influence of different treatments on solid-state fermentation of uncut natural grass as described by dry mass loss (□), corrected digestibility (◇), digestibility of the remaining residue (△), and pH (▽).

- **Fine steamed grass**

Dry mass

Dry mass in the steamed grass control experiment decreased from 100% to 54.3% (45.7%) over the experimental period (Figure 3.6). Dry mass loss was very slow during the first 5 weeks but increased rapidly during the sixth week of the experimental period. Dry mass in steamed grass inoculated with *S. commune* decreased from 100% to 97.6% (2.4%) over the experimental period (Figure 3.6). Dry mass increased 104.6% (4.6%) within the first week of the experimental period. Dry mass in steamed grass inoculated with *P. ostreatus* decreased from 100% to 87.6% (12.4%) over the experimental period (Figure 3.6). Dry mass increased after the first 2 weeks to 107.7% (7.7%) after which it again decreased. Dry mass in steamed grass inoculated with *P. chrysosporium* decreased from 100% to 71.4% (28.6%) over the experimental period (Figure 3.6). No increase in dry mass was observed.

Digestibility

The adjusted digestibility of the control experiment grass increased from 39.2% to 61.6% (22.4%) over the experimental period (Figure 3.6). The digestibility initially decreased within the first 2 weeks (11.6%) after which it again increased. The residue digestibility followed the same trend. The adjusted digestibility of steamed grass inoculated with *S. commune* decreased from 39.2% to 36.0% (3.2%) over the experimental period (Figure 3.6). The digestibility increased to 41.7% (2.5%) within the first 2 weeks after which it again decreased. The residue digestibility followed the same trend. The adjusted digestibility of steamed grass inoculated with *P. ostreatus* increased from 39.2% to 46.2% (7%) over the experimental period (Figure 3.6). The digestibility decreased within the first 3 weeks to 26.8% (12.4%) after which it again increased. The residue digestibility followed the same trend. The adjusted digestibility of steamed grass inoculated with *P. chrysosporium* increased from 39.2% to 47.8% (8.6%) over the experimental period (Figure 3.6). The adjusted digestibility decreased within the first 2 weeks to 27.7% (11.5%) after which it again increased. The residue digestibility followed the same trend up to week 4 from where it continued its downward trend.

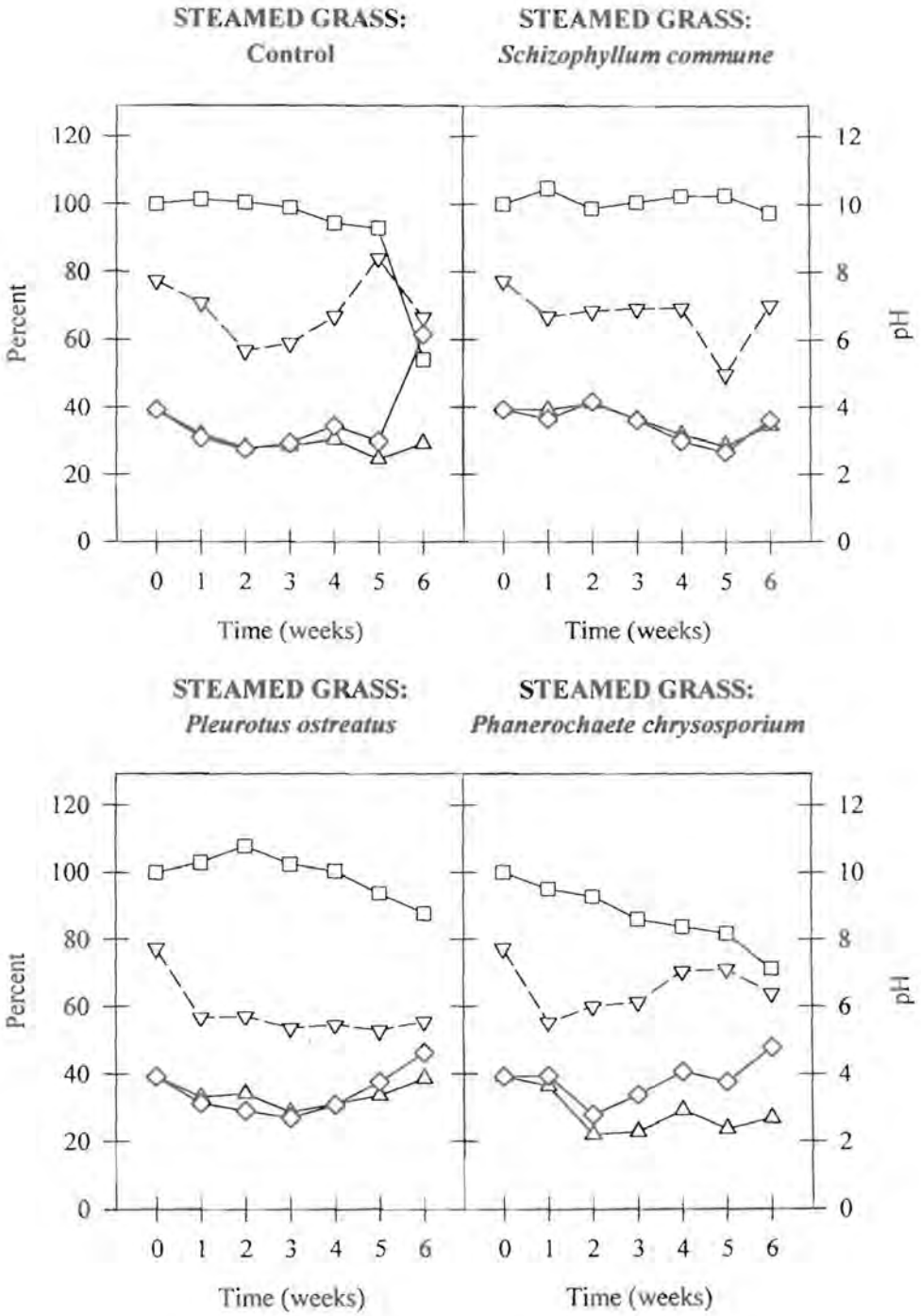

Figure 3.6: Influence of different treatments on solid-state fermentation of fine steamed grass as described by dry mass loss (□), corrected digestibility (◇), digestibility of the remaining residue (△), and pH (▽). Digestibility of the remaining residue was similar to the corrected digestibility, except for *P. chrysosporium*, for which a downward trend was maintained.

pH

The pH of the control experiment grass decreased to from 7.73 to 6.66 over the experimental period (Figure 3.6). The pH decreased to pH 5.68 after the first 2 weeks, after which it increased to 8.40 after the fifth week of the experimental period. The pH of steamed grass inoculated with *S. commune* decreased from 7.73 to 7.03 over the experimental period (Figure 3.6). The pH decreased to 5.00 after the fifth week of the experimental period. The pH of steamed grass inoculated with *P. ostreatus* decreased from 7.73 to 5.53 over the experimental period (Figure 3.6). The pH decreased to 5.25 within the fifth week of the experimental period. The pH of steamed grass inoculated with *P. chrysosporium* decreased from 7.73 to 6.38 over the experimental period (Figure 3.6). The pH decreased to 5.51 within the first week of the experimental period, after which it increased again.

3.2 Microbiological analysis

Complex and dynamic fungal communities exist on decaying plant material consisting mainly of common saprophytes (Figure 3.7). Dominant species in this study were selected based on their numerical superiority on agar plates and were identified using light microscopy as *Aspergillus niger*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Fusarium equiseti*, *Fusarium chlamydosporum* and *Acremonium kiliense* (Figure 3.8). *Scopulariopsis brevicaulis* was the dominant organism isolated from uncut natural grass treatments. At least four different yeast genera were isolated. *Coprinus* spp. was visible on uncut grass inoculated with *S. commune* and *P. ostreatus* after 4 weeks (Figure 3.9). Slime moulds were detected on natural fine-cut grass inoculated with *P. ostreatus*.

Succession amongst the different fungi was observed (Figure 3.7). *Aspergillus* spp., *Rhizopus/Mucor* spp. and various yeast genera dominated the early stages of fine and medium natural grass treatments. Initially, these "sugar fungi" utilized easily metabolizable sugars that was present in the decaying grass. The later stages were then dominated by *Fusarium* spp. and *Geotrichum* spp.

Key

- *Fusarium* spp.
- Yeast spp. (various genera)
- *Scopulariopsis brevicaulis*
- *Geotrichum* spp.
- *Penicillium* spp.
- *Aspergillus* spp.
- *Trichoderma* spp.
- *Mucor* spp. / *Rhizopus* spp.
- *Memnoniella* spp.
- *Acremonium* spp.

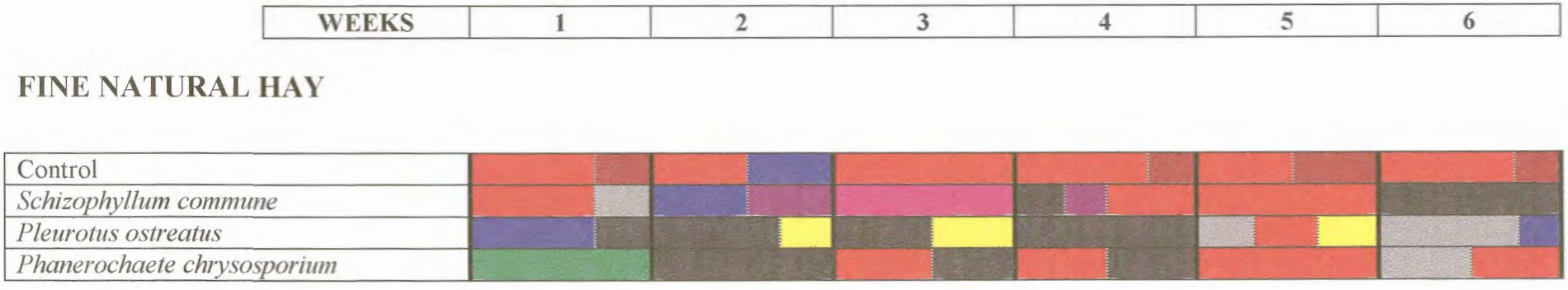

Figure 3.7: Schematic representation of the fungal community structure showing the dominant fungi present on decaying grass.

WEEKS	1	2	3	4	5	6

FINE STEAMED HAY

Control	
Schizophyllum commune	
Pleurotus ostreatus	
Phanerochaete chrysosporium	
P. chrysosporium (Internal surfaces)	

MEDIUM NATURAL HAY

Control	
Schizophyllum commune	
Pleurotus ostreatus	
Phanerochaete chrysosporium	

UNCUT NATURAL HAY

Control	
Schizophyllum commune	
Pleurotus ostreatus	
Phanerochaete chrysosporium	

Figure 3.7 (continued): Schematic representation of the fungal community structure showing the dominant fungi present on decaying grass.

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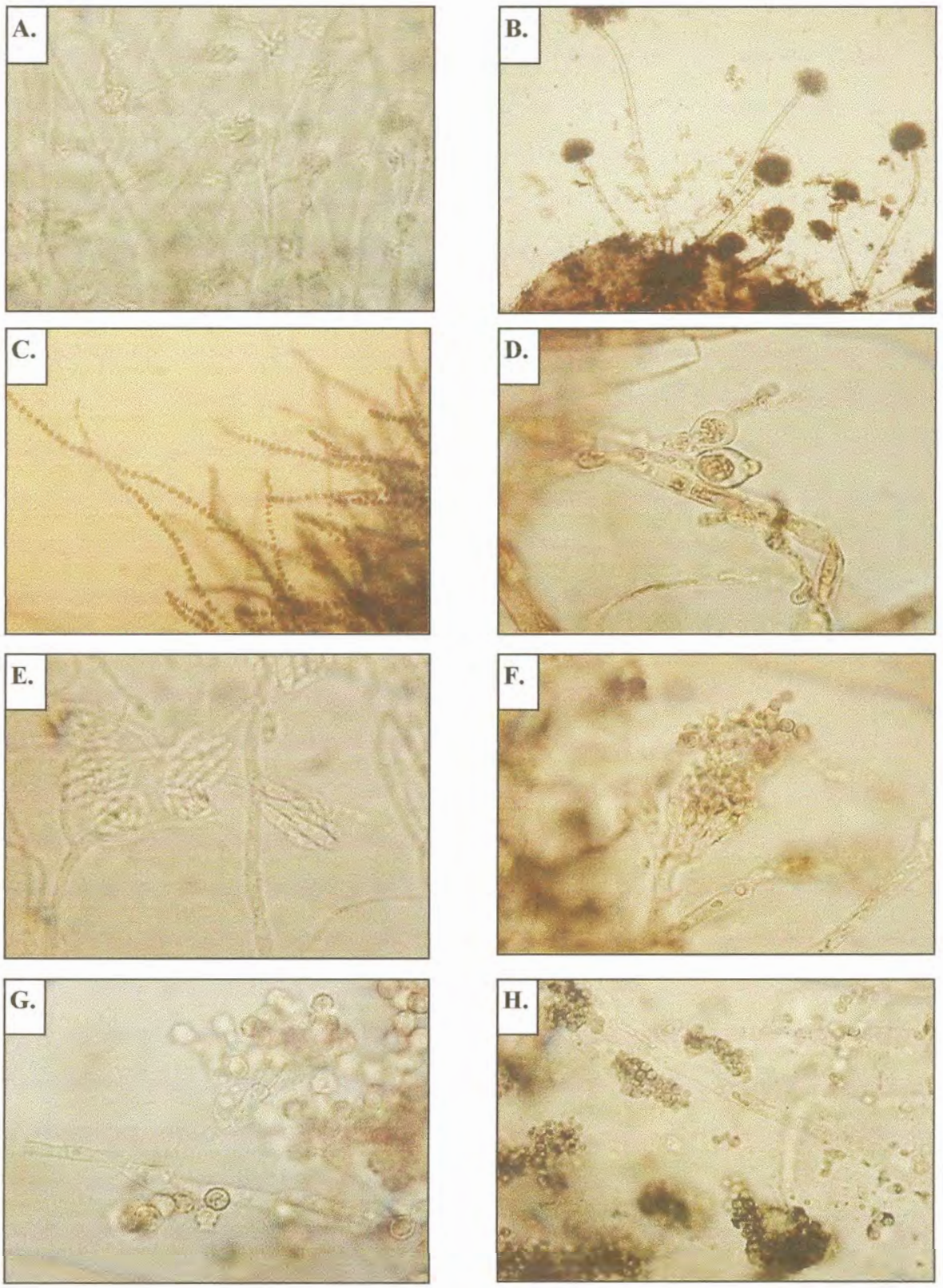

Figure 3.8: Light microscope images of some of the dominant fungi isolated from the decaying plant material. A) *Acremonium kiliense*, B) *Aspergillus niger* , C) *Candida* spp. (10X magnification of culture plate), D) *Fusarium chlamydosporum*, E) *Fusarium moniliforme*, F) *Penicillium* spp., G) *Scopulariopsis brevicaulis* , and H) *Trichoderma* spp.

These organisms are known for their ability to produce toxic compounds and that probably enabled them to survive on the decaying substrate. *S. brevicaulis* was not a dominant organism, but was present on the grass for the entire experimental period. A similar succession pattern was observed for steamed grass treatments.

A large number of genera were present on the steamed grass during the first two weeks of the experimental period. *Trichoderma* spp., *Fusarium* spp. and *Penicillium* spp. became the dominant genera on the steamed grass during the later stages of the experiment. Medium natural grass treatments maintained active populations consisting mainly of various yeast genera, *Geotrichum* spp. *Fusarium* spp. and to some extent *Aspergillus* spp. Uncut natural grass treatments were completely dominated from the onset of the experiment by *S. brevicaulis*, but this changed by week 5 and 6 when various genera were able to suppress *S. brevicaulis*.

Scanning electron microscopy revealed extensive colonization of the substrate surfaces by various fungi, yeasts and bacteria (Figure 3.10).

Within 1 week, inoculated steamed grass was completely colonised by *Pleurotus ostreatus* and *Phanerochaete chrysosporium*. After 2-3 weeks, the steamed grass inoculated with *P. chrysosporium* showed severe secondary colonisation by another fungi (*Trichoderma* spp.).

Figure 3.9: *Coprinus* spp. mushrooms on uncut natural grass inoculated with *P. ostreatus* (week 5). Broad substrate specificities and significant lignolytic enzyme activities at alkaline pH are some of the unique characteristics of this genus (Heinzkil *et al.*, 1998).

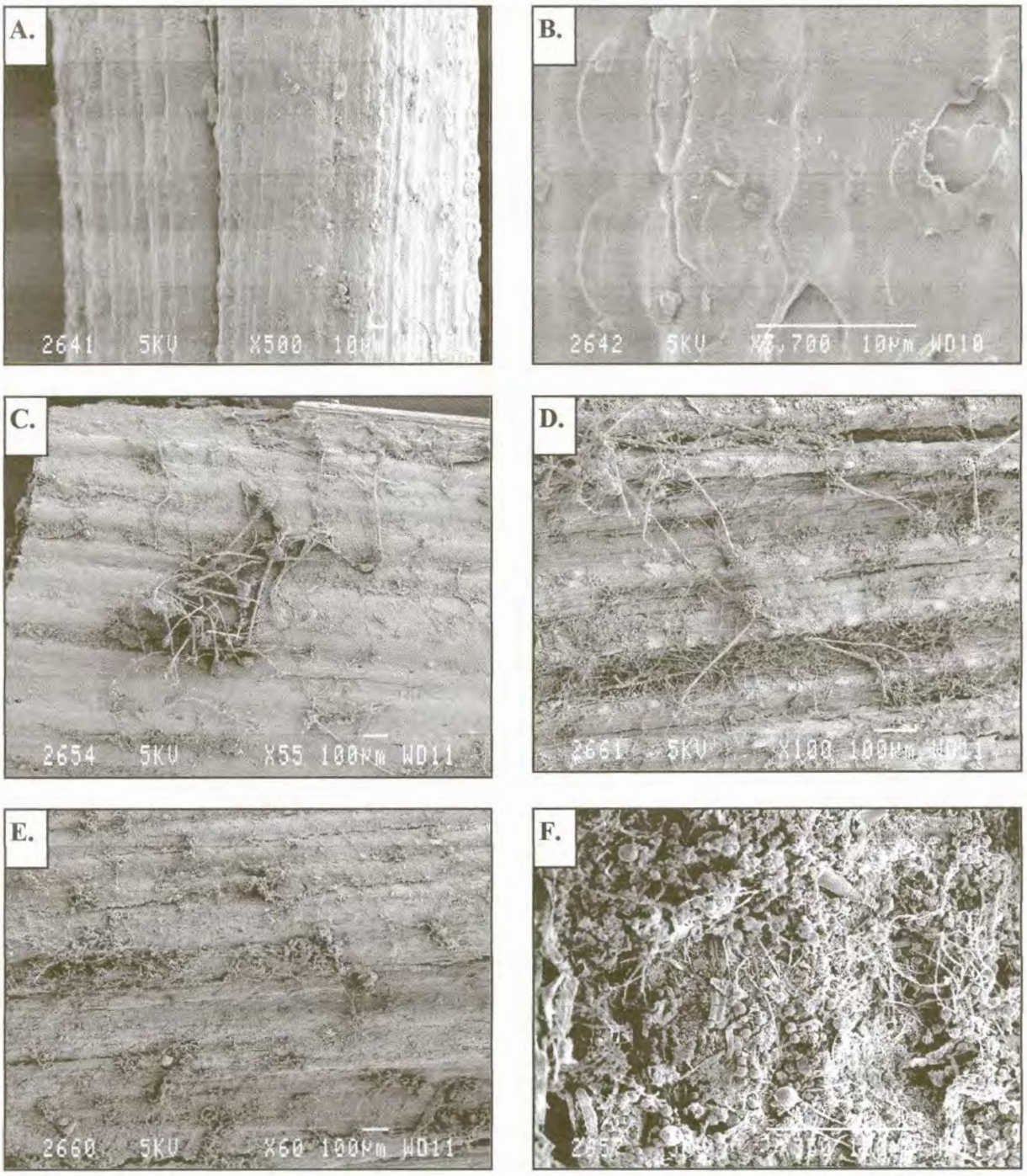

Figure 3.10: Scanning electron microscope images of control (autoclaved) and uncut natural grass treatments. The autoclaved grass seen here has an intact structure (A-B). The natural substrates were colonised to different extents. Localised (C), moderate (D-E), and severe surface colonisation (F) were noticed. Figure 3.10C represents uncut natural grass inoculated with *P. chrysosporium* after 6 weeks. The relative degradation of the substrate can be followed from week 1, control (E); week 3, *S. commune* (D) and week 5, *P. ostreatus* (F).

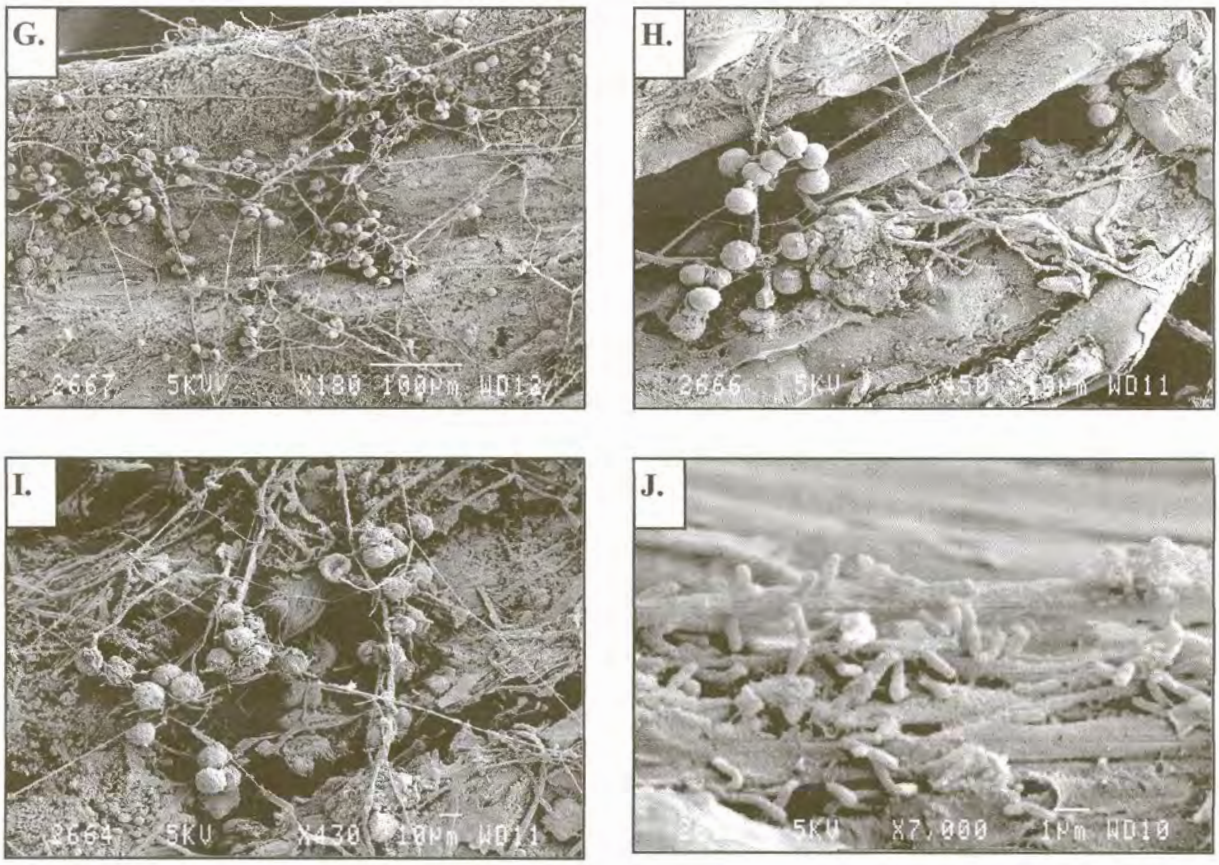

Figure 3.10 (continued): Scanning electron micrographs of uncut natural grass. Uninoculated uncut natural grass have been densely colonised by fungi, bacteria and yeasts (Figure 3.10 (G-I). Figure 3.10J shows large numbers of bacteria attached to the substrate.

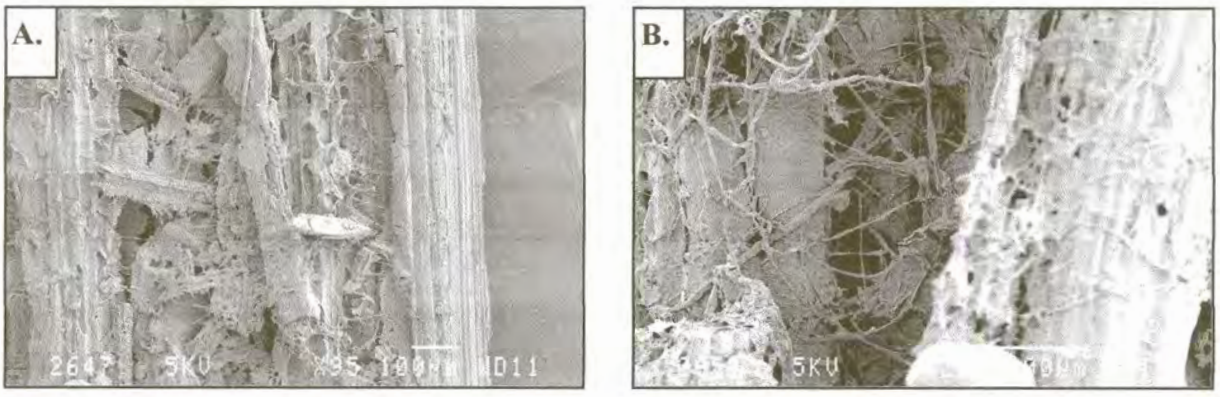

Figure 3.11: Scanning electron micrographs of fine steamed grass inoculated with *S. commune*. Damage to the fiber structure and evidence of fungal colonisation were apparent after 1 week, compared to the control (Figure 3.10A-B). Yeasts and bacteria were also observed but not to the same extent when compared to uncut natural grass treatments.

4. DISCUSSION

4.1 Influence of biological pretreatment on digestibility of Buffelsgrass

Dry mass loss for the control experiments was gradual, whereas the inoculated treatments showed a rapid dry mass loss within the first week, after which the process slowed down substantially. This was attributed to degradation of readily biodegradable compounds and the simultaneous leaching of water-soluble compounds. This was also indicated by the leachate that already formed within the first week. Further weight loss was attributed to fungal activity. This resulted in the increase of leachate volume over time. This may be exactly what is required, since degradation is taking place at a slow rate, whilst leachate is being produced.

The digestibility results confirm the conclusions drawn from the dry mass results, that readily biodegradable material was utilized initially, after which degradation slowed down as a result of a more recalcitrant substrate. This is in agreement with Valmaseda *et al.* (1991) who indicated that during the initial colonization phase of wheat straw, free sugars were available, but were rapidly metabolized by the fungi.

4.2 The effect of substrate particle size on Buffelsgrass degradation

Particle size influenced the ability of the naturally occurring microflora to degrade natural grass (Figures 3.3 - 3.6). The natural consortia, consisting of bacteria, yeasts and fungi, better degraded medium sized natural grass than fine-cut natural grass. Although dry mass increased for uncut natural grass treatments, the digestibility decreased for all treatments. This is an indication that fungal degradation occurred while biomass accumulated. The extent of degradation of uncut natural grass was not more extensive than medium-cut natural grass treatments. Also, the fungal community structure (Figure 3.7) showed that a higher degree of diversity existed on fine- and medium-cut natural grass compared to the lower degree of diversity on uncut natural grass. The spaces between the uncut natural grass particles allowed for sufficient aeration and this favoured substrate colonization by fungi. The spaces between the fine-cut particles were much smaller and were water clogged. This prevented

sufficient gas transfer, removal of volatile metabolic products and facilitated the spread of bacteria (Reid, 1989). Therefore, the fine-cut particle sizes, and to some extent the medium-cut particle size, created favourable conditions for extensive substrate colonization by fungi and bacteria. The colonization and degradation of steamed fine-cut grass by *P. ostreatus* was not impeded. This implies that the substrate particle size did not influence colonization and degradation of the natural substrate by *P. ostreatus*, but was rather influenced by competition with the microflora or the alkaline pH.

The fine- and medium-cut particle sizes facilitated the continuous degradation of the Buffelsgrass. The uncut grass was also degraded but showed dry mass increases due to biomass accumulation. Therefore, particle size influenced substrate degradation in terms of the fungal community structures but did not influence the overall extent of degradation by the inoculated fungi and the naturally occurring microflora.

4.3 The influence of pH on the Buffelsgrass digestibility

The pH of all treatments increased rapidly within one or two weeks to an alkaline pH in excess of 9.8. The origin of the alkali conditions remains unknown. Alkali treatment has been used for years to increase the digestibility of forages (Jackson, 1977). Alkali treatment produces a number of substantial changes in cell wall composition and organization i.e. the partial solubilization of hemicellulose, lignin and silica; the hydrolysis of uronic and acetic acid esters (Chesson, 1981). In grasses, a high proportion of ferulic and *p*-coumaric acids are esterified to hemicellulose and lignin (Jeffries, 1990). Ester linkages are readily cleaved by low levels of alkali that may account for the substantial solubilization of phenolic material (Chesson, 1981). Increased cellulose digestibility was attributed to the removal of phenolics and not the weakening of the cellulose structure (Chesson, 1981). Alkali resistant material showed considerable resistance to microbial action and was not released from cell walls until over 50% of the cellulose and hemicellulose was degraded (Chesson, 1981). More recent studies on this phenomenon showed that polyphenols (pyrogallol, gallic acid and 1,2,4-trihydroxybenzene) oxidize rapidly under alkaline conditions (Armstrong *et al.*, 1994), forage upgrading by NaOH and $Ca(OH)_2$ treatment proved viable (Haddad

et al., 1994), and that industrial alkaline pulping removed lignin from the neighboring polysaccharide moieties (Sun *et al.*, 1998).

We propose that the alkaline pH decreased the substrate residue digestibility by leaching potentially digestible compounds. The mechanisms of creating and maintaining the alkaline pH are unknown, nor whether intrinsic chemical reactions or microbial metabolic actions are responsible.

4.4 Microbiology of Buffelsgrass degradation

Microbial consortia degraded lignocellulose faster than pure cultures of white-rot fungi (Blanchette and Shaw, 1978). Significant increases in weight loss and stimulation of mycelial growth was observed in wood decay treatments combining bacteria and yeasts with white-rot fungi (Blanchette and Shaw, 1978). Our results indicated that the natural populations of microflora present of the Buffelsgrass caused continuous degradation of the substrate over time. The naturally occurring microflora increased the rate of substrate degradation in the presence of the inoculated fungi, but substrate degradation proceeded very slowly after a threshold level was reached within 1 or 2 weeks. Therefore, the naturally occurring microflora on Buffelsgrass and the inoculated fungi synergistically degraded the substrate.

The micrographs presented here (Figures 3.10 and 3.11) suggest a mutualistic association among bacteria, yeasts, and fungi during lignocellulose decay. This is in agreement with the observations by Blanchette and Shaw (1978). The fungi isolated from the decaying grass are classified as primary saprophytes that metabolize easily degradable sugars and other compounds. These fungi may not directly cause decomposition, but can synergistically increase rates of decay when they develop mutualistic relationships with, and flourishing alongside species decomposing cellulose and lignin (Dix and Webster, 1995). Microorganisms unable to degrade the ligninpolymer can obtain energy from the low molecular weight intermediates released from the degradation of lignin by true white-rot fungi. A similar situation arises where competent fungi degrade cellulose.

Trichoderma spp. is common to soil and grow very rapidly, producing a large amount of conidia and they have the ability to secrete a range of enzymes, including cellulases. They are able to withstand severe nutrient stress and are known as highly combative fungi, being able to repel attacks by other microorganisms and capture nutrient resources. *Trichoderma hirzianum* was identified as the causal agent of green mould disease that plague mushroom farms worldwide (Castle *et al.*, 1998). The colonization of steamed grass by *P. chrysosporium* was severely interrupted by *Trichoderma* spp. This is an example of a negative interaction the white rot fungus encountered on the steamed substrate surfaces. The *Trichoderma* spp. was able to displace *P. chrysosporium* from the substrate. This is also a classic example of how lignocellulose degradation by the white rot fungus was exploited by an organism unable to degrade lignin. The white rot fungus removed the lignin shield and was subsequently displaced from the substrate by the more aggressive microorganism.

Fungi are known for their ability to survive and grow at extreme pH ranges. *Coprinus* spp. comprises lignolytic enzymes characterized by broad substrate specificities and significant enzyme activities at alkaline pH (Heinzkill *et al.*, 1998). Lignin model compounds were efficiently degraded by species of this genus (Guiraud *et al.*, 1999). The microfungi isolated from the decayed grass were able to survive the alkaline conditions on the Buffelsgrass. The ability of the fungi to survive at the alkaline pH gave them an advantage over fungi that could not. The introduction of urea to forest litter induced the appearance of unusual fungi (Lehman, 1976). Urea treatment of pine needles stimulated the development of some fungi, but suppressed others (Lehman and Hudson, 1977). Lehman and Hudson (1977) described the effect of alkali on the appearance of the leaf litter: darkening, water soaking and a rise in pH. This is in agreement with our own observations of the reactor contents. The action of the urea remained unclear, but it was considered unlikely to be merely an effect of adding a nitrogenous compound. The release of ammonia was also observed in our study and the study by Lehman and Hudson (1977). The origin of the alkaline conditions remains unknown

The nutritional state of the steamed substrate, not the particle size, influenced its colonization and degradation by the white-rot fungi. *S. commune* comprises potent

cellulose degrading enzymes as well as esterases which can disrupt the lignin-hemicellulose linkages, thereby solubilizing the lignocellulose matrix into smaller lignin-carbohydrate complexes (Fang *et al.*, 1998; Haltrich *et al.*, 1995; Jeffries, 1990; Mankel and Kothe, 1999; Willick *et al.*, 1984). The nutrient deficient substrate (in terms of the lack of enzyme substrates) influenced the ability of *S. commune* to effectively colonize this substrate. *S. commune* removes lignin through solubilization and modification and does not comprise the powerful lignin oxidizing enzymes described for *P. ostreatus* and *P. chrysosporium*. The latter two organisms were able to colonize and degrade the substrate because their action of lignin degradation is based on potent lignin oxidizing enzymes.

Various interactions occurred between the fungal populations on the decaying grass. Patterns of succession were observed and mutualistic and antagonistic interactions between these populations were described. The inoculated fungi increased the degradation rate of natural grass. *P. ostreatus* was the only fungus capable of improving the starting digestibility of the steamed grass by the end of the experiment.

4.5 Leachate origin and significance

Hot water treatment of wheat and cotton straw yielded solutions containing sugars and phenolics (Milstein *et al.*, 1981; Vered *et al.*, 1981). Most of the sugars were bound to either polysaccharides or phenolics, indicating that hot water treatment released lignin-carbohydrate complexes and sugar containing polymers. The time of steam exposure affected the extent of nutrient loss. Rapid release of phenolics and carbohydrates into liquor took place within 5 h of hot water treatment and increased steadily thereafter (Vered *et al.*, 1981). Thus, although the microbiological obstacles were partially removed during the 4 h steaming of the Buffelsgrass, much of the potentially digestible nutrients were lost. The remaining fraction contained a higher proportion of recalcitrant polymers in the form of lignin-carbohydrate complexes. The low starting digestibility of the steamed grass can therefore be ascribed to the steam pasteurization of the substrate.

The production of a leachate can be viewed two ways. The negative implication of leaching is that potentially digestible compounds are released from the lignocellulose matrix and is either lost by seeping or through mineralization by the naturally occurring microflora. This leachate can also contain antimicrobial compounds that might be inhibitory when applied to a biologically driven process. However, literature indicated that microbial consortia in various environments were capable of detoxifying potentially toxic lignocellulose degradation intermediates (Cornu *et al.*, 1994; Egland *et al.*, 1997; Wilson and Mertens, 1995). Sulphate reducing bacteria are also able to degrade lignin degradation products either in pure culture or synergistically (Boopathy, 1997; Lovley *et al.*, 1995). The advantages of leaching are that it can provide microbial consortia with easily fermentable substrates that can be fermented to low molecular weight organic acids. These fermentation products can be utilized by sulphate reducing bacteria as a carbon and nutrient source.

5. CONCLUSIONS

- In all treatments, the initial digestibility was high, rapidly decreasing after 1 to 2 weeks. This was attributed to readily biodegradable substrates being utilised during the first 2 weeks, leaving a more recalcitrant substrate which was slowly digested. None of the treatments directly increased the digestibility of the substrate. However, the leachate which was produced, could be a direct result of the breakdown of the substrate yielding potentially available carbon.

- The pH of all the un-steamed grass treatments increased rapidly to an alkaline pH in excess of 9. However, the origin of the alkaline conditions remains unknown. The pH of the steamed grass did not increase.

- The microbial community structure was very dynamic. This was attributed to the changes in the substrate composition over time and possibly interspecific competition.

- The fact that the digestibility was not increased, is not necessarily a negative factor. In AMD treatment a sustainable substrate is required, an a rapid increase in digestibility would reduce availability of substrate over an extended period of time. The leachate, as a by-product could actually be more significant, if it can be sustained. In this study, leachate was produced throughout the 6 week experimental period.

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CHAPTER 4

SULPHATE REDUCTION USING FUNGAL HYDROLYSIS BY-PRODUCTS (LEACHATE) OF *CENCHRUS CILIARIS* CV. MOLOPO (BUFFELSGRASS)

ABSTRACT

Removal of sulphate from sulphate-rich waters through biological sulphate reduction has the potential to be an effective procedure for bioremediation of acid mine drainage (AMD). A suitable carbon source that can sustain sulphate reducing bacteria (SRB) growth remains a major obstacle in using SRB in biotechnological applications. Previous studies indicated that organic carbon was possibly leached from *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) by chemical and microbiological mechanisms. Using a filter paper disk assay, the leachate did not exhibit antimicrobial activity when tested against Gram negative or Gram positive organisms. The effect of lignocellulose leachates on sulphate reduction by SRB was investigated using drip bag bioreactors. An SRB-enriched inoculum was produced by incubating anaerobic digester sludge with artificial AMD. Reactors containing artificial AMD, 3% inoculum and 3% or 6% of the leachates were constructed. The chemical complexity of the substrate and its biodegradation influenced sulphate reduction. Metalsulphides contributed to fluctuations in the chemical oxygen demand (COD). Overall sulphate reduction varied from 27.7% to 44.9% and long-term sulphate reduction rates of 9.4 mg/l/d to 15 mg/l/d were observed. Sulphate removal efficiencies (in terms of mg SO_4^{2-} reduced / mg COD consumed) of 27% to 52% were obtained. No biological sulphate reduction was observed in the control reactors. Sulphate removal was comparable to literature values, but sulphate reduction rates were considerably lower in this study. Near neutral pH was maintained in all leachate amended reactors, unlike leachate-free controls. Both total anaerobic bacteria and sulphate reducing bacteria benefited from leachate addition. Therefore, leachate buffering capacity could enhance SRB survival and consequently expedite biological sulphate reduction.

1. INTRODUCTION

1.1 The carbon source dilemma

Removal of sulphate from sulphate-rich waters through biological sulphate reduction (BSR) has the potential to be an effective and cost-effective procedure for bioremediation of acid mine drainage (AMD). Sulphate reducing bacteria (SRB) will form the core component of such an approach. A suitable carbon source that can sustain SRB growth remains a major obstacle in using SRB in biotechnological applications (Béchard *et al.*, 1994). Various carbon sources have been evaluated for use in passive treatment of AMD under laboratory and field conditions (Table 4.1).

Defined carbon substrates do not provide a cost-effective means of stimulating sulphate reduction by sulphate reducing bacteria. Cost of acquirement, transport, handling difficulties etc. prevent these carbon substrates from being applied on-site in large-scale passive AMD treatment systems. Undefined carbon substrates typically are agricultural residues and other lignocellulose wastes. These carbon substrates are cheap sources of fermentable organic carbon. However, in its natural form, lignocellulose is a highly recalcitrant heteropolymer. The intricate associations between the dominant polymers (cellulose, hemicellulose and lignin) creates a physical and chemical barrier that prevents the rapid and complete degradation of the organic carbon. Therefore, efforts to improve lignocellulose digestibility aim at removing the lignin barrier, thereby releasing carbohydrate polymers for hydrolysis and fermentation.

Table 4.1: Defined and undefined carbon sources evaluated for use in AMD treatment systems.

Carbon source	References
Wood dust	Tuttle *et al.*, 1969a; Tuttle *et al.*, 1969b
Wood dust and various defined organic nutrients	Wakao *et al.*, 1979
Digester slurry of cattle waste	Ueki *et al.*, 1988
Spent mushroom compost	Dvorak *et al.*, 1992
Alfalfa hay, timothy hay, and cereal straw	Béchard *et al.*, 1994
Whey and cow manure	Christensen *et al.*, 1996
Rice stalks and cow manure	Cheong *et al.*, 1998
Lactate	Elliott *et al.*, 1998
Cow manure, sawdust, and cheese whey	Drury *et al.*, 1999
Methanol, lactate, and horse manure	Tsukamoto and Miller, 1999
Various undefined carbon sources	Chang *et al.*, 2000

1.2 Lignocellulose leachate: characteristics and significance

Leaching of nutrients from crop residues left on the field after harvest is a natural phenomenon (Schreiber, 1999). Previous studies indicated that organic carbon was possibly leached from Buffelsgrass by chemical and microbiological mechanisms (Chapter 3)

Our knowledge on the leachate can be summarised as follows:

- *The chemical composition of the leachate is complex.* Hot water extracts of wheat straw contained phenolic compounds and carbohydrates (Galletti *et al.*, 1990; Milstein *et al.*, 1981; Vered *et al.*, 1981). Larger and more complex lignocellulose degradation products like lignin-carbohydrate complexes (LCC) might also be present (Cornu *et al.*, 1994; Jeffries, 1990). Other compounds including proteins, nucleic acids and lipids or

Jeffries, 1990). Other compounds including proteins, nucleic acids and lipids or degradation intermediates of these compounds might be present in the leachate. Therefore, a wide range of compounds might be present in the leachate but their effect on sulphate reduction by sulphate reducing bacteria remains uncertain.

- *Leachate might stimulate bacterial growth due to the presence of nutrients.* Water extract from wheat straw residues had no inhibitory effects on the tested microbes (Abdel-Nasser *et al.*, 1983). High bacterial cell yields were obtained when *Arthrobacter* spp. was grown on wood leachate produced from pentachlorophenol (PCP) treated wood poles (Mollah and Allen, 1999). In this leachate PCP contributed to about 5% of the total COD. Bacterial growth on this leachate was attributed to the presence of other carbon sources in the leachate. Therefore, leachate might be advantageous by stimulating sulphate reduction by sulphate reducing bacteria.

- *Microbial consortia might neutralise toxicity of the leachate.* Extractions of wheat straw with hexane, ethyl ether and ethanol indicated the presence of antibacterial substances (Abdel-Nasser *et al.*, 1983). Based on *Daphnia magna* toxicity tests, the biodegradation process detoxified the wood leachate produced from pentachlorophenol (PCP) treated wood poles (Mollah and Allen, 1999). Biodegradation resulted in an 18-fold reduction in toxicity. No phytotoxicity of field leachates (leachates that originated from decomposing agricultural material left on the ground after harvest) was observed (Breakwell and Turco, 1989). Chang *et al.* (2000) found that compounds inhibitory to sulphidogenesis contained in raw agricultural biomass was degraded under anaerobic conditions. SRB can utilise an impressive number of carbon compounds, and their diverse metabolic capabilities enhances their survival in natural and man-made environments (Hansen, 1994). Therefore, SRB can overcome any inhibitory effects contained in the leachate by cooperative degradation within an anaerobic environment. The question of whether SRB can benefit from lignocellulose leachate remains unanswered.

Akhtar *et al.* (1997) reduced the amount of fungal inoculum required for biopulping by adding low cost nutrients in the form of corn steep liquor (CSL). These nutrients stimulated

fungal growth and colonisation of wood chips. CSL is produced during the wet milling process when dry corn is soaked in a warm dilute acid solution. CSL and lignocellulose leachate are similar in the following aspects:

1. Both originate from natural and/or aided breakdown of lignocellulose.
2. Both are by-products from a primary process – leaching occurs during natural degradation of plant material and CSL is produced during wet corn milling.
3. Both offer cheap sources of nutrients

Therefore, lignocellulose leachate can be a source of readily available organic carbon and other growth factors.

1.3 Objectives

The effect of the leachate on aerobic bacteria was investigated, but the effect of the leachate on sulphate reduction by sulphate reducing bacteria remained or primary concern. This project was divided into three phases (Figure 4.1). The first phase comprised the production of the inoculum to be used in the follow-up phases. The objective of the first phase was to ensure that the SRB depleted the residual carbon contained within the digester sludge. The content of this drip bag was used as the inoculum to evaluate the effect of the leachate as the sole carbon source on sulphate reduction by the SRB. The second phase consisted of a trial run using only one leachate in a drip bag experiment. The objective of the second phase was to ensure that the experimental setup would yield the expected results. The third phase started once the trial run was successfully completed. The objective of the third phase was to evaluate all the available leachates in a similar experimental setup as described for phase two.

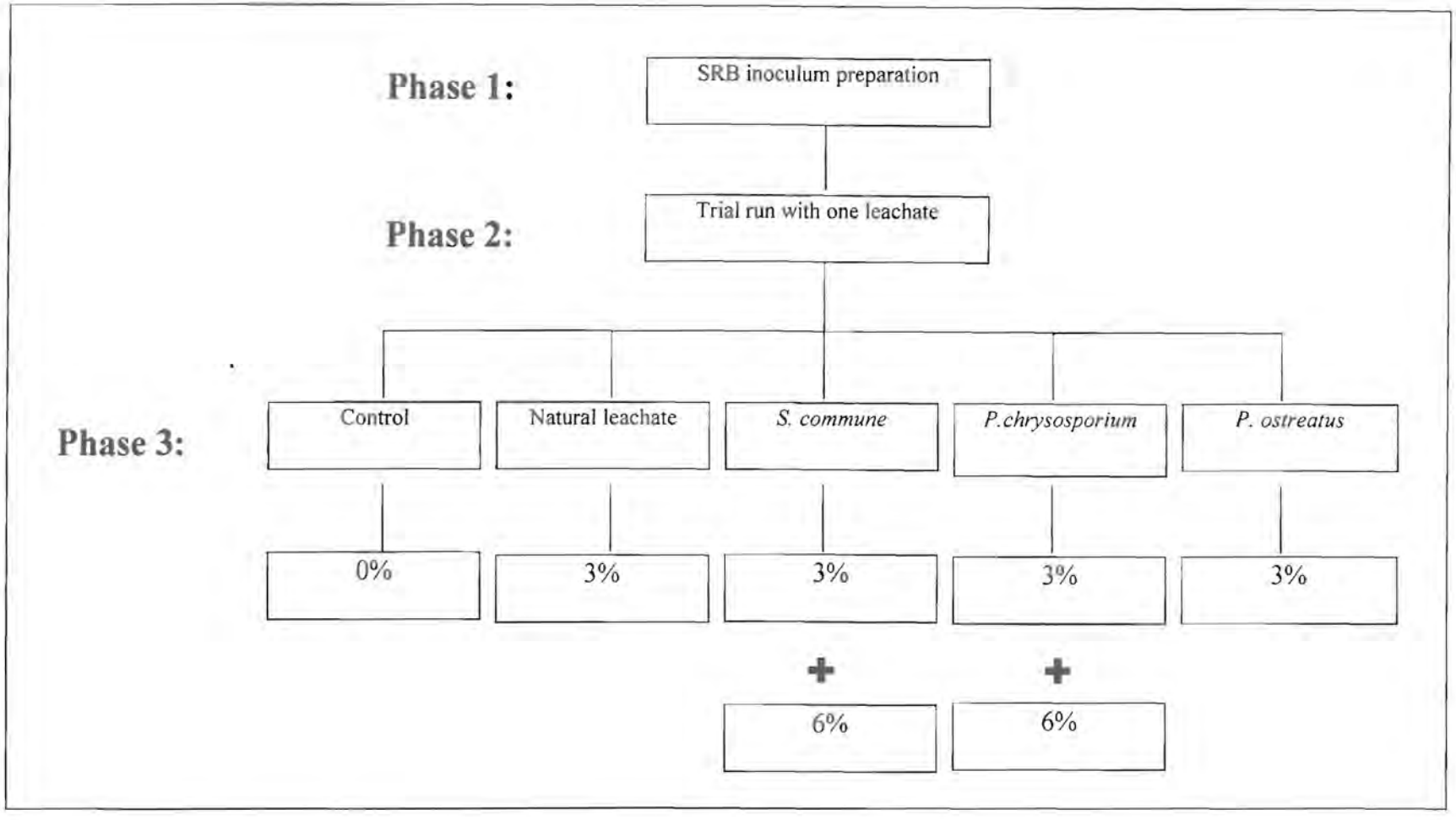

Figure 4.1: Summary of the experimental layout for evaluating sulphate reduction using the leachate as carbon source.

2. MATERIALS AND METHODS

2.1 Leachate antimicrobial activity assay

The antimicrobial activity of the leachates from fine natural grass treatments (weeks 1, 3, and 5) was tested against 2 Gram positive bacteria species (*Bacillus subtilis, Staphylococcus aureus*) and 2 Gram negative bacteria species *(Pseudomonas putida, Pseudomonas alcaligenes*). A number of pretreatment methods were evaluated to determine the best leachate sterilisation procedure (Table 4.2). Raw leachate (no pretreatment) was also evaluated. Sterile water containing chloramphenicol (250 mg/l) was included as a control.

Table 4.2: Pretreatment methods evaluated to determine the best leachate sterilization procedure.

- Ultracentrifugation for 10 min at 2 500 rpm, 5 000 rpm and 10 000 rpm
- Filter sterilisation through 0.2 μm porosity membranes
- Autoclaving

0.1 ml of an actively growing bacterial culture was inoculated into an empty petri dish. Nutrient Agar at 45°C was poured into the petri dish, rotated to ensure good mixing and distribution of bacterial cells, and allowed to cool and set. Pretreated leachate was pipetted onto sterilized filter paper disks (10 mm diameter) until the disks were saturated. Leachate impregnated filter paper disks were transferred aseptically onto the agar plates. Two disks were placed on each plate. The plates were incubated at 37°C and monitored after 24, 48 and 72 h for the formation of inhibition zones.

2.2 Preparation of inoculum

Anaerobic digester sludge, obtained from Daspoort Water Treatment Facility in Pretoria, was used as inoculum. 600 ml of digester sludge was incubated with 1400 ml of artificial acid mine drainage (AMD) without the addition of leachate (Table 4.3).

Table 4.3: Chemical composition of artificial acid mine drainage.

Component	Final concentration (mg/l) or volume added per liter (ml/l)
H_3PO_4 (85%)	0.02 ml/l
$MgSO_4.7H_2O$	1310 mg/l
H_2SO_4	519 mg/l
$FeSO_4.7H_2O$	4557 mg/l
NH_4Cl	190 mg/l
The final pH was adjusted to 7.0 using 1M NaOH.	

The reactor was filled with the AMD/digester mixture and sealed. Excess gas inside the reactor was removed with a sterile syringe and needle. The inoculum reactor was incubated at room temperature (17°C - 23°C) for 21 d. After 21 d, the incubation temperature was increased to 30°C. The reactor was shaken every day and excess gas was removed daily with a sterile syringe and needle. Samples were taken aseptically (using a sterile syringe and needle) from the reactor once a week until the desired SRB / total anaerobic bacteria ratio was obtained.

Following sulphate depletion, 1310 mg/l $MgSO_4.7H_2O$ and 4557 mg/l $FeSO_4.7H_2O$ were added to the inoculum reactor after 63 d. Samples were taken once a week and analysed for sulphate (Spectroquant SQ 118, Merck), total anaerobic bacteria, SRB and pH. COD was not determined.

The vitality of the inoculum was tested using 2 reactors containing 1 l artificial AMD and 30 ml (3%) of inoculum reactor content. 30 ml (3%) of a 3 month old Buffelsgrass leachate was added to one of the reactors. Samples were collected once a week and sulphate (Spectroquant SQ 118, Merck), pH, total anaerobic bacteria and SRB were determined.

2.3 Leachate origin and application strategy

A dark brown leachate was collected from plastic-tubing reactors containing moist Buffelsgrass incubated for 6 weeks with natural or added fungi (Chapter 3). The leachates from fine and medium cut natural grass (unsteamed) were pooled for each treatment. The volume of available leachate dictated the amount of replicates that could be included in this experiment and the concentrations at which the leachates could be applied (Table 4.4).

Table 4.4: Leachate application strategy.

Treatment	Concentration	No. of replicates
1. Control	None	2
2. Natural leachate	3%	1
3. *P. ostreatus*	3%	2
4. *S. commune*	3%	2
5. *P. chrysosporium*	3%	2
6. *S. commune*	6%	1
7. *P. chrysosporium*	6%	1

Therefore, 4 leachate pools were evaluated: leachate from uninoculated unsteamed Buffelsgrass (1), and leachates from unsteamed Buffelsgrass inoculated with *Schizophyllum commune* (2), *Pleurotus ostreatus* (3) or *Phanerochaete chrysosporium* (4).

2.4 Leachate amended experiments

A total of eleven 2 l reactors were constructed for the final phase of the study (Figure 4.1; Table 4.4). All reactors contained 1800 ml of artificial acid mine drainage (AMD) (Table 4.3) and 60 ml (3%) of the inoculum. The appropriate volume of leachate was added to yield a final concentration of either 3% or 6% (Table 4.4). Reactors were sealed, excess gas removed and incubated at 30°C. The contents were mixed daily and excess gas was removed aseptically using sterile syringes and needles.

2.5 Physical and chemical analyses

Unless otherwise specified, 100 ml sample was removed from the reactors once weekly and sent to Waterlab Research (Pty) Ltd for chemical oxygen demand (COD) (dichromate method; APHA-AWWA-WPCF, 1995) and sulphate determinations (turbidimetric method; APHA-AWWA-WPCF, 1995). The ratio of H_2S and HS^- in the samples depended on the pH and were calculated using the following equation:

$$H_2S = [1 + 1.02 \times 10^{(pH-7)}]^{-1}$$

From these fractions, the total COD was corrected by substracting the COD equivalent of the FeS content. During the determination of the COD, the oxidation reaction using $Cr_2O_7^{2-}$ as oxidant occurred as follows:

$$Cr_2O_7^{2-} + 14H^+ + 6e^- \rightleftarrows 2Cr^{3+} + 7H_2O$$

The remaining dichromate was determined titrimetrically and the amount of oxidation was determined by difference. Stoichiometrically, 3.5 moles O_2 is required to oxidise 1 mole HS^-. Therefore, 3.39 g of O_2 is required to oxidise 1 g of HS^-.

During the third phase of the experiment, 5 d biochemical oxygen demand (BOD_5) was determined for all samples after 42 d until the end of the experiment after 70 d. The pH was determined using a Ross model 8102 electrode (Orion Research).

2.6 Microbiological analyses

1 ml sample was taken from the reactor once a week and used for the microbiological analyses. The total anaerobic bacteria were enumerated on nutrient agar (Biolab) incubated under anaerobic conditions in an anaerobic jar (48 h at room temperature). Gas generating kits (Oxoid) were used to generate hydrogen and carbon dioxide in the sealed anaerobic jar. Anaerotest^R test strips (Merck) were included as indicators of anaerobiosis in the anaerobic jar. The total anaerobic plate counts (TAPC) were conducted in duplicate. SRB were enumerated in duplicate using modified Postgate's medium B (Table 4.5). SRB were enumerated after 21 d incubation at 30°C.

Table 4.5: Composition and preparation of modified Postgate's medium B for the enumeration of sulphate reducing bacteria (SRB).

Solution A		Solution B		Solution C	
	900 ml distilled H_2O		**100 ml distilled H_2O**		**50 ml distilled H_2O**
KH_2PO_4	0.5 g	$MgSO_4.7H_2O$	2.0 g	Tri-sodium citrate	2.94 g
NH_4Cl	1.0 g	$FeSO_4.7H_2O$	0.5 g		
Yeast extract	1.0 g			Titanium-chloride	5 ml
Resazurin	0.01 g				
Agar	10 g				
Sodium lactate (50%)	7 ml				
Adjust pH to 7.0 with 1 M NaOH prior to the addition of the agar. Autoclave at 121°C for 20 minutes.		Filter sterilise into solution A after it has been autoclaved.		Adjust to pH 7.0 with $NaHCO_3$. Autoclave at 121°C for 20 minutes. Add 200 µl to the A+B mixture already aseptically dispensed into sterile Hungate tubes.	

3. RESULTS

3.1 Leachate antimicrobial activity

None of the different pretreatment methods (Table 4.2) influenced the outcome of the antimicrobial activity experiment. Leachate pretreated with ultrafiltration through 0.2μm porosity membranes, high-speed centrifugation (2500 rpm, 5000 rpm and 10000 rpm for 10 min), and autoclaving showed no antimicrobial activity. Instead, the opposite occurred with zones of increased bacterial density within the leachate diffusion zone. No inhibition was caused by the leachates recovered from week 1, 3 and 5 of all treatments of fine natural grass (Figure 4.2).

3.2 Vitality of inoculum

Visual observations

Gas was removed from the inoculum reactor on a daily basis to prevent the build-up of inhibitory volatile compounds. However, gas production ceased after 21 days and no black precipitate formed. It was decided to incubate the reactor at 30°C for the remainder of the experimental period.

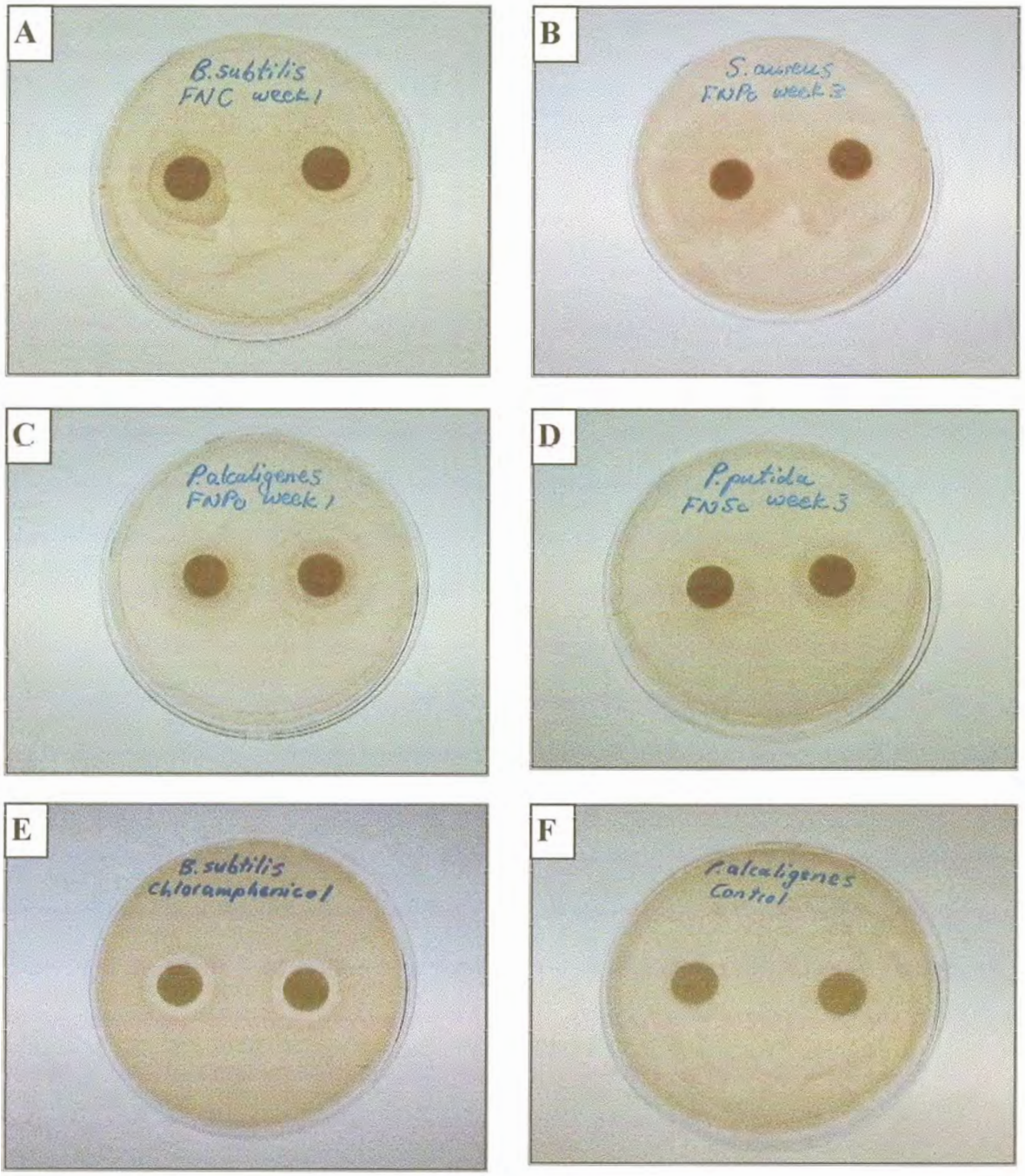

Figure 4.2: Antimicrobial activity assay for leachates derived from different weeks of fine natural grass treatments: A) Control (FNC), B) *Phanerochaete chrysosporium* (FNPc), C) *Pleurotus ostreatus* (FNPo), and D) *Schizophyllum commune* (FNSc). Heavy bacterial growth was observed within close proximity of the leachate saturated disks (A and B). Diffusion zones were clearly visible in some cases (C) and bacterial growth was visible within these zones. The controls contained either chloramphenicol (E) or distilled water (F) and showed clear inhibition zones or none respectively.

The entire reactor blackened after 2 days at 30°C. Therefore, the elevated incubation temperature had a positive effect on the overall sulphate reduction process due to the rapid formation of the black precipitates. H_2S was also detected by smell in subsequent samples.

Physical and chemical analyses

Active biological sulphate reduction occurred in the inoculum reactor. The reduced sulphate was converted to H_2S (odour detectable after 19 d) and metal sulphides (formation of black precipitates). The production of metal sulphides influenced COD determinations. The organic COD content was sufficient to facilitate complete sulphate reduction after 56 d at 31.2 mg SO_4^{2-}/l/d (Figure 4.3).

Stoichiometrically, 1171.8 mg/l COD was required for complete sulphate reduction. Experimentally, 1624 mg/l COD was consumed which represented a COD demand of 452.1 mg/l. The increase in the pH from 6.97 to 8.09 after 56 d was evidence of sulphate reducing bacterial activity. Sulphate was reintroduced into the reactor after 63 d and decreased over 14 d from 1778 mg/l to 1428 mg/l at 25 mg/l/d (Figure 4.3). Theoretically, H_2S accumulated to 405.3 mg/l in the inoculum reactor after 56 d. Reis *et al.* (1992) found that H_2S produced from sulphate reduction had a direct and reversible toxicity effect on SRB. A H_2S concentration of 547 mg/l completely inhibited culture growth. However, H_2S toxicity at concentrations lower than 200 mg/l has been reported (Hilton and Oleszkiewicz, 1988; Hulshoff Pol *et al.*, 1998). Unionised H_2S is toxic to microorganisms because neutral molecules can permeate well through the bacterial cell membrane. Inside the cell, H_2S can react with proteins to form new bisulfide bonds and can also affect the intracellular pH (Hulshoff Pol *et al.*, 1998).

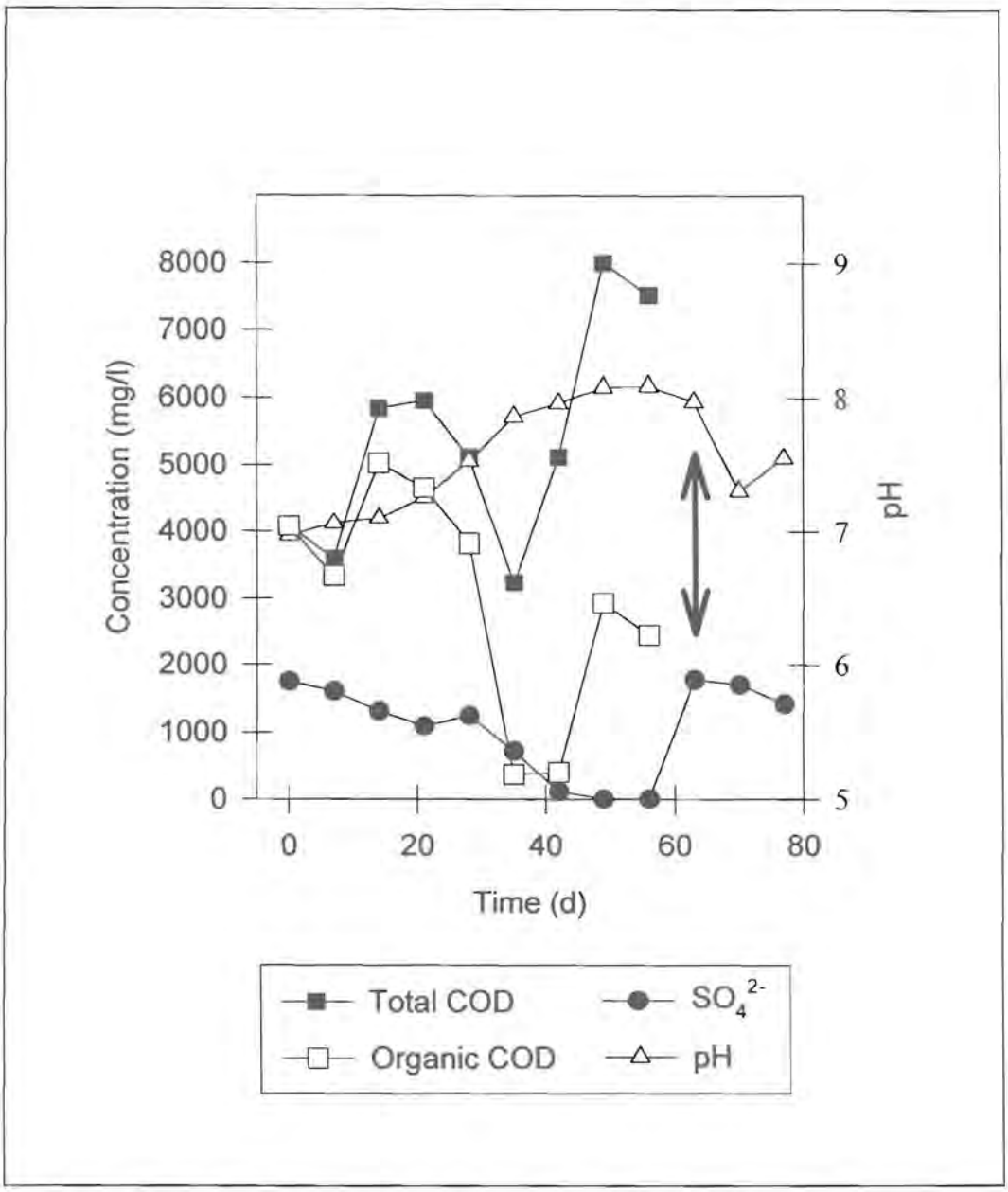

Figure 4.3: Change in chemical parameters monitored during inoculum preparation. The arrow indicates when sulphate was supplemented. COD was not determined after 63 d.

Microbiological analyses

Chemical analyses indicated that active sulphate removal occurred in the inoculum reactor (Figure 4.3). Microbiological results indicated that an active consortium of SRB was present in the inoculum reactor. The total number of anaerobic bacteria increased over 21 d from 10^3 colony forming units per ml (cfu/ml) to 10^7 cfu/ml (Figure 4.4). Anaerobic bacteria proliferated during the hydrolysis of the anaerobic

digester sludge. However, once the hydrolysis stage was completed, SRB effectively outcompeted the fermentative bacteria for nutrients in the form of fermentation intermediates. This was evident in the decline of the total number of anaerobic bacteria during the following 3 weeks to 10^5 cfu/ml. SRB maintained their dominance in the inoculum reactor at SRB/TAPC ratios of 0.18 to 2.96 (after 35 to 56 d) until addition of unsterile sulphate after 56 d (Figure 4.4). We concluded that an active consortium of SRB was dominant in the reactor. Hence, phase 2 of this study was initiated to determine the vitality of this inoculum.

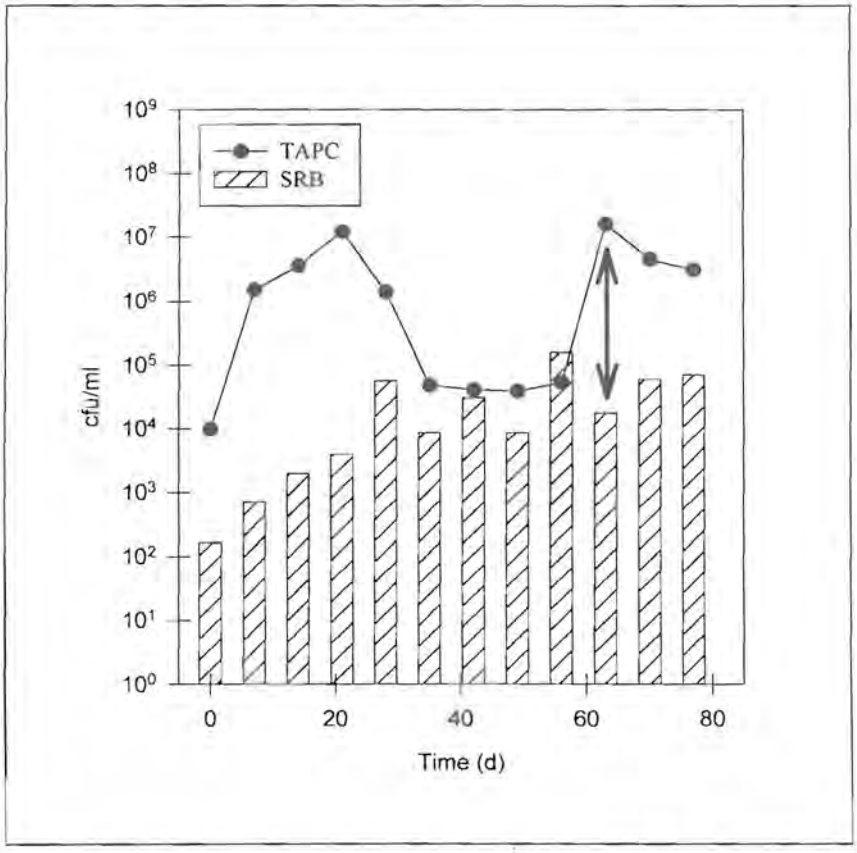

Figure 4.4: Development of microbial population structure during inoculum preparation. The arrow indicates when sulphate was supplemented. TAPC = total anaerobic bacteria count; SRB = sulphate reducing bacteria

Inoculum evaluation experiment

The inoculum responded well when it was reintroduced into fresh artificial AMD (pH ± 7) with a 3-month old Buffelsgrass leachate as carbon source. The greatest difference between the reactors was evident when they were compared visually

between the reactors was evident when they were compared visually (Figure 4.5). The sulphate concentration in the drip bag with the 3% leachate decreased over 14 d from 2833 mg/l to 1763 mg/l at 76.4 mg/l/d (Table 4.6). The leachate contained appreciable amounts of sulphate (2324 mg/l) and this facilitated the onset of biological sulphate reduction (Ueki *et al.*, 1988). Artificial AMD was unstable and this was reflected by the pH decrease and the release of sulphate over 14 d (Table 4.6). SRB attained dominance within 14 d in the leachate amended reactor, but SRB numbers decreased in the control reactor (Table 4.6). The pH decrease in the control reactor contributed to the decline in SRB numbers since optimum SRB growth occurred at pH 5-9 (Postgate, 1979).

Figure 4.5: Comparison of the reactor that received 3% leachate (left) with the control reactor (right). Both reactors were inoculated with the inoculum produced in this study. Gas production was detected after 7 d in the leachate amended reactor, but not in the leachate-free control reactor.

Table 4.6: Chemical and microbiological results from preliminary drip bag experiment evaluating effectiveness of inoculum in the presence and absence of leachate.

Time (d)	3% leachate[a]				Leachate-free control			
	pH	SO_4 (mg/l)	TAPC[b] (cfu/ml)	SRB[c] (cfu/ml)	pH	SO_4 (mg/l)	TAPC (cfu/ml)	SRB (cfu/ml)
0	6.98	2833	0	0	6.94	1977	2150	0
7	6.67	1957	$3.0x10^4$	$1.9x10^5$	5.42	2388	$1.2x10^6$	$3.2x10^4$
14	6.90	1763	1050	$8.5x10^4$	4.64	2127	$2.2x10^7$	900

a. 3 month old leachate produced from *Cenchrus ciliaris* cv. Molopo. Initial sulphate concentration: 2324 mg/l; b. Total anaerobic plate count; c. Sulphate reducing bacteria.

3.3 Leachate amended experiments

Visual observations

Contents of all 11 reactors were dark blue-green at the start of the experiment. The content of the control reactors became red-brown after 3 d while the leachate amended reactor contents turned black after 3 d. H_2S odour were not detected in the samples over the experimental period of 70 d. One of the control reactors turned dark grey after 49 d.

Characteristics of biological sulphate removal

Biological sulphate reduction took place in all the leachate amended reactors (Figures 4.6-4.8 and Table 4.7). Overall sulphate reduction rates of 9.4 mg/l/d (Natural leachate) to 15.1 mg/l/d (6% *S. commune* leachate) were observed (Table 4.7). However, when the sulphate increases after 63-70 d were ignored, the sulphate reduction rates improved to a maximum of 17.7 mg/l/d (natural leachate) (Table 4.7).

No sulphate reduction occurred in the control reactors. Again this can be attributed in part to low pH. However, once the pH increased to around 6 after 42 d, sulphate reduction could still not commence due to the absence of sufficient organic carbon. Near-neutral pH values were maintained within leachate amended reactors. Lignocellulose leachate probably has a buffering mechanism which facilitate decomposition of lignocellulose in any environment.

Closer inspection of the sulphate reduction curves revealed that sulphate reduction was dependent upon the release/bioavailability of organic carbon (Figures 4.6-4.8). The first phase involved rapid utilisation of organic matter (simple sugars, proteins, etc.) by fermentative microorganisms. The pH in all the leachate amended reactors decreased slightly by 0.1 - 0.5 pH units within the first 7 d, but increased thereafter. This adaptation phase was characterised by sulphate reduction within the first 3 to 7 d in some of the leachate amended reactors.

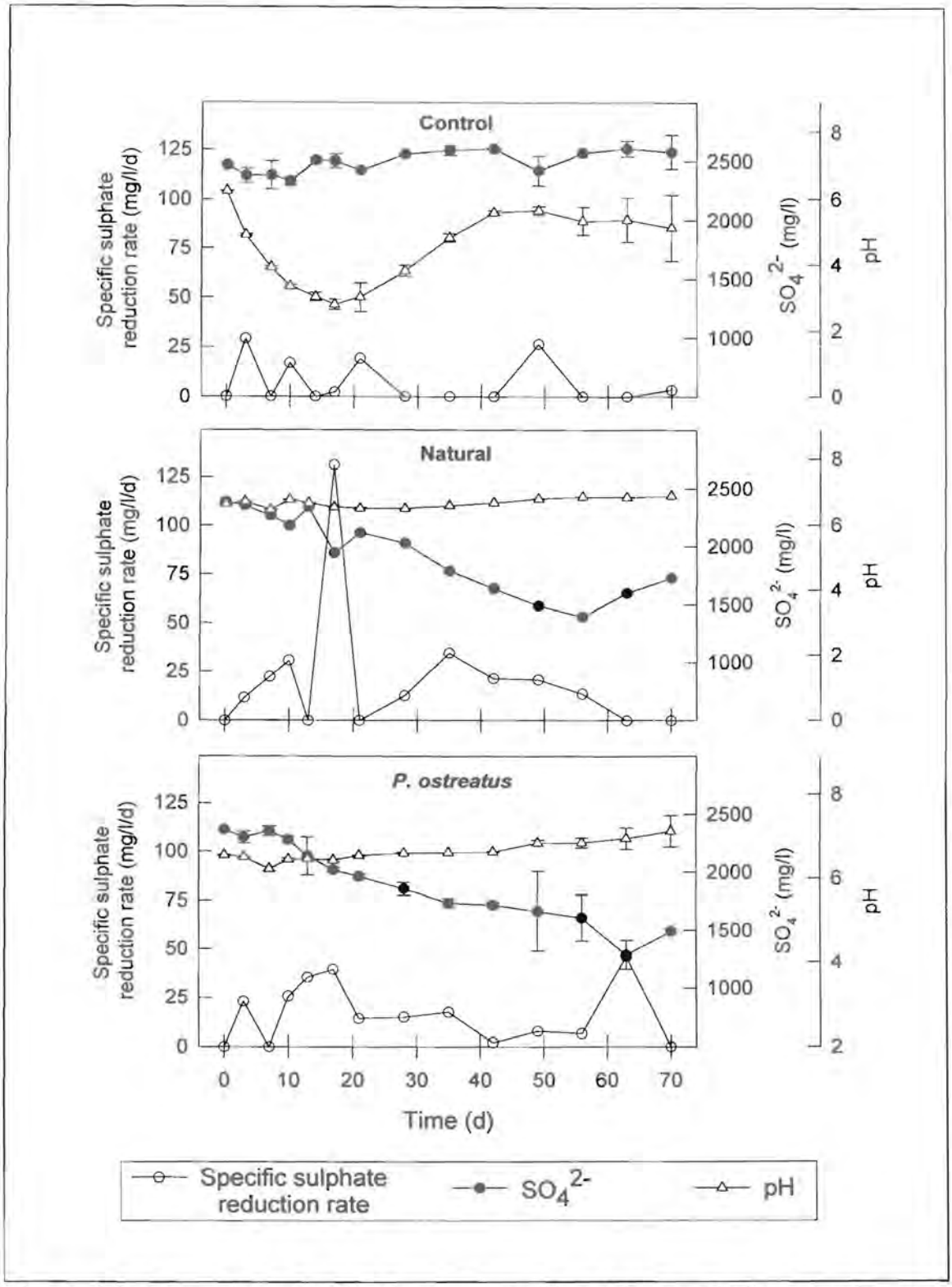

Figure 4.6: Comparison of pH values and specific sulphate reduction rates between sampling events for leachate-free controls, natural and *P. ostreatus* leachate treatments. Sulphate increases were interpreted as zero sulphate reduction.

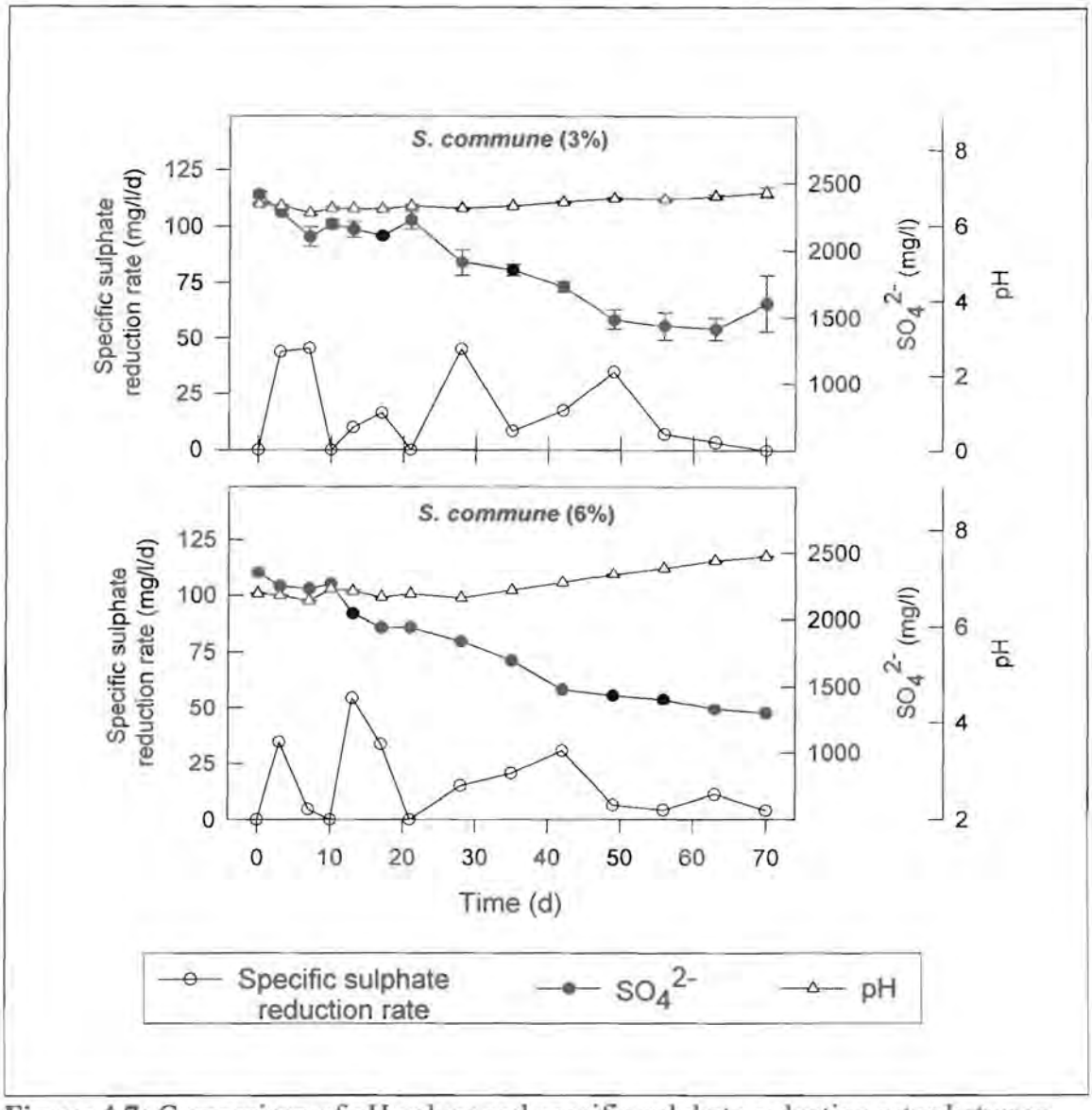

Figure 4.7: Comparison of pH values and specific sulphate reduction rates between sampling events for *S. commune* leachate treatments. Sulphate increases were interpreted as zero sulphate reduction.

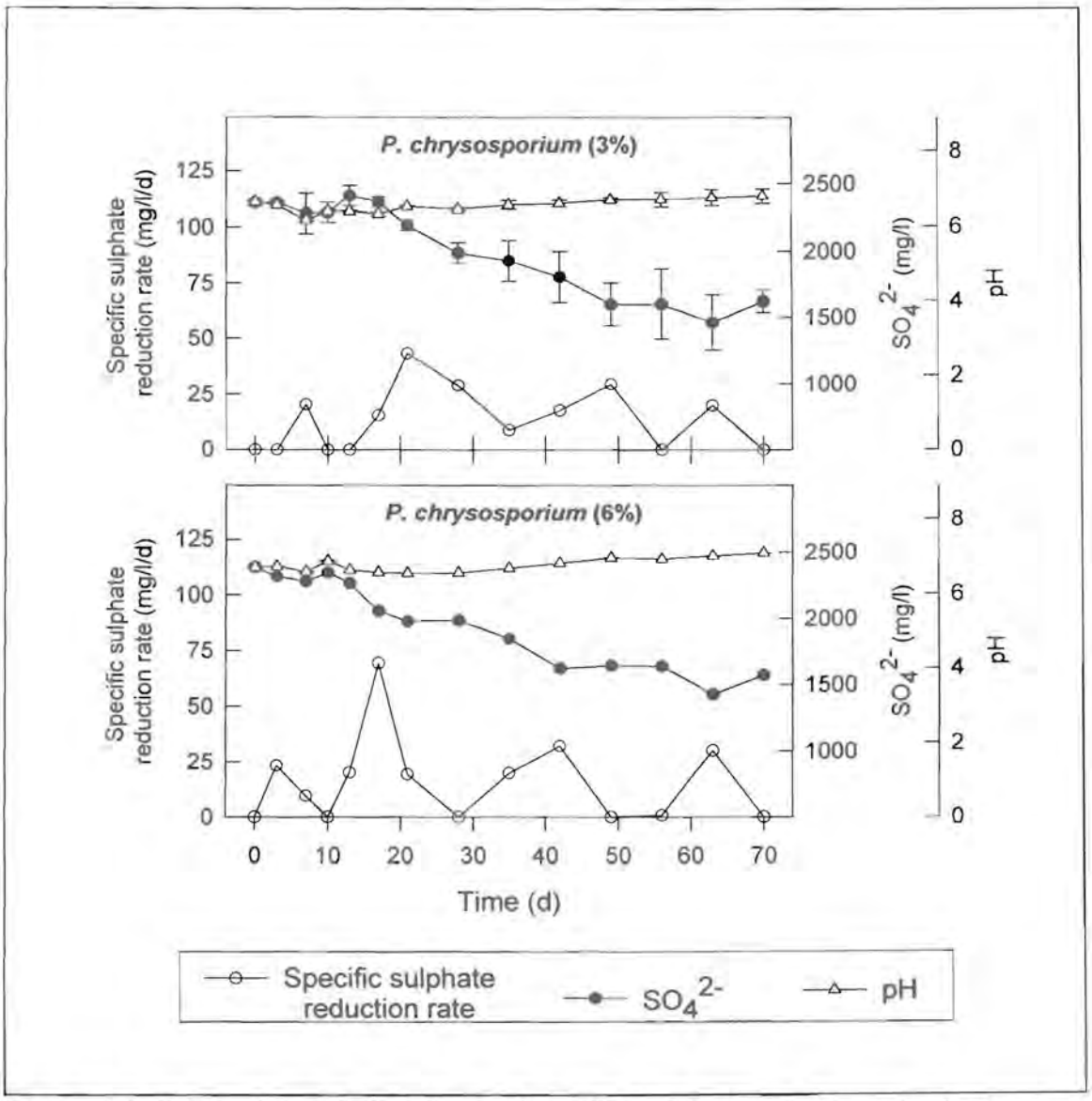

Figure 4.8: Comparison of pH values and specific sulphate reduction rates between sampling events for *P. chrysosporium* leachate treatments. Sulphate increases were interpreted as zero sulphate reduction.

Table 4.7: Overall sulphate removal efficiencies for leachate amended treatments after 70 d. Most of the sulphate values increased after 56 or 63 d. Therefore, the best sulphate reduction rates and percentage sulphate removed are indicated in brackets.

Treatment	Initial SO_4^{2-} (mg/l)	Final SO_4^{2-} (mg/l)	SO_4^{2-} reduction rate (mg/l/d)	SO_4^{2-} reduction rate after 35 d (mg/l/d)	% SO_4^{2-} removed
Control	2466	2579	0	0	+ 4.6
Natural	2386	1725 (1395 after 56 d)	9.4 (17.7)	17.2	27.7 (41.5)
P. ostreatus	2368	1493 (1287 after 63 d)	12.5 (17.2)	18.1	36.9 (45.7)
S. commune (3%)	2413	1604 (1411 after 63 d)	11.6 (15.9)	16.1	33.5 (41.5)
P. chrysosporium (3%)	2350	1621 (1461 after 63 d)	10.4 (14.1)	12.3	31.0 (37.8)
S. commune (6%)	2359	1299	15.1	18.7	44.9
P. chrysosporium (6%)	2386	1573 (1429 after 63 d)	11.6 (15.2)	15.3	34.0 (40.1)

Following the adaptation phase, the rate of sulphate reduction decreased after 10 to 15 d. The duration of this lag phase differed between treatments. Sulphate reduction resumed after the lag phase and introduced a period of sulphate reduction characterised by sequential starts and stops in terms of the rate of sulphate reduction (Figures 4.6-4.8).

At least two periods of sulphate reduction separated by a lag phase can be distinguished in the data of all leachate treatments (Figures 4.6-4.8). The trends observed in the variable phase are reminiscent of diauxic bacterial growth (Figure 4.9) (Caldwell, 1995). When *E. coli* was transferred to a medium containing glucose and lactose, glucose was utilised first and lactose was utilised only after glucose exhaustion. Therefore, once the monosaccharides were exhausted, a short stationary phase followed during which the enzymatic systems needed to utilise the disaccharides were induced. The exponential growth phase continued albeit at a slower rate, because more metabolic energy was required to utilise the now abundant, but more complex carbon source.

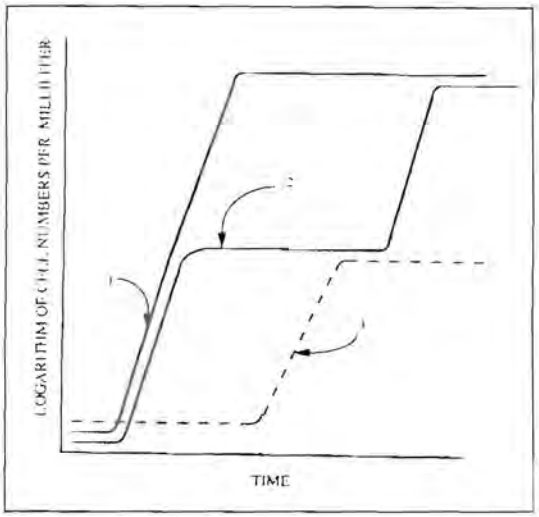

Figure 4.9: Diauxic growth of *E. coli*. (1) Lactose-grown cells transferred into a medium containing a mixture of glucose and lactose. (2) Glucose-grown cells transferred into a medium containing glucose and lactose. (3) Glucose-grown cells transferred into a medium containing only lactose as a carbon source (Caldwell, 1995)

Therefore, the specific sulphate reduction rates indicated that complex organic compounds are being degraded under anaerobic conditions, yielding degradation intermediates that could be utilised by the sulphate reducing consortium to sustain sulphate reduction. The bioavailability of these degradation intermediates regulated the initiation and rate of sulphate reduction.

Relative biodegrability potential

In this study, the BOD_5 results were an indicator of the relative potential of the reactor contents to be biodegradable. The BOD_5 analysis was performed using an aerobic sludge seed. Under aerobic conditions, a COD:BOD_5 ratio of 2:1 is considered as optimal. However, this relationship might not be relevant for anaerobic environments. In this study, and under anaerobic conditions, the COD:BOD_5 ratio varied approximately between 1:0.13 to 1:4 (Figure 4.10-4.12). The worst ratio was 1:4 (Control after 56 d, *P. ostreatus* after 49 d) and the best ratio was 0.5 (*P. ostreatus* after 63 d). For all treatments in general, these ratios decreased from 1:3 to 1:1 or less, indicating that biodegradability increased over time.

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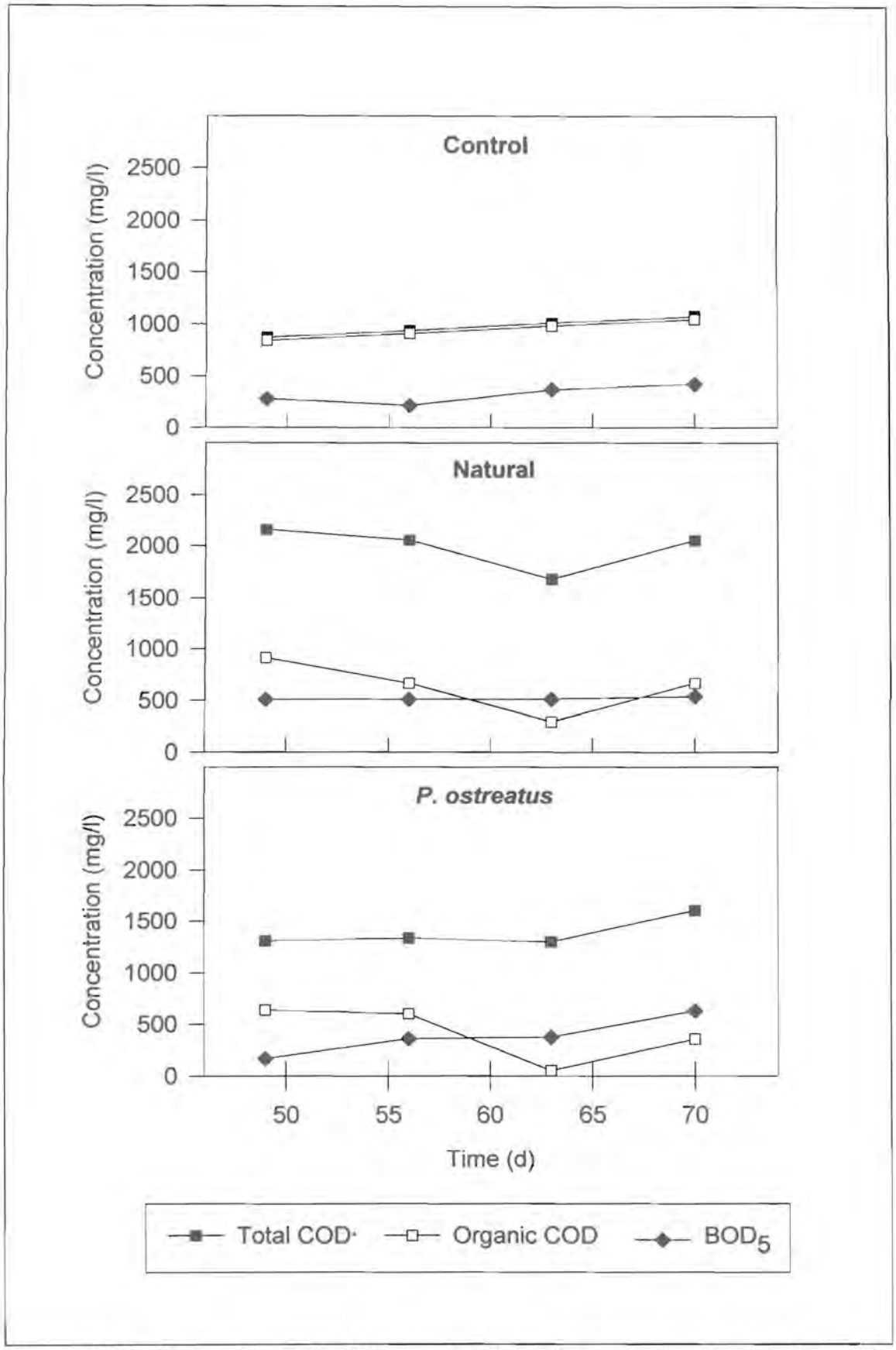

Figure 4.10: Comparison of BOD_5 results from the control, and natural and *P. ostreatus* leachate treatments.

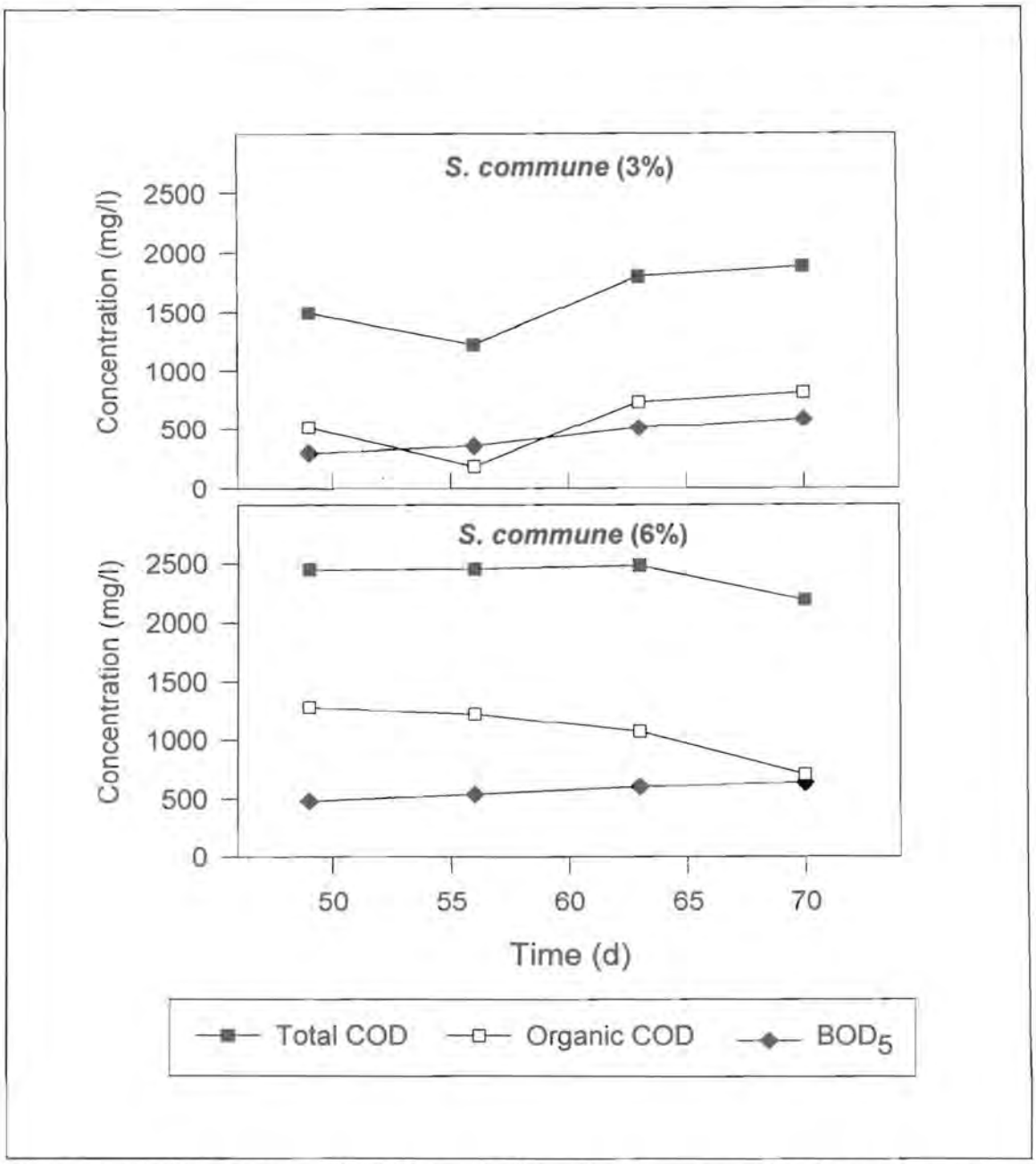

Figure 4.11: Comparison of BOD_5 results from the *S. commune* leachate treatments.

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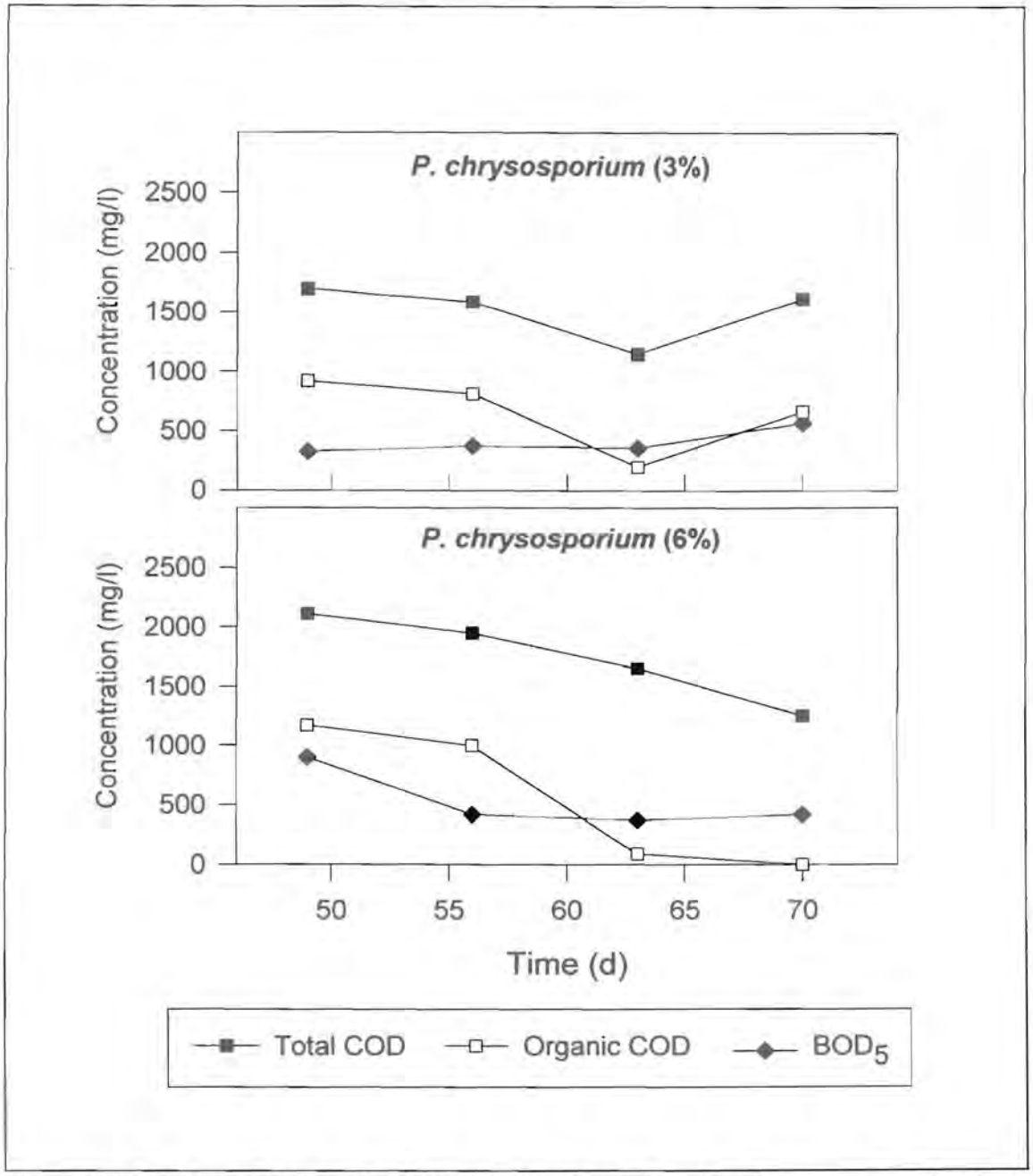

Figure 4.12: Comparison of BOD_5 results from the *P. chrysosporium* leachate treatments.

Sulphide toxicity

Predicting H_2S production stoichiometrically and theoretically, based on the solution pH, we have concluded that H_2S toxicity did pose a threat to the viability of SRB in this study (Figures 4.13-4.15). In this study, the total sulphide theoretically present in the control reactors after 70 d, 98.7% was in the form of un-ionised H_2S. Stoichiometrically, 424.1 mg/l H_2S accumulated in the control reactors after 70 d. However, process failure in the control reactors can also be attributed to insufficient organic carbon concentration, low pH, and low SRB numbers.

In the leachate amended treatments, stoichiometric H_2S concentrations varied from 648 mg/l (*S. commune*, 6%) to 920 mg/l (natural). Specific sulphate reduction rates decreased over time (35 d to 70 d) in most of the leachate amended reactors with the theoretical accumulation of H_2S (Figures 4.6-4.8, 4.13-4.15).

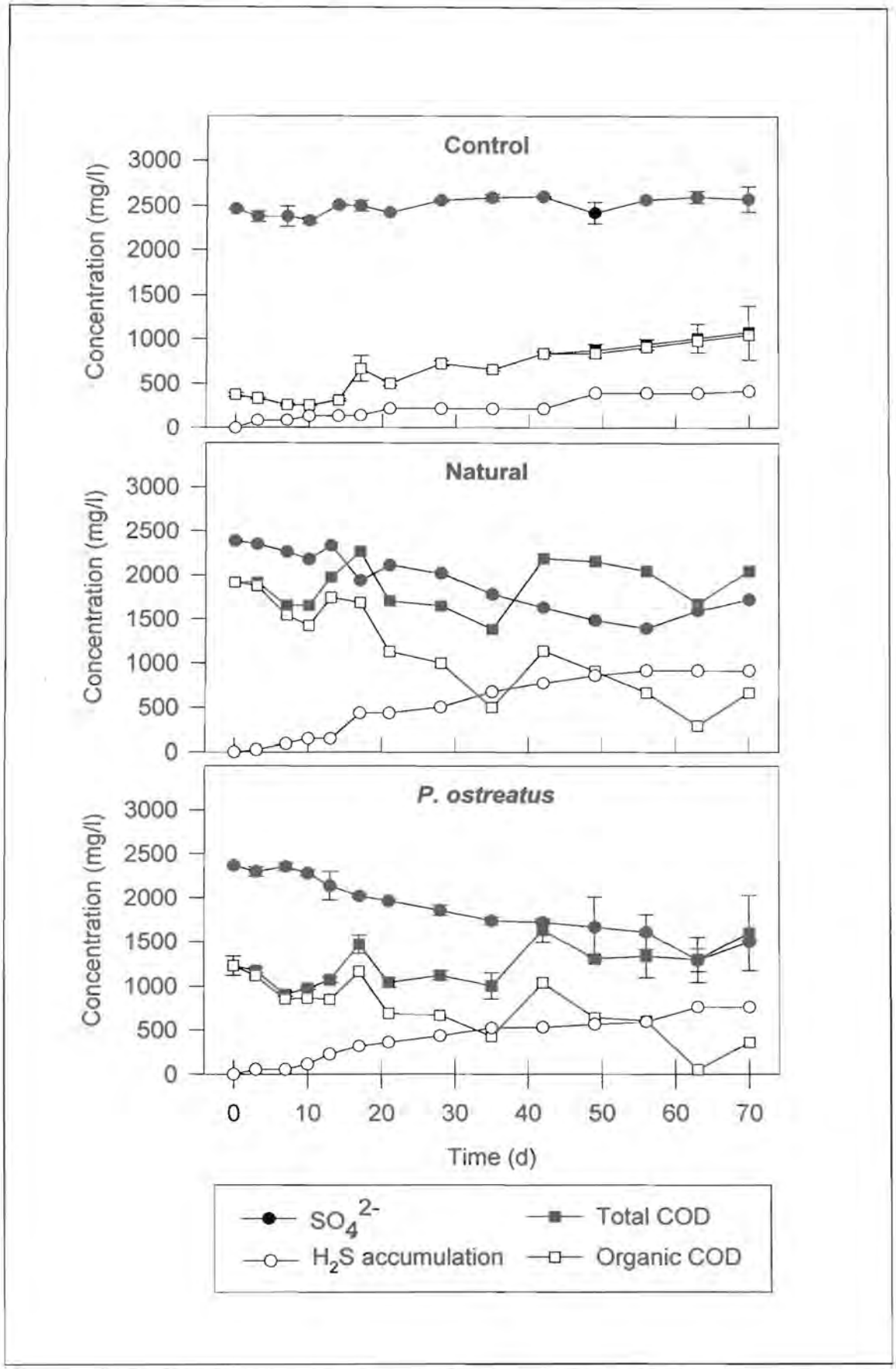

Figure 4.13: Relationship between observed sulphate reduction and theoretical FeS/H_2S accumulation for the controls, and natural and *P. ostreatus* leachate.

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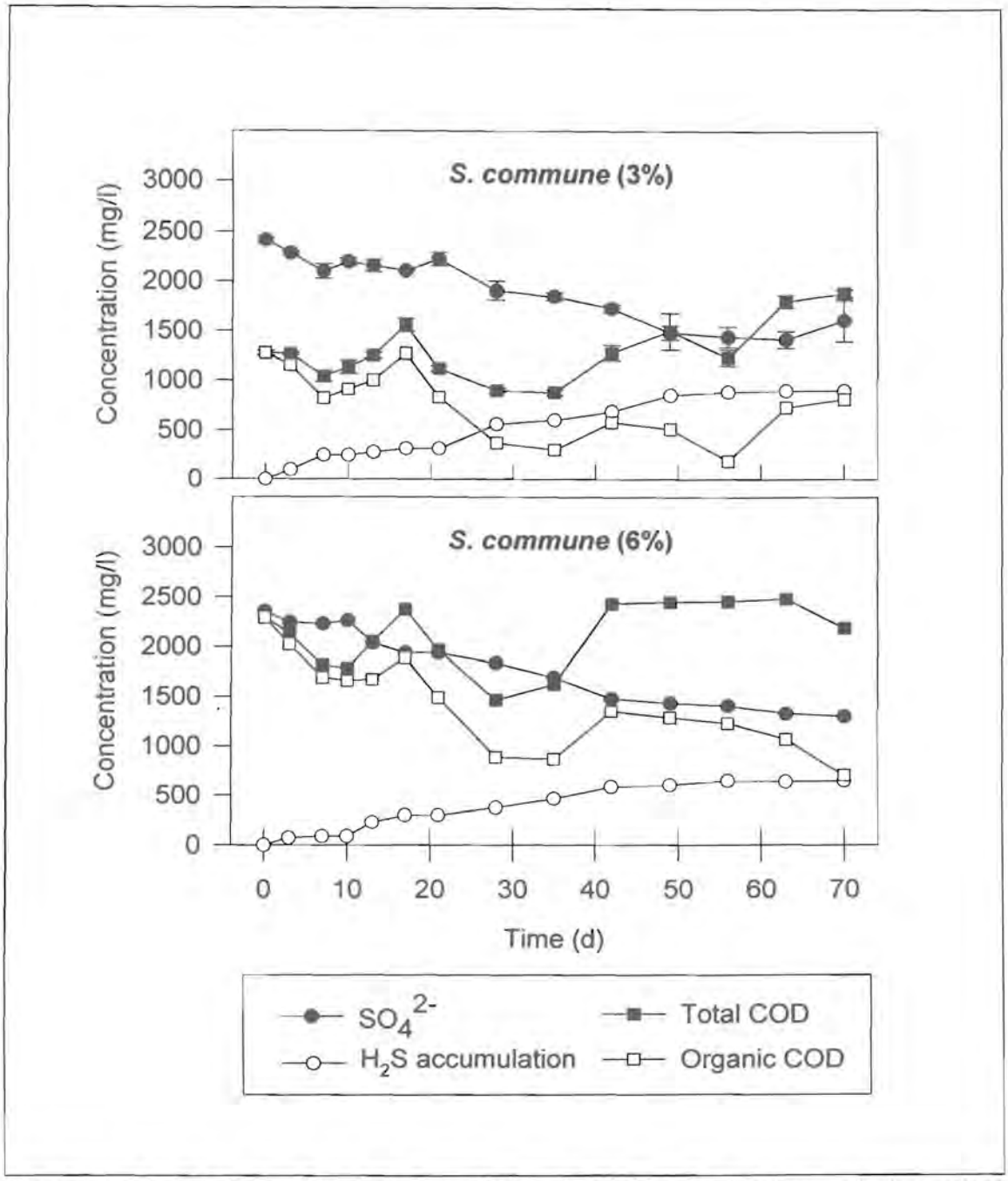

Figure 4.14 : Relationship between observed sulphate reduction and theoretical FeS/H_2S accumulation for *S. commune* leachate treatments.

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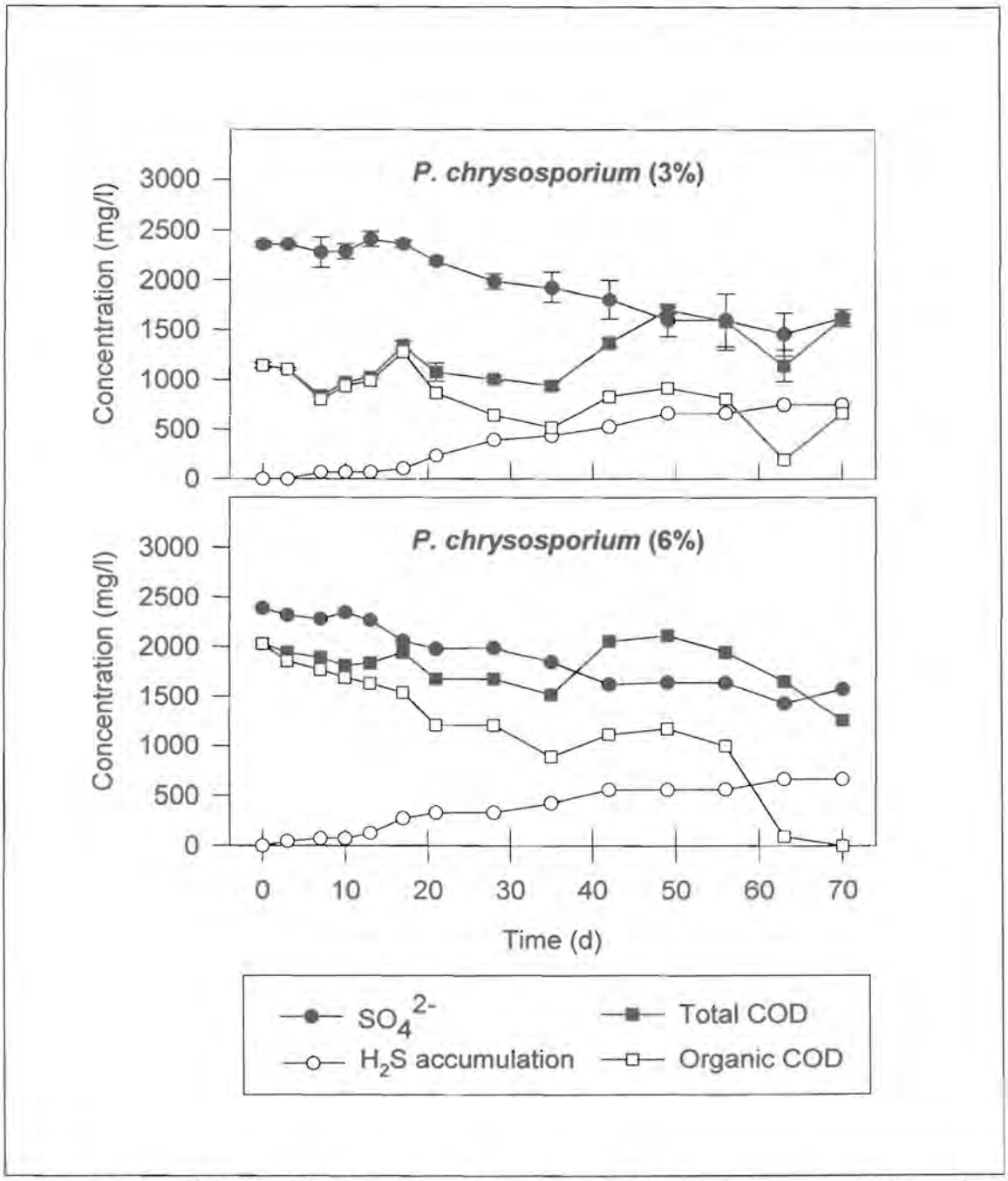

Figure 4.15: Relationship between observed sulphate reduction and theoretical FeS/H_2S accumulation for *P. chrysosporium* leachate treatments.

Microbiological results

The leachate not only sustained SRB activity in the leachate amended units, but the anaerobic microbial population in general also benefited (Figures 4.16-4.18). Relatively high numbers of total anaerobic bacteria was maintained in the leachate amended units throughout the experimental period.

SRB numbers increased from 10^4 cfu/ml to more than 10^5 cfu/ml within 7-20 d (Figures 4.16-4.18). These numbers remained constant for the remainder of the experiment between 10^4 and 10^5 cfu/ml in most of the leachate amended treatments, reaching 10^5 to 10^6 cfu/ml in the units amended with 3% of *S. commune* and *P. ostreatus* leachates (Figures 4.17 and 4.18). However, a gradual decrease in the SRB numbers was observed after 30-40 d in the treatments amended with 3% of *S. commune* and *P. ostreatus* leachates. It appears as if SRB viability is decreasing in the 3% *S. commune* treatment because sulphate reduction rates decreased over the same time period (Figure 4.7). The reasons for this could be greater sensitivity to sulphide inhibition or poor biodegradability of the remaining COD (Figure 4.11).

The numbers of total anaerobic bacteria also remained constant around 10^6 cfu/ml during the experimental period (Figures 4.16-4.18). The number of total anaerobic bacteria and SRB declined in the control treatments from the outset of the experiment. However, these numbers increased after 30 d (Figure 4.16). This sudden proliferation of microorganisms was directly related to the increase in pH. Even though SRB numbers increased to 10^4 cfu/ml, no continuous sulphate reduction occurred. The increasing differences between the duplicate control reactors were evident in the increasing standard deviations by the end of the experiment (Figures 4.6 and 4.16). One reactor remained red-brown while the other was dark-grey in colour. Reactor A (final pH 4.10) supported 5.7×10^4 cfu/ml total anaerobic bacteria while reactor B (final pH 6.12) supported 1.26×10^7 cfu/ml total anaerobic bacteria at the end of the experiment. Both reactors showed a sulphate increase of 7.9% (A) and 3.3% (B) respectively (Figure 4.6).

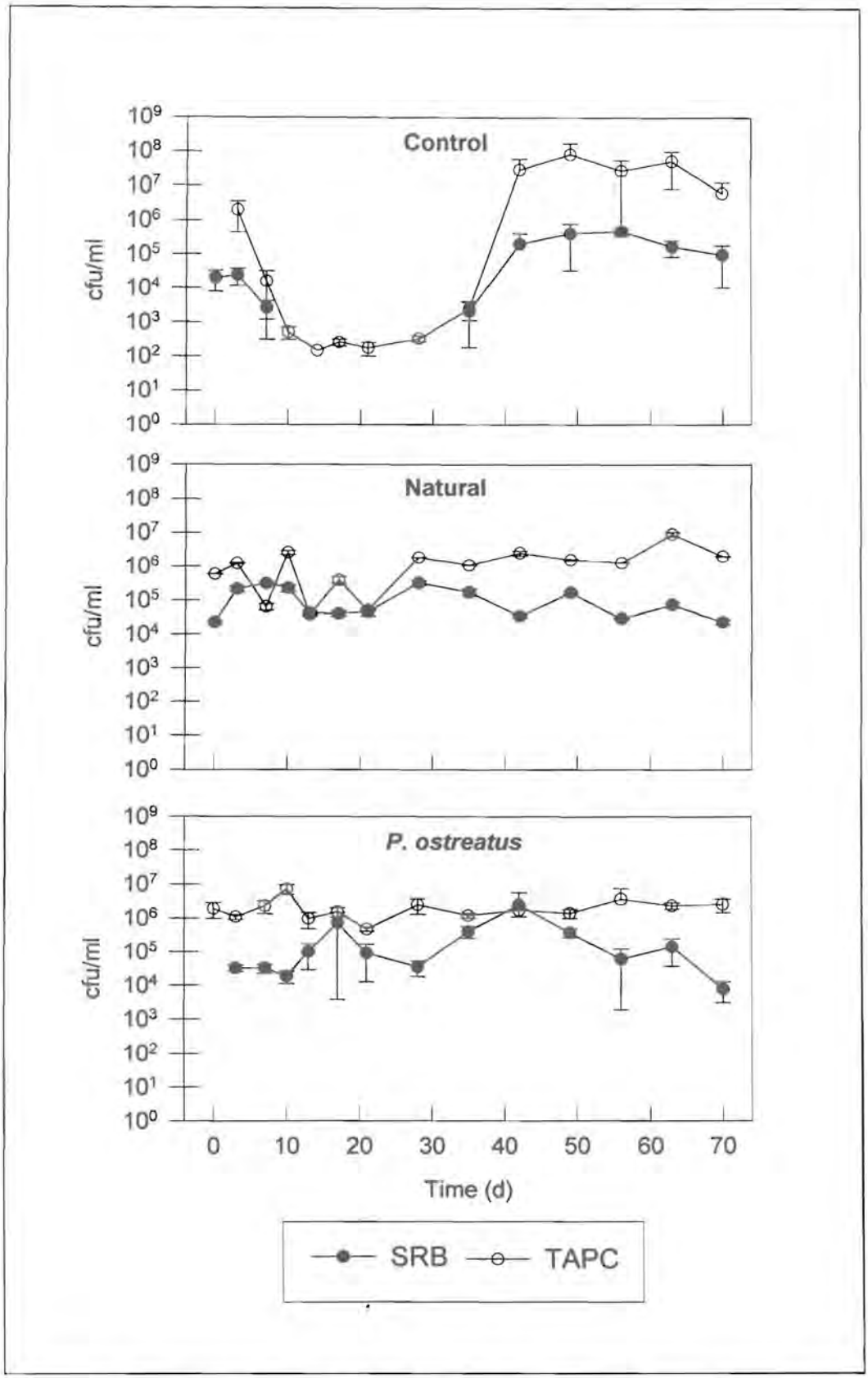

Figure 4.16: Change in microbiological parameters of leachate-free controls, natural and *P. ostreatus* leachate treatments. SRB = sulphate reducing bacteria; TAPC = total anaerobic plate count.

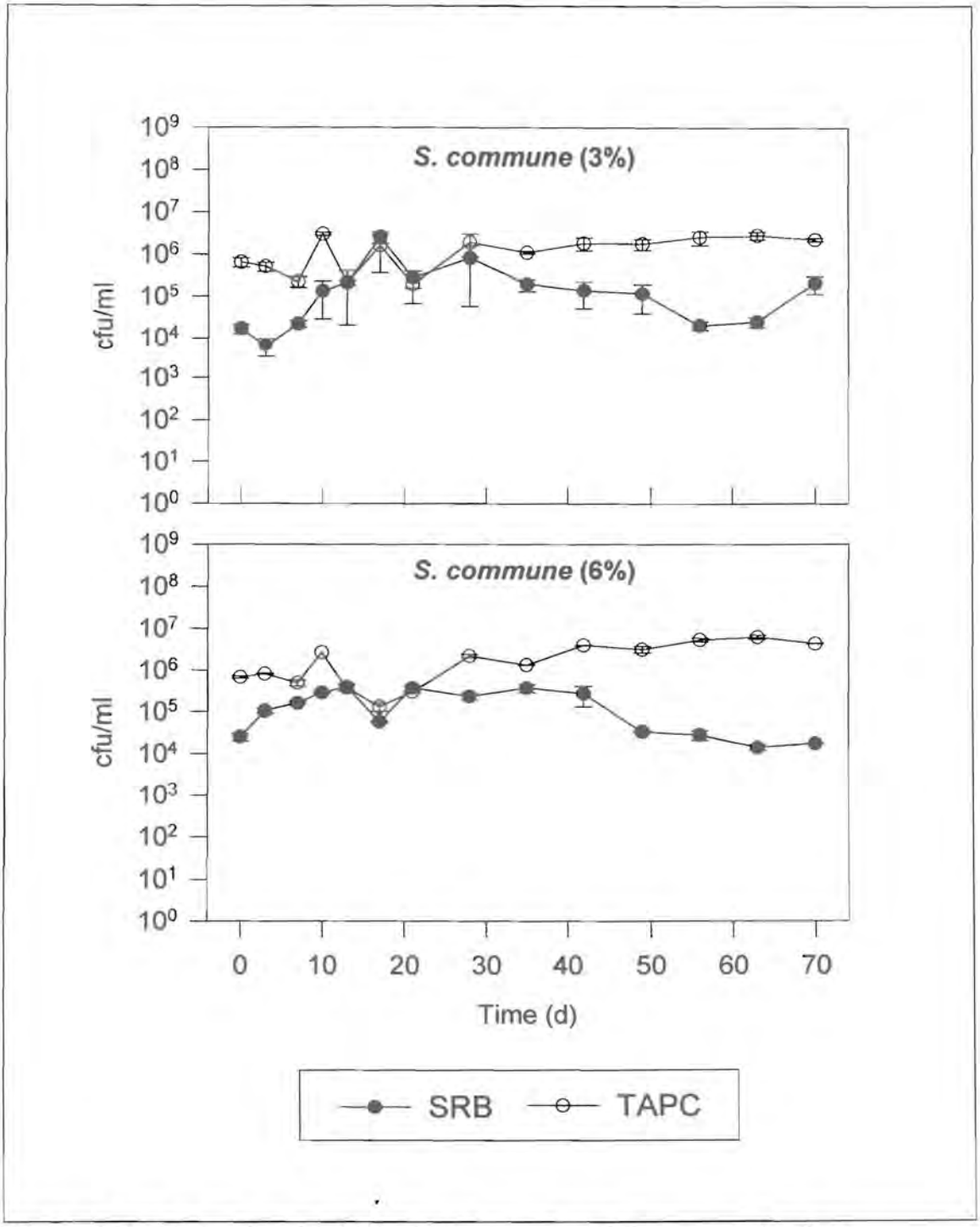

Figure 4.17: Change in microbiological parameters of *S. commune* leachate treatments. SRB = sulphate reducing bacteria; TAPC = total anaerobic plate count.

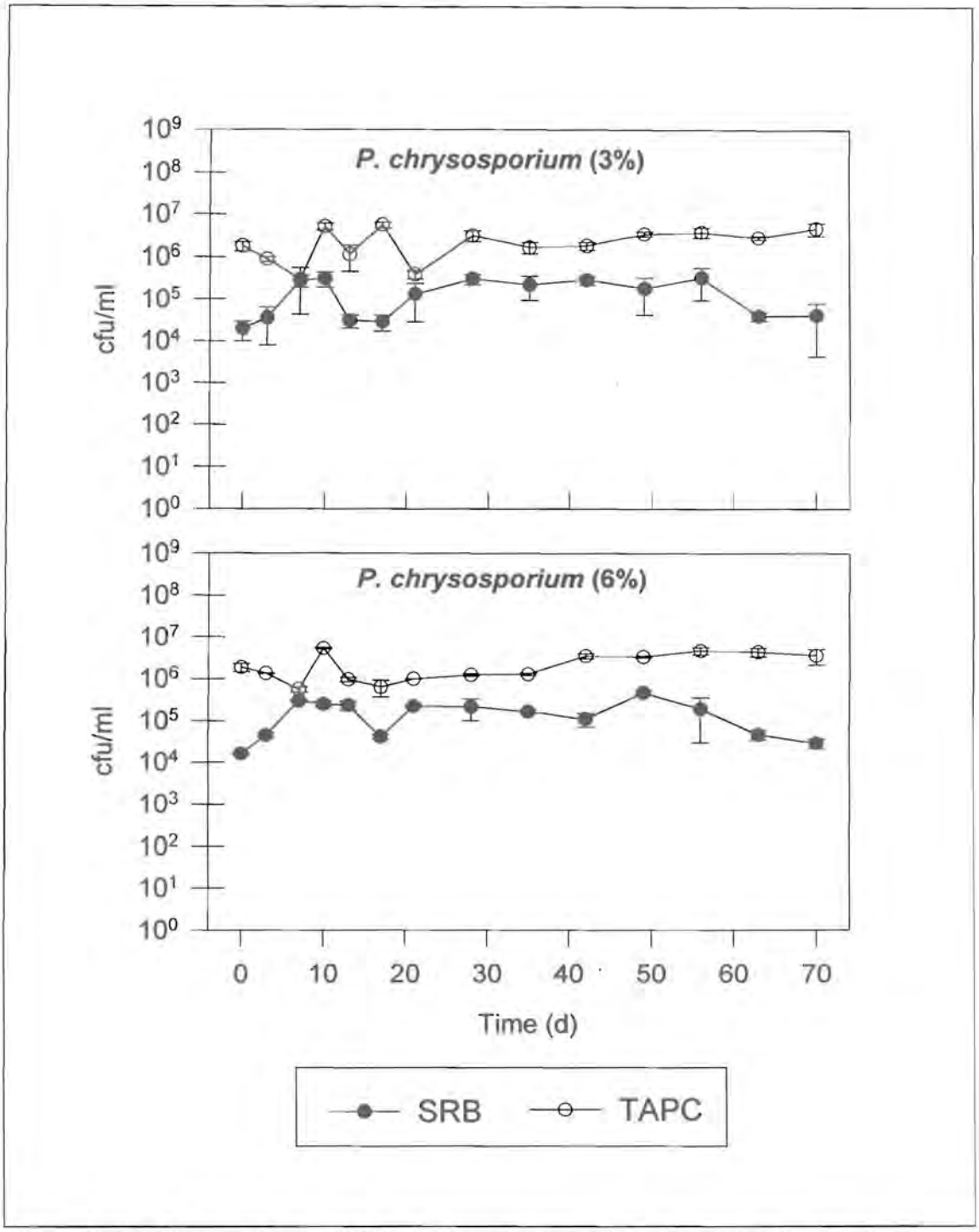

Figure 4.18: Change in microbiological parameters of *P. chrysosporium* leachate treatments. SRB = sulphate reducing bacteria; TAPC = total anaerobic plate count.

Overall process performance

Comparison of experimental to theoretical sulphate removal efficiencies indicated that biological sulphate reduction was supported to different degrees (Table 4.8). Sulphate removal efficiencies in excess of 100% were calculated for *S. commune* (3%) and *P. chrysosporium* (3%) treatments. Although the total COD data were corrected for the sulphide content, COD accumulation was still observed (Figures 4.13-4.15). If the lowest COD values at the end of the experiment were used to calculate the sulphate removal efficiency, *S. commune* (3%) and *P. chrysosporium* (3%) treatments showed 50% and 52% efficiency respectively, while natural and *P. ostreatus*_treatments showed 27% and 50% efficiency respectively.

The COD increases at the end of the experiment could be attributed to accumulation of SRB metabolic end-products and the failure of the methanogens to remove this COD (Polprasert and Hass, 1995). However, reported sensitivity of methanogens to sulphide toxicity varies (Hilton and Oleszkiewicz, 1988; Hulshoff Pol *et al.*, 1998).

Table 4.8: Efficiency of biological sulphate removal process based on lignocellulose leachate carbon source. Efficiencies calculated using the lowest COD value are indicated in brackets.

Treatment	SO_4^{2-} removed (mg/l)	COD consumed (mg/l)	Theoretical COD requirement (mg/l)	SO_4^{2-} removed (mg SO_4^{2-} / mg COD)	% SO_4^{2-} removal efficiency
Control	0	0	0	0	0
Natural	661	1253	443	0.53	35 (27)
P. ostreatus	875	870	586	1.01	67 (50)
***S. commune* (3%)**	809	463	542	1.75	117 (50)
***S. commune* (6%)**	1051	1589	704	0.66	44
***P. chryso* (3%)**	738	481	494	1.53	103 (52)
***P. chryso* (6%)**	813	2027	545	0.40	27

4. DISCUSSION

4.1 Process performance

The ultimate aim of this work was to determine whether lignocellulose leachate could be used to stimulate full-scale biological sulphate reduction. A comparison of the current process performance to theoretical yields will determine process efficiency and ultimately cost-effectivity.

We have provided possible explanations for the accumulation of COD in all of treatments. Standard methods and literature cite several sources of interference (APHA-AWWA-WPCF, 1960; Methods for the examination of waters and associated materials, 1986). We have indicated that FeS accumulation influenced the COD results. Other researchers made similar observations and corrected COD values for sulphide and other reduced inorganic species (Maree and Strydom, 1987; Polprasert and Haas, 1995). After correction of our data for sulphide interference, an overall COD decrease was observed.

Other sources of interference emanate from the chemical complexity of the leachate. The brown colour of the leachate was an indication of a high phenolic content in the form of lignin or lignin degradation products (Eaton *et al.*, 1980). The organic contents can therefore interfere with the COD analysis, especially organic molecules with ring structures (humic substances, lignin degradation intermediates, and phenolic compounds). Accumulation of SRB metabolic end-products and the failure of methanogens to remove this COD could be an explanation for the COD accumulation at the end of the leachate experiment (Polprasert and Hass, 1995).

Process performance was estimated by comparing experimental and theoretical sulphate reduction efficiencies. If the COD accumulation at the end of the experiment was ignored, sulphate removal efficiencies of 27% to 52% were calculated. Reactors amended with 3% *P. ostreatus*, *S. commune* and *P. chrysosporium* leachates performed better compared to the other leachate treatments. These fungi have powerful polymer degrading enzymes (notably cellulases, hemicellulases and lignin

degrading enzymes) that facilitate lignocellulose degradation. Lignocellulose degradation intermediates released by the action of these enzymes are probably present in the leachates in greater quantities compared to the leachate obtained from natural fungal degradation of Buffelsgrass.

The overall sulphate reduction rates obtained in this study were lower than rates reported in literature (Compare Table 4.7 and Table 4.9). Sulphate reduction rates or efficiencies found in literature were not reported in terms of sulphate removed per reducing equivalent of substrate (COD). Overall long-term sulphate reduction rates of 9.4 mg/l/d to 15.1 mg/l/d were obtained in this study (Table 4.7), which was lower than the rates obtained by Dvorak *et al.* (1992). The short-term sulphate reduction rates observed in this study were considerably lower than rates reported in Table 4.9 and by Zdyb (1999) (Table 4.10). However, the final sulphate reduction rates reported in this study compare favourably with the final rates obtained by Drury (1999) (Table 4.9).

In this study, the overall sulphate removal varied from 27.7% to 44.9% (Table 4.7). Comparing the results from Table 4.7 with the literature values in Table 4.9, overall sulphate removal percentages comparable to all undefined carbon source-based systems were achieved in this study (Béchard *et al.*, 1994; Chang *et al.*, 2000; Tsukamoto and Miller, 1999).

4.2 Characteristics of organic COD

According to Hulshoff Pol *et al.* (1998), addition of organic carbon to a sulphate treatment system should be cost effective and not contribute to pollution of the receiving waters. Cost effectivity of undefined carbon sources could be easily estimated, but the down-stream effects could be easily overlooked. The characterisation and conversion of inert COD or non-biodegradable COD is becoming more important to industry because of ever increasing effluent discharge limits set by water control agencies (Archibald *et al.*, 1998). These inert COD fractions have been identified as humic substances (Namour and Müller, 1998) and soluble microbial products (Barker and Stuckey, 1999).

Table 4.9: Examples of defined and undefined carbon sources evaluated for use in active or passive AMD treatment systems.

Carbon sources	Laboratory/ Field experiment	Significance and/or mean sulphate reduction rates (mg/l/d) / efficiencies (%)	Duration of study (d)	References
Wood dust	Both	Origin of concept of treating AMD through biological sulphate reduction. Lab study utilising partially degraded wood dust: • 53.2 mg/l/d (acid mine water) @ 37°C • 58.3 mg/l/d (artificial AMD) @ 37°C	Lab study: 14 d	Tuttle *et al.*, 1969a; Tuttle *et al.*, 1969b
Wood dust and various defined organic nutrients	Lab	• Glucose (0.1%) ◊ 22.8 mg/l/d • Acetic acid (0.5%) ◊ 6.5 mg/l/d • Na-lactate (0.5%) ◊ 58 mg/l/d	Overall: 30 d	Wakao *et al.*, 1979
Digester slurry of cattle waste	Lab	• 48.95 mg/l/d Rate of sulphate reduction was enhanced by increasing the sulphate concentration.	Overall: 20 d	Ueki *et al.*, 1988
Spent mushroom compost	Field	• Pittsburgh system ◊ 32 mg/l/d • Palmerton system ◊ 21 mg/l/d	Pittsburgh system: 350 d Palmerton system: 112 d	Dvorak *et al.*, 1992
Alfalfa hay, timothy hay, and cereal straw	Lab	4.7% (cereal straw), 31% (timothy hay), and 34.2% (alfalfa hay) overall sulphate reduction. Long-term stability could not be achieved. Supplementation with sucrose and urea was required.	Overall: 107 d Process failure after 21 d	Béchard *et al.*, 1994
Whey and cow manure	Lab	Sulphate increased in reactors inoculated with SRB for reasons unknown. Oversaturation with ferric ion minerals caused rapid chemical precipitation during setup and reduced starting concentrations of metals as well as sulphate.	Overall: 203 d	Christensen *et al.*, 1996
Rice stalks and cow manure	Field	Good initial metal removal, but operating problems affected metal removal efficiency. No data on sulphate removal available.	Unknown	Cheong *et al.*, 1998
Lactate	Lab	• 38.3% sulphate removal at pH 3.25 • 14.4% sulphate removal at pH 3.0 Viable SRB could be isolated after 64 d from pH 3.0 environment.	⇒ 42 d ⇒ 64 d	Elliott *et al.*, 1998
Cow manure, sawdust, and cheese whey	Lab	Whey addition increased effluent alkalinity, sulphate removal (98% vs. 60%), and metals removal. Sulphate removal rate decreased over time from 24mg/l/d to 12 mg/l/d (with whey) or 24 mg/l/d to 4 mg/l/d (without whey).	Overall: 644 d (23 months)	Drury, 1999
Methanol, lactate, and horse manure	Both	Field study: • Spent manure ◊ 7% sulphate removal • Spent manure + methanol ◊ 31% "	⇒ Process failure after 230 d ⇒ Overall: 140 d	Tsukamoto and Miller, 1999
Various undefined carbon sources	Lab	32% sulphate removal on average for all carbon sources.	Overall: 35 d Greater sulphate removal efficiency after 14 d than 35 d	Chang *et al.*, 2000

Table 4.10: Summary of results obtained from AMD treatment experiments using undefined carbon sources (Zdyb, 1999). Reactors were inoculated with 30% anaerobic digester sludge.

Carbon sources	Experimental period (d)	Sulphate reduction rate (mg/l/d)	% sulphate removal
Digester sludge[a]	10	0	0
Molasses	10	52.6	12.02
Cow manure (composted)	10	2.0	0.76
Hay	22	103.4	98.96
Fly ash	25	86.3	86.67
Mushroom compost	25	95.2	92.18
Cow manure (fresh)[b]	28	104.6	89.05
Kikuyu (fresh)[b]	28	138.3	97.84
Whey[b]	28	82.7	77.87
Chicken manure (composted)	30	41.8	66.19
Citrus compost	30	45.7	52.75
Silage	30	92.7	93.71

a. Net sulphate increase was reported.
b. Reactors were recharged with the same amount of carbon source after 19 d of operation. Stationary phase in sulphate reduction after 19 d prompted carbon recharge. Sulphate reduction resumed and continued until end of experiment.

The leachate is chemically complex and can contain a mixture of carbohydrates, phenolic compounds, and complex combinations of both. Also, these inert fractions might be insignificant in the beginning of the experiment, but accumulate when biodegradation of readily available residues takes place. Lignin and lignin degradation intermediates can be degraded in anaerobic environments at slow but environmentally significant rates (Zehnder, 1988). Ziomek and Wiliams (1989) found that *Desulfovibrio desulfuricans* modified lignins under anaerobic conditions with a release of low molecular weight phenolic compounds. Therefore, in this study the organic matter in the COD was biodegradable at first, but recalcitrant residues accumulated with time. These inert fractions can be degraded by anaerobic consortia, albeit at a slow rate. However, accumulation of inert COD would indirectly result in organic carbon depletion and inevitable process failure. This could explain why the reactors amended with 6% leachate managed lower sulphate removal efficiencies (Table 4.8).

4.3 Implications of leachate buffering capacity

A problem with biological treatment of AMD is that extremely acidic pH can inhibit biological sulphate reduction (Elliott *et al.*, 1998). The optimum pH for SRB growth is between 5 and 9 (Postgate, 1979). However, viable SRB have been isolated from a very acidic environment (pH 3.0) (Elliott *et al.*, 1998). The initiation of sulphate reduction in AMD treatment systems required an increase in pH to a threshold level of 4-5.2, either by activity of other fermentative bacteria or addition of neutralizing agents (Wakao *et al.*, 1979). A higher initial pH increased the sulphate reduction rate by decreasing the lag phase (Ueki *et al.*, 1988; Wakao *et al.*, 1979). That is why laboratory experiments are conducted using artificial AMD formulations that have been neutralized (Chang *et al.*, 2000). However, pre-adjusting the pH of AMD chemically is not realistic and reproducible in large scale operations. In this study, addition of lignocellulose leachate to the artificial AMD/inoculum mixture prevented a decrease in pH compared to the control reactors.

5. CONCLUSIONS

- Sulphate removal efficiencies of 27% to 52% (mg SO_4^{2-} reduced / mg COD consumed) were obtained in leachate amended reactors. Reactors amended with 3% *P. ostreatus*, *S. commune* and *P. chrysosporium* leachates performed better compared to the other leachate treatments.

- Overall sulphate removal varied from 27.7% to 44.9%, and long-term sulphate reduction rates of 9.4 mg/l/d to 15.1 mg/l/d were observed. Although sulphate removal was comparable to literature values, the sulphate reduction rates were considerably lower.

- Accumulation of inert COD in the form of lignin degradation intermediates could affect process performance of future studies using lignocellulose leachate as carbon source for biological sulphate reduction.

- Near-neutral pH were maintained in all leachate amended reactors. Therefore, leachate buffering capacity/potential could enhance SRB survival and consequently expedite biological sulphate reduction.

- The analytical approach toward following biological sulphate reduction should incorporate additional chemical analyses (iron and sulphide) to accurately model the dynamics of FeS formation and dissolution.

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CHAPTER 5

GENERAL CONCLUSIONS

- The biological treatment of acid mine drainage using sulphate reducing bacteria (SRB) is an alternative to chemical treatment. However, substrate availability normally becomes the limiting factor for sustaining sulphate reduction.

- Biological pretreatment of *Cenchrus ciliaris* cv. Molopo (Buffelsgrass) by white rot fungi was investigated to enhance the biodegradability of this lignocellulose substrate. *In vitro* dry matter digestibility was used to measure the effectiveness of fungal delignification. Although the initial digestibility was high (± 60%), none of the inoculated or control treatments increased the digestibility of the natural substrate.

- A brown leachate was produced in all treatments over the experimental period. Literature indicated that such a leachate could contain inhibitors or stimulants of bacterial growth. An antimicrobial activity assay indicated that the leachate did not inhibit aerobic Gram negative or Gram positive bacteria.

- The effect of the leachate on sulphate reduction by SRB was investigated at 3% and 6% concentrations, using 3% preconditioned inoculum. No biological sulphate reduction was observed in the control (leachate-free) reactors. Reactors amended with 3% *P. ostreatus*, *S. commune* and *P. chrysosporium* leachate performed better compared to other leachate treatments. Sulphate removal was comparable to literature values (27.7% to 44.9%), but the sulphate reduction rates obtained in this study (9.4 mg/l/d to 15 mg/l/d) were considerably lower. Near-neutral pH was maintained in all leachate amended reactors, unlike the controls. Therefore, leachate buffering capacity could enhance SRB survival and consequently expedite biological sulphate reduction.

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