PRODUCTION OF CELLULOLYTIC
ENZYMES USING IMMOBILISED
ANAEROBIC FUNGI

Bernadette K. McCabe (née Thomson)
B.Sc. (Hons.), University of Sydney

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**PLEASE NOTE**

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
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STATEMENT OF AUTHENTICATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to, a substantial extent, has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgement is made in the text of the thesis.

...........

B. K. McCabe

17th June 1998
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<tr>
<td>ABTS</td>
<td>di-ammonium 2,2’-azino-di-(3-ethyl-benzothiazoline-6-sulfonate)</td>
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<td>ADS</td>
<td>anaerobic dilution solution</td>
</tr>
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<td>BMC</td>
<td>ball-milled cellulose</td>
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<td>cellobiose</td>
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<tr>
<td>CBG</td>
<td>Coastal Bermuda Grass</td>
</tr>
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<td>CELB</td>
<td>cellobiose broth</td>
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<td>CSM</td>
<td>cellulose sloppy medium</td>
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<td>Commonwealth Scientific Industrial Research Organisation</td>
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<td>°C</td>
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ABSTRACT

An investigation was made into the isolation and screening of highly cellulolytic anaerobic fungi and their production of cellulolytic enzymes using immobilised rhizomycelia.

A total of 46 anaerobic fungi were isolated on cellulosic substrates from ruminant and non-ruminant herbivores. Primary screening of these isolates was performed using dye release from cellulose-azure which qualitatively detected cellulolytic activity. A quantitative approach was performed in an assay for cellulolysis using U-[14C]-labelled cellulose. Twelve isolates were chosen on the basis of their maximum solubilisation rates of the labelled cellulose and then subjected to secondary screening which involved the quantification of enzyme activity. The enzyme mixtures were characterised by carboxymethylcellulase, xylanase, β-glucosidase, β-xylosidase and cellobiase assays, measured by the production of either reducing sugars, p-nitrophenol or glucose. All strains produced a number of enzymes that allowed them to hydrolyse straw and highest enzyme activity was measured in static cultures grown on 0.5% straw.

A monocentric isolate, *Pirromyces* strain KSX1 from a red kangaroo, and a cattle polycentric isolate, *Orpinomyces* strain 478P1, were selected for study of cellulolytic enzyme production on the basis of high fibre digestion capability and amenability toward encapsulation. A procedure for the immobilisation of anaerobic fungi in calcium alginate was developed allowing for the repeat-batch culture for enzyme synthesis. β-glucosidase production was maintained for 45 days over six consecutive repeat-batch cultivations in immobilised KSX1 cultures using 0.25% cellobiose in the production medium, although fungal rhizomycelium accumulated in the culture liquor throughout the incubation. The basis of sustainability of enzyme production in immobilised KSX1 cultures was undetermined and it is likely that biomass in the bead did contribute in part to enzyme activity in earlier batches before losing viability as the fermentation proceeded. The ability of immobilised cells of strain 478P1 to produce significant amounts of carboxymethyl cellulase, Avicelase and β-glucosidase
activity on a long-term basis was demonstrated. In repeat-batch cultivations, the immobilised fungus could be successfully used for four consecutive batches totalling 30 days of incubation using 0.25% cellobiose in the production medium. The immobilised polycentric strain proved to be operationally superior to strain KSX1 as strain 478P1 did not produce any viable growth in the culture liquor.

Studies into single batch cultures of free cells of strains KSX1 and 478P1 revealed that the maximum specific rate of β-glucosidase production occurred concomitantly with maximum specific growth rate suggesting that the immobilised fungus must grow for continuous enzyme production to occur. Although the physiology of cellulase synthesis in strains KSX1 and 478P1 was found to be growth-associated, immobilisation of the fungus offered the advantage of the repeat-batch use of cells with the accumulation of extracellular enzymes after each batch. Thus, operational gains were the key issues in assessing the potential application of immobilised anaerobic fungi in the production of cellulolytic enzymes. The repeat-batch system was operationally more efficient than the free cell batch cultures because immobilisation removed the need of reculturing the cells for every single batch.
CHAPTER ONE

Introduction and Review of the Literature

1.1 General Introduction

1.1.1 Cellulose and cellulases

Plant cell walls comprise three major groups of polymers: cellulose, hemicellulose and lignin. Cellulose is a homopolymer whereas hemicellulose shows great variability in both structure and composition. Hemicelluloses are particularly heterogeneous polymers in that they can be composed of xylans, mannans or galactans (Dekker and Richards, 1976). Lignin is a complex high molecular mass polymer composed of repeating phenylpropane units linked by a variety of complex chemical bonds (Kirk, 1971).

Bioconversion of cellulose offers an attractive alternative for utilisation of urban and industrial wastes, agricultural and forest residues and for the growth of plant biomass as an energy feedstock. Cellulose amounts to about 20-40% of the dry matter of all higher plants making it the most abundant polysaccharide on earth. It has enormous potential as an alternative source of fuel, feed-stock for the chemical industry and conversion to edible biomass (Coughlan, 1985).

For these reasons, a variety of strategies for the degradation of cellulose to its component sugar have been proposed and explored. These range from purely chemical means, such as acid hydrolysis and pyrolysis, to biological methods such as enzymatic hydrolysis and fermentation. Enzymatic hydrolysis seems preferable, having several important advantages over acid hydrolysis. These include mild operating conditions and higher D-glucose yield.
1.1.1.1 Structure

Cellulose is a regular linear homopolymer of repeating D-glucose units held together by $\beta(1\rightarrow4)$ linkages. Each residue is rotated $180^\circ$ around the main axis with respect to its neighbouring residues (Figure 1.1). This results in an unstrained linear configuration with cellobiose as the basic repeating unit. Cellulose chains, arranged in parallel and staggered with respect to their partners, associate to form insoluble fibrils in which the chains are held together by hydrogen bonding. The hydrogen bonding network consists of inter- and intramolecular bonds between successive and adjacent glucose residues. The intramolecular bonds help to maintain the rigidity of the cellulose chain. Within the cellulose fibres there are areas of complete order, that is, crystalline areas, and also less well ordered or amorphous regions. The degree of crystallinity within fibres varies with the source of the cellulose and the chemical treatment to which it has been subjected.

![Image of cellulose structure]

**Figure 1.1** Cellulose chains showing the $\beta$-1,4-linked residues rotated through $180^\circ$ with respect to their neighbours in the chain. Intra-molecular hydrogen bonds tightly crosslink adjacent chains within a microfibril (Liang and Marchessault, 1959)
1.1.1.2 The cellulase complex

The generally accepted mechanism of cellulose degradation is that catalysed by the synergistic action of at least three enzymes which together comprise the cellulase complex: endoglucanase (endo-1,4-β-D-glucanase, EC 3.2.1.4), exoglucanase (cellbiohydrolase, exo-1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91 or exo-1,4-β-D-glucosidase, exo-1,4-β-D-glucan glucohydrolase EC 3.2.1.74) and β-glucosidase (cellobiase, β-D-glucoside glucohydrolase, EC 3.2.1.21) (Coughlan and Ljungdahl, 1988; Wood, 1989). Some of the enzymes in the cellulase complex are named after the substrates they attack. For example, endoglucanase and exoglucanase are also referred to as carboxymethyl-cellulase (CM-cellulase) and Avicelase, respectively, when the substrates carboxymethyl cellulose and Avicel are used to measure their activity. Figure 1.2 outlines the sequential and synergistic action of enzymes of the cellulase complex. Endoglucanase initiates hydrolysis by randomly cleaving the internal β-1,4 linkages. Cellbiohydrolase then sequentially removes cellobiose from the non-reducing end of the chain. The combined action of the endoglucanase in concert with cellbiohydrolase yields cellobiose and oligosaccharides. β-glucosidase acts on the cellobiose and other oligomers to produce glucose.

The above simplified mechanism is of a non-aggregating cellulase system. In several cellulytic anaerobic bacteria, secreted cellulases associate into high molecular mass multienzyme complexes. The complexes are generally associated with the cell and insoluble substrate. The most extensively studied aggregating system is that of Clostridium thermocellum (Lamed and Bayer, 1988). The complex, termed the ‘cellulosome’, comprises at least 14 distinct polypeptides including numerous endoglucanases and xylanases and at least one β-glucosidase. These enzymatically active polypeptides are associated with a 210 kDalton non-catalytic scaffolding protein which mediates adherence to either the cell surface and/or the substrate (Bayer and Lamed, 1986; Wu et al., 1988). Individual components of the cellulosome display little activity against crystalline cellulose. A model for the action of this cellulase complex (Mayer et al., 1987) proposes that
cellulose is attacked simultaneously by regularly spaced catalytic subunits which are lined up along the cellulose molecule.

It is apparent that the molecular architecture of cellulases can be of two types; single domain and multidomain. In the former, the enzymes comprise a single catalytic domain. Most cellulases, however, consist of multiple domains which are joined by characteristic linker sequences. Numerous multidomain cellulases contain cellulase-binding domains (CBD). Considerable homology exists between these domains in which the following features are highly conserved: (i) two cysteines are present close to the N- and C- termini respectively; (ii) there are four very highly conserved tryptophan residues in addition to glycine and asparagine residues which also show identity in the various domains; (iii) there is a marked lack of charged amino acids (Ferreira et al., 1991). By analogy with other sugar binding proteins, it has been proposed that the interaction of the CBD with cellulose is mediated by the conserved aromatic residues, either through hydrogen-bonding or hydrophobic interactions (Béguin, 1990). In fungi, CBDs appear to be essential for the respective enzymes to attack crystalline cellulose, however, the precise role of bacterial CBDs in plant cell wall hydrolysis is a matter of some debate (Shen et al., 1991; Ferreira et al., 1991). The properties and aggregation of cellulases produced by anaerobic fungi are dealt with in Section 1.2.1.6.

1.1.2 Sources of cellulase

Many microbes are capable of degrading crystalline cellulose but relatively few yield culture broths containing enzymes that promote complete hydrolysis of crystalline cellulose (Sternberg, 1976). While many bacteria utilise cellulose by cell-bound enzymes, many fungal cellulases are extracellular. Therefore, most interest in enzymatic hydrolysis of cellulose has involved the use of fungal cellulases in particular those from Trichoderma reesei strains. The enzymes from the fungi T. reesei (Mandels, 1975; Nevalainen et al., 1980; Ryu and Mandels, 1980), T. koningii (Wood et al., 1988), T. viride (Gritzali and Brown, 1979),
Figure 1.2  *Schematic outline of the sequential and synergistic action of enzymes of the cellulase complex (Montenecourt and Eveleigh, 1979)*

Perhaps the most conventional approach to cellulose utilisation is the production and harvesting of cellulase enzymes, followed by the hydrolysis of cellulosic materials to produce glucose. In such a process the cost of enzyme production accounts for some 50% of the total cost of producing glucose (Perez *et al.*, 1980). Therefore, considerable effort is being made to make cellulase production more efficient. The cellulase complex consists of an interacting battery of enzymes that are produced in low yield. A major disadvantage of *T. reesei* is the low specific activity of the enzymes obtained from this organism and the enzymes are often subject to end-product inhibition (Mandels *et al.*, 1975). Furthermore, their synthesis is subject to catabolite repression. Until new sources of cellulases that are less subject to end-product inhibition and with greater specific activities can be found, the enzymatic hydrolysis process is likely to remain commercially unviable.

1.1.2.1 Anaerobic fungi as sources of cellulosytic enzymes

The limitations connected with the production of cellulase can be improved by searching for new sources of microorganisms with desirable properties and to gain an understanding of the physiology of the strain. The rumen is a good source of mesophilic microorganisms that can hydrolyse crystalline cellulose, particularly the bacteria *Ruminococcus albus*, *R. flavefaciens* and *Fibrobacter (Bacteroides) succinogenes*. However, the enzymes they release into the culture medium have little capacity to degrade hydrogen bond-ordered cellulose (Wood, 1984). On the other hand, growing anaerobic fungi release into the medium all of the cellulosolytic enzyme activities needed for complete cellulose degradation, that is, endoglucanases, exoglucanases and β-glucosidases (Trinci *et al.*, 1994).
Until recently the most active cellulolytic enzymes were reported for *Trichoderma reesei*. The cellulase from a co-culture of *Neocallimastix frontalis* (strain RK21) with a rumen methanogen (*Methanobrevibacter smithii*) was found to be more active than that from *T. reesei* (strain C-30) (Wood *et al.*, 1986). Enzymes in the co-culture were able to solubilise 98% of the highly ordered cotton cellulose. The remarkable efficiency with which the cell-free enzyme from the co-culture could solubilise crystalline cellulose in the form of cotton fibre is shown in Figure 1.3, which compares the activity of the co-culture with that of *T. reesei*, each at the optimal conditions for pH and temperature.

![Figure 1.3](image_url)  
*Figure 1.3*  
*Rate of hydrolysis of crystalline cellulose by cellulases produced by N. frontalis (RK21) and T. reesei (C30). (Wood et al., 1986)*
Since then, Wood and Wilson (1995) have reported that monocultures of another rumen fungus, *Piromyces communis*, contain an extracellular cellulase that can solubilise hydrogen-bond-ordered cellulose at a rate that is even greater than that shown by the cellulase of the *N. frontalis/M. smithii* co-culture when grown on cotton fibre, and one that was very much better than that shown by the aerobic fungal cellulase.

The study of aerobic fungal cellulases has been proceeding for many years, but investigations of anaerobic fungal cellulases have only recently been initiated. As a consequence very little is known as yet about anaerobic fungal cellulases but knowledge of the aerobic systems is extensive. The finding that the cellulose solubilisation capabilities of *N. frontalis* and *P. communis* are better than that from *T. reesei* provides confidence that the potential for converting cellulose to fermentable sugar may lie in the enhanced cellulytic activity of anaerobic fungi.

### 1.1.3 Cellulase production using immobilised fungi

Immobilised microbial cells can be defined as cells which are physically confined or localised in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously (Chibata, 1978). Physical confinement is achieved by attachment to an inert immobilising material. Reuse is made simple because the aggregations of cells and immobilising material is much denser than the medium and is easily separated from it.

The study of immobilised cells to produce various primary and secondary metabolites has expanded rapidly in the last two decades. Although the first and main interest has been in the immobilisation of bacteria, this has been extended to filamentous microorganisms such as fungi over the last 15 years.

The most studied method of cellulase production by *T. reesei* has been batch production, however, batch production is limited by relatively low productivity and fairly low enzyme titre owing to the low substrate concentrations that can be
used. Another option is the use of immobilised cell cultivation. This approach has been little exploited in cellulase production (see Section 1.2.3.3) but it appears to hold considerable potential for improving cellulase production.

1.1.3.1 Operational advantages

Processes using immobilised growing cells seem to be more promising than traditional microbial processes with free cells (see reviews by Tanaka and Nakajima, 1990; Furasaki and Seki, 1992) since the immobilisation enables the microbial cells to be used repeatedly and continuously. A high density of cells can be obtained and cells can be easily separated from the culture liquor. Therefore, immobilisation can offer the advantage of the continuous or repeat-batch use of cells. With immobilisation, the culture liquor can be removed at the end of a batch and the vessel refilled with fresh medium, and reuse of the same population of cells occurs. This removes the need for reculturing of cells for each new batch (Webb, 1987). Furthermore, techniques for the immobilisation of microbial cells allows the exploitation of the synthesis of secondary metabolites. Thus, cells can be cultured in a medium that encourages rapid biomass accumulation and then transferred in an immobilised form to a medium that is optimum for product formation.

1.1.3.2 The relationship between mycelial growth and synthesis of cellulase in *Trichoderma* sp.

Studies on *Trichoderma* sp. have revealed that the physicochemical requirements for optimum growth and maximal synthesis of cellulases are different (Mandels *et al.*, 1975) and that a growth rate close to zero is preferred for maximal enzyme synthesis (Ryu *et al.*, 1979). When maximal synthesis of a microbial product occurs after completion of the growth phase of the microorganism, the latter can be said to exhibit "growth-dissociated" synthesis and the product is a "non-growth associated" metabolite (Enatsu and Shinmyo, 1978).
1.1.3.2.1 Exploiting the growth-dissociated nature of enzyme synthesis by using repeat-batch culture

The physiology of microorganisms exhibiting growth-dissociated synthesis cannot be fully exploited using traditional batch culture. Growth dissociated synthesis makes it advantageous to divide a production process into two separate sequential phases (referred to as “phase separation“): (i) accumulation of biomass, and (ii) repeated use of the same biomass for synthesis of the growth-dissociated product (Kuek, 1986). Techniques for the immobilisation of microbial cells allows for the exploitation of the synthesis of growth-dissociated products because the technique enables the easy recovery of biomass for re-use. Given the correct culture conditions, it is possible that cellulases of anaerobic fungi may be produced in a growth-dissociated manner.

1.1.4 Objectives of the study

The findings that the physiology of cellulase synthesis in Trichoderma sp. can be exploited by immobilisation of the fungus indicates that this technique may also be suitable in the production of cellulase using immobilised anaerobic fungi. Hence, the research described in this thesis was concerned with the evaluation of the potential use of immobilised anaerobic fungi to produce cellulolytic enzymes.

The scope of this investigation encompassed two main objectives:

- The first objective was to find isolates of anaerobic fungi from a range of mammalian herbivores that have the potential to produce high levels of cellulase. The procedure applied was to isolate cultures from rumen fluid and faecal samples, and then to perform primary screening to identify cellulolytic isolates, and secondary screening to identify those with the best potential for producing high enzyme levels. Since the anaerobic fungi compared with the aerobic fungi such as Trichoderma sp. have only recently been investigated as sources of cellulase, this approach may result in the finding of either novel
producers or those with cellulase levels superior to those commonly achieved to date.

- The second objective was to investigate the use of immobilisation of fungal rhizomycelia and repeat-batch culture as a novel and potentially advantageous approach to cellulase production in the anaerobic fungi. These investigations will necessarily include the production of immobilised rhizomycelia, elucidation of the production conditions for repeat-batch culture and the relationship between rhizomycelial growth and the synthesis of cellulase in the isolate(s) found.
1.2 Review of the Literature

Prior to the discovery of anaerobic fungi a little over two decades ago, it was assumed that only anaerobic bacteria and protozoa were involved in the hydrolysis of plant biomass in the rumen. But now it is acknowledged that anaerobic fungi participate in this process in both ruminant and non-ruminant herbivores. Consequently, the use of these organisms in the production of cellulase is a relatively new area. This literature review sets out to examine aspects of anaerobic fungi and cell immobilisation that pertain to this study. It describes the life cycles of anaerobic fungi, an issue that will become important in selecting suitable propagules for immobilisation, and assesses their contribution to fibre digestion in herbivores. Current published studies concerning the production of extracellular fibre-degrading enzymes by anaerobic fungi are reviewed and provide a basis for comparison with enzyme yields obtained by anaerobic fungi in this project. An examination of cell immobilisation details such issues as types of matrices and propagule choice and compares the types of culture strategies that are available in the culture of immobilised cells to optimise cellulase synthesis. These issues contribute to the development of suitable protocols that are necessary to meet the project objectives.

1.2.1 Anaerobic fungi

1.2.1.1 Historical background

The earliest studies of the anaerobic zoosporic fungi can be traced back to the work of Liebetanz (1910) (as cited by Wubah et al., 1993), who described four uniflagellate organisms he believed were protozoa and assigned them to four genera: Cercomonas, Oikomonas, Sphaeromonas, and Piromonas. Weissenberg (1912) (as cited by Wubah et al., 1993) described a multflagellate organism that was parasitic on freshwater copepods and named it Callimastix cyclops. Weissenberg. Subsequently, new species of multflagellated organisms that were believed to be Callimastix were observed and described. Braune (1913) (as cited
by Wubah et al., 1993) named a polyflagellate rumen inhabitant, *Callimastix frontalis*, and Hsuing (1930) (as cited by Wubah et al., 1993) named *C. equi* from the horse caecum.

Weissenberg (1950) (as cited by Wubah et al., 1993) suggested that *C. cyclopsis* might be a zoospore of a fungus, not a protozoan, and Vavra and Joyon (1966) (as cited by Wubah et al., 1993) substantiated this hypothesis when they discovered the vegetative thallus of the fungus. As a result of this discovery, the remaining polyflagellate species of *Callimastix* were grouped into a new ‘protozoan’ genus, *Neocallimastix*, with *N. frontalis* (formally *Callimastix frontalis*) as a type species (Vavra and Joyon, 1966).

Studies of sheep rumen flagellated microorganisms showed that there were diurnal fluctuations in some populations. Motile multiflagellate organisms that were similar to *C. frontalis* showed a significant increase in population density within 1 hour after feeding the sheep (Warner, 1966). In 1974, Orpin set out to examine the factors that controlled these fluctuations and found greater changes than those reported by Warner and that the population increase occurred between 15 and 30 minutes after feeding. Further studies led to the conclusion that either the flagellates sequestered on or within the plant fragments in the rumen, or a multiple reproductive phase, such as a sporangium, was present in the life cycle of *N. frontalis* (Orpin, 1974). Subsequently, Orpin identified three anaerobic fungi in the rumen of sheep, calling them *Neocallimastix frontalis*, *Sphaeromonas communis* and *Piromonas communis*, each of which had a motile stage (the zoospore) and a non-motile zoosporangium (Orpin, 1975, 1976, 1977b).

### 1.2.1.2 Life cycle

The life cycle of monocentric fungi consists of an alteration between a motile, zoosporic stage and a vegetative, zoosporangial stage (Figures 1.4 a and b). Flagellate zoospores are released from a sporangium and encyst by shedding their flagella. The cyst germinates to produce a germ tube, which later develops into
Figure 1.4 a and b  Life cycle of anaerobic fungi

1. zoospore; 2. germinating zoospore; 3. sporangium; 4. vegetative rhizomycelium. Monocentric fungi must complete a life cycle regularly in order to grow (A) whereas polycentric fungi (B) grow vegetatively as well as completing their life cycle. (Phillips and Gordon, 1995a).
rhizoids (Orpin, 1977a; 1977b). Mitosis takes place in the cyst, which then develops into a cell body (Gold et al., 1988). The cell body develops into a sporangium in which cytokinesis occurs to produce the uninucleate zoospores, which are released from the sporangium to complete the cycle.

From in vitro and in vivo studies of Neocallimastix species, it has been established that the life cycle lasts about 24-32 hours (Joblin, 1981; Bauchop, 1983; Lowe et al., 1987a). The development of zoospores from young sporangia may occur within 8 hours after encystment under appropriate conditions (Orpin, 1977a, 1977c).

Unlike monocentric fungi, polycentric fungi have indeterminate life cycles and are not dependent upon the formation of zoospores for their continued survival (Figure 1.4 b). Their growth pattern resembles that of higher fungi, that is, they are propagated by hyphae (Ho and Bauchop, 1991). Zoospores are produced infrequently or zoosporogenesis is even absent (Phillips, 1989). Differences in the life cycle between monocentric and polycentric fungi will become important when considering the choice of suitable propagules for immobilisation in calcium alginate gel.

1.2.1.3 Classification

It is generally agreed that anaerobic fungi are zoospore-producing fungi and should be assigned to the class Chytridiomycetes. The Chytridiomycetes, which are thought to be the ancestral group for higher fungi, are subdivided into four orders, the Blastocladiales, Monoblepharidales, Chytridales and the Spizellomycetales (Barr, 1980). Based on ultrastructural characteristics of the zoospores, anaerobic fungi were assigned to the order of the Spizellomycetales in a new family, the Neocallimasticaceae (Barr, 1988). Gold et al (1988) suggested the subdivision of this family into three genera containing monocentric species, Neocallimastix, Piromyces (previously Piromonas) and Caecomyces (previously Sphaeromonas). Since then two polycentric genera have been described,
Orpinomyces (Barr et al., 1989) and Anaeromyces (Breton et al., 1990). Table 1.1 shows the classification and morphological characteristics of anaerobic fungi. Presently, 17 species of anaerobic fungi have been described and classified (Table 1.2).

**Table 1.1**  
*Classification and morphological characteristics of anaerobic fungi*

Division: Eumycota  
Subdivision: Mastigomycotina  
Class: Chytridiomycetes  
Order: Spizellomycetales  
Family: Neocallimasticaceae  
Genera: monocentric:  
Caecomyces (zoospore has one or two flagella and thallus with a globular rhizoid)  
Neocallimastix (zoospore has four to twenty flagella and thallus with filamentous branching rhizoids)  
Piromyces (zoospore has one to four flagella and thallus with filamentous branching rhizoids)  
polycentric:  
Orpinomyces (zoospore is multiflagellate)  
Anaeromyces (zoospore has one flagellum)
<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Source of isolate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caecomycetes</td>
<td><em>C. communis</em>¹</td>
<td>Sheep</td>
<td>Gold et al. (1988)</td>
</tr>
<tr>
<td></td>
<td><em>C. equi</em></td>
<td>Horse</td>
<td>Gold et al. (1988)</td>
</tr>
<tr>
<td>Piromycetes</td>
<td><em>P. communis</em>²</td>
<td>Sheep</td>
<td>Gold et al. (1988)</td>
</tr>
<tr>
<td></td>
<td><em>P. mae</em></td>
<td>Horse</td>
<td>Li et al. (1990)</td>
</tr>
<tr>
<td></td>
<td><em>P. dumbonica</em></td>
<td>Elephant</td>
<td>Li et al. (1990)</td>
</tr>
<tr>
<td></td>
<td><em>P. rhizinflata</em></td>
<td>Saharan ass</td>
<td>Breton et al. (1991)</td>
</tr>
<tr>
<td></td>
<td><em>P. minutus</em></td>
<td>Deer</td>
<td>Ho et al. (1993c)</td>
</tr>
<tr>
<td></td>
<td><em>P. spiralis</em></td>
<td>Goat</td>
<td>Ho et al. (1993d)</td>
</tr>
<tr>
<td></td>
<td><em>P. citronii</em></td>
<td>Horse</td>
<td>Gaillard-Martinie et al. (1995)</td>
</tr>
<tr>
<td>Neocallimastix</td>
<td><em>N. frontalis</em></td>
<td>Sheep</td>
<td>Heath et al. (1983)</td>
</tr>
<tr>
<td></td>
<td><em>N. patriciarum</em>³</td>
<td>Sheep</td>
<td>Orpin and Munn (1986)</td>
</tr>
<tr>
<td></td>
<td><em>N. hurleyensis</em></td>
<td>Sheep</td>
<td>Webb and Theodorou (1991)</td>
</tr>
<tr>
<td></td>
<td><em>N. variabilis</em></td>
<td>Cow</td>
<td>Ho et al. (1993a)</td>
</tr>
<tr>
<td>Anaeromyces</td>
<td><em>A. elegans</em>⁴</td>
<td>Cow</td>
<td>Ho et al. (1993b)</td>
</tr>
<tr>
<td></td>
<td><em>A. mucronatus</em></td>
<td>Sheep</td>
<td>Breton et al. (1990)</td>
</tr>
<tr>
<td>Orpinomyces</td>
<td><em>O. joyonii</em>⁵</td>
<td>Sheep</td>
<td>Breton et al. (1989)</td>
</tr>
<tr>
<td></td>
<td><em>O. intercalaris</em></td>
<td>Cow</td>
<td>Ho et al. (1994)</td>
</tr>
</tbody>
</table>

Originally called: ¹*Sphaeromonas communis* (Orpin, 1976); ²*Piromonas communis* (Orpin, 1977b); ³*Neocallimastix frontalis* (Orpin, 1975); ⁴*Ruminomyces elegans* (Ho et al., 1990); ⁵*Orpinomyces bovis* (Barr et al., 1989); and *Neocallimastix joyonii* (Breton et al., 1989).
1.2.1.4 Distribution and establishment

Since their first isolation in the UK from the rumen of sheep (Orpin, 1975), anaerobic fungi have been found on all continents (except for Antarctica) and in all of the geographical regions where they have been sought. Anaerobic fungi are ubiquitous among foregut fermenters, ruminants such as cattle, red deer and impala (Bauochop, 1980; 1983), as well as from ruminant-like animals such as the grey kangaroo, wallaroo, swamp wallaby (Bauochop, 1980) and camel (Milne et al., 1989). Anaerobic fungi have also been isolated from faecal samples of the ass (Breton et al., 1991), horse (Orpin, 1981), Indian elephant and zebra (Milne et al., 1989) all of which are hindgut fermenters. Recently, Wubah and Kim (1994) described the first isolation and morphological characterisation of an anaerobic fungus from the anoxic layers of a pond.

Fonty et al. (1987) found that anaerobic fungi became established in normal young lambs within 8-10 days of birth in the presence of adult animals, although the survival of the fungus was not guaranteed, as young animals may not have a fully functional rumen. Lowe et al. (1987d) demonstrated that anaerobic fungi could be isolated from saliva and faeces in sheep, indicating that the mode of transmission of the fungi could be by direct oral contact, by aerosol or by faecal contamination. Wubah et al. (1991b) isolated species of Neocallimastix, Piromyces, Orpinomyces, and Caecomyces from both fresh and dry faeces of a cow; the same isolates were obtained from the rumen of the cow. Each of these isolates produced a melanised resting stage in vitro and similar melanized sporangia were observed in faecal smears, which supports the hypothesis that faeces are the major route for transfer of anaerobic fungi.
1.2.1.5 Plant cell wall degradation

Both monocentric and polycentric species are able to extensively degrade plant cell walls (Borneman and Akin, 1994) and grow on a range of structural carbohydrates present in forage fibre (Gordon and Phillips, 1989; Lowe et al., 1987b; 1987c). Their role in plant fibre degradation has been extensively examined (Bauchope, 1981; 1983; Windham and Akin, 1984; Akin and Rigsby, 1987; Akin and Benner, 1988; Akin et al., 1988; Joblin and Naylor, 1989). The rhizoids of vegetative thalli penetrate plant tissue better than bacteria and protozoa, so they can gain access to plant material that is unavailable to other rumen microorganisms (Orpin 1977a; Orpin and Joblin, 1988). Bauchope and Mountfort (1981) have suggested that this penetration leads to a more rapid and complete degradation of forage entering the rumen.

1.2.1.6 Enzymology

To degrade and utilise plant cell walls anaerobic fungi produce a wide range of hydrolytic enzymes including cellulases (Lowe et al., 1987c; Barichievich and Calza, 1990), hemicellulases (Lowe et al., 1987d; Mountfort and Asher, 1989), proteases (Wallace and Joblin, 1985), amylases, amyloglycosidases (Pearce and Bauchope, 1985; Mountfort and Asher, 1988), feruloyl and p-coumaryl esterases (Borneman et al., 1990; 1991), various disaccharidas (Hébraud and Fèvre, 1988; Chen et al., 1994) and pectinases (Gordon and Phillips, 1992). Some of these enzymes are constitutive whereas others are apparently induced by their substrates (Lowe et al., 1987a; Gordon and Phillips, 1989). Digestive enzyme activities have been detected in zoospores, vegetative thalli, and culture supernatant and are regulated by the growth substrate (Williams and Orpin, 1987a; 1987b; Barichievich and Calza, 1990; Morrison et al., 1990). For example, endoglucanase production by N. frontalis was highest after growth on cellulose while synthesis was totally repressed by the addition of glucose to the culture, indicating that the enzyme was subject to regulation (Mountfort and Asher, 1985).
It is generally accepted that anaerobic fungi produce cellulolytic enzymes not only as individual proteins, but as proteins associated in a multiprotein complex of high molecular weight (Wood et al., 1988; Teunissen et al., 1992b; Wilson and Wood 1992a; 1992b; Ali et al., 1995; Dijkerman et al., 1996b; 1997b). Wood et al. (1988) were the first to suggest that the endoglucanase, β-glucosidase, and another factor, (thought to be responsible for crystalline cellulose hydrolysis), of *N. frontalis* existed in solution as a multicomponent enzyme complex. It was found that activity towards Avicel (crystalline cellulose) was associated almost entirely with the high molecular weight enzyme complex, called the `crystallinesolubilising factor, or CCSF (Wood et al., 1988). There is similarity with the cellulases of the aerobic fungi in that there is some synergism when the high molecular weight CCSF acts in concert with that part of the endoglucanase and β-glucosidase that does not exist as a complex in solution (Wilson and Wood, 1992a; 1992b). Gene cloning of cellulases of *N. patriciarum* (Xue et al., 1992a; 1992b) and studies involving the synergistic action of parts of the enzyme system with cellobiohydrolase I and cellobiohydrolase II from the aerobic fungus *Trichoderma koningii* (Wood et al., 1994) have also provided some evidence that the cellulase system of *Neocallimastix* species may resemble the cellulases of the aerobic fungi in containing cellobiohydrolase enzymes.

Five different cellulase genes have been cloned from *N. patriciarum* (Gilbert et al., 1992; Xue et al., 1992a; 1992b). All these genes expressed proteins in *Escherichia coli* that have cellodextrinase and endo-1,4-β-glucanase activities. Three of them (*CelA, CelD* and *CelE*) also have significant cellobiohydrolase activity and release cellobiose from crystalline cellulose, while *CelD* also has xylanase and xylo-oligosaccharidase activity. While *CelA, CelB, and CelC* are inducible by the presence of cellulose, *CelD* is produced constitutively. This confirms the results of Lowe et al. (1987c), who observed some constitutive xylanase production, and Barichievich and Calza (1990), who detected a low level of cellulase activity in glucose-grown cultures. Constitutive cellulases are rare, and the enzyme expressed by *CelD* may be of major importance to the fungus during initial colonisation of a plant fragment (Orpin, 1993).
1.2.1.7 Extracellular cellulolytic and xylanolytic enzyme yields produced by anaerobic fungi

The biotechnological potential of the cellulolytic enzymes of anaerobic fungi has made them the subject of a number of studies over recent years. These studies have focused on the optimisation of production of cellulolytic and xylanolytic enzymes by anaerobic fungi grown on a range of cellulosic substrates (Pearce and Bauchop, 1985; Lowe et al., 1987c; Williams and Orpin, 1987a; 1987b; Borneman et al., 1989; Gordon and Phillips, 1989; Barichievich and Calza, 1990; Bernalier et al., 1992; Teunissen et al., 1991; 1992a; 1993; Tsai and Calza, 1993; Williams et al., 1994; Dijkerman et al., 1996a; 1997a; Gerbi et al., 1996; Zhu, et al., 1996; 1997; Bata and Gerbi, 1997; also see reviews Wubah et al., 1993; and Teunissen and Op den Camp, 1993).

Recent reports have focussed on modifying the culture medium and growth conditions in an attempt to increase product yield. In some of these investigations the same strain of anaerobic fungus has been utilised making it possible to assess the success of the different culture conditions in terms of enzyme yield. The following Tables 1.3, 1.4 and 1.5 provide a review of traditional fermentation techniques for the production of cellulolytic and xylanolytic enzymes and will constitute the baseline values that will be used to assess achievement of the project objective to find superior cellulase producers.

A comparison of cellulolytic and xylanolytic enzyme activities of \textit{N. patriciarum} (N2), \textit{Neocallimastix} sp. N1 and \textit{Piromyces} sp. E2 and R1 in various batch and semi-continuous systems is given in Table 1.3. A first approach at optimisation was made by Teunissen \textit{et al.} (1992a), who reported on the cultivation of \textit{Piromyces} strain E2 in a 0.8 L semi-continuous culture system. In this study it was observed that xylanase and \(\beta\)-glucosidase activities were 10-fold and 16-fold higher, respectively, when compared to an earlier batch study conducted by Teunissen \textit{et al.} (1991) using the same organism. However, two disadvantages of this semi-continuous system were the relatively long time required for stabilisation.
Table 1.3  *Comparison of cellulolytic and xylanolytic enzyme activities by N. patriciarum (N2), Neocallimastix sp. N1 and Piromyces sp. E2 and R1 in various batch and semi-continuous culture systems*

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Substrate</th>
<th>Culture system</th>
<th>Enzyme activity (mIU/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Avicel</td>
<td>Endoglucanase</td>
</tr>
<tr>
<td><em>Neocallimastix patriciarum</em> N2</td>
<td>Avicel</td>
<td>Batch (20 mL)</td>
<td>14</td>
<td>120</td>
</tr>
<tr>
<td><em>Neocallimastix</em> sp. strain N1</td>
<td></td>
<td></td>
<td>14</td>
<td>120</td>
</tr>
<tr>
<td><em>Piromyces</em> sp. strain E2</td>
<td></td>
<td></td>
<td>22</td>
<td>180</td>
</tr>
<tr>
<td><em>Piromyces</em> sp. strain R1</td>
<td></td>
<td></td>
<td>13</td>
<td>110</td>
</tr>
<tr>
<td><em>Piromyces</em> sp. strain E2</td>
<td>Avicel</td>
<td>Semi-continuous (0.8L)</td>
<td>27</td>
<td>140</td>
</tr>
<tr>
<td><em>Piromyces</em> sp. strain E2 (ATCC 76762)</td>
<td>Avicel</td>
<td>Batch (20 mL)</td>
<td>48 (0.18)</td>
<td>1570 (5.84)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Batch (10 L)</td>
<td>48 (0.03)</td>
<td>1870 (10.11)</td>
</tr>
<tr>
<td><em>Neocallimastix patriciarum</em> N2</td>
<td></td>
<td>Batch (20 mL)</td>
<td>62 (0.28)</td>
<td>1780 (8.13)</td>
</tr>
<tr>
<td>(ATCC 76761)</td>
<td></td>
<td>Batch (10 L)</td>
<td>39 (0.27)</td>
<td>1510 (10.34)</td>
</tr>
</tbody>
</table>

( )\(^1\) Specific activity calculated as IU/mg protein
of the culture and clogging of the filter unit used to prevent washout of the fungus. More recently, Dijkerman et al. (1996a) reported on the cultivation of the anaerobic fungi *Piromyces* strain E2, and *Neocallimastix patriciarum* strain N2 in a 10 L batch fermentor with filter paper cellulose as the carbon source for the production of hemicellulolytic enzymes. In this study they found that the specific activities of Avicelase, endoglucanase, β-glucosidase and xylanase were up to threefold higher compared to small batch cultures used in the same study. Twenty millilitre batch cultures of *Piromyces* sp. strain E2 and *Neocallimastix patriciarum* strain N2 produced higher extracellular enzyme levels in the study by Dijkerman et al. (1996a) when compared to an earlier study by Teunissen et al. (1991). Different assay conditions used in the two studies may account for the difference in enzyme activities for the same fungi. Dijkerman et al. (1997c) investigated the degradation capacity of extracellular enzyme preparations from strains E2 and N2 and found that the performance of these enzymes surpassed that of a combined preparation of the commercial enzymes Celluclast (Avicelase) and Novozyme (β-glucosidase) in batch degradation of 2% (w/v) Avicel. This applied study (Dijkerman et al., 1997c) provides confidence for the use of anaerobic fungi as sources of cellulolytic enzymes.

Further research into the use of continuous flow culture was performed using *Neocallimastix hurleyensis* (Table 1.4). Lowe et al. (1987c) initiated investigations into the production of cellulolytic and xylanolytic enzymes by *Neocallimastix* sp. R1 in batch culture. This strain was later classified as *N. hurleyensis* (Webb and Theodorou, 1991). Zhu et al. (1996) were the first to observe the growth and survival of anaerobic fungi in continuous-flow cultures. In this study they found that the growth of *N. hurleyensis* in continuous-flow culture enabled the production of greater quantities (up to 20 times) of cell-wall degrading enzymes compared to batch cultures performed in the same study. While examining the effects of increased dilution rates in batch and continuous flow cultures of *N. hurleyensis* on wheat straw, Zhu et al. (1997) found increased enzyme yields of up to 30 times compared to batch cultures. However, enzyme activities in batch cultures of the 1997 study were lower compared to the previous
Table 1.4  Comparison of cellulolytic and xylanolytic enzyme production by Neocallimastix hurleyensis (Neocallimastix sp. R1) in various batch and continuous-flow culture systems

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Substrate</th>
<th>Culture system</th>
<th>Enzyme production (mIU/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CM-cellulase</td>
<td>β-glucosidase</td>
</tr>
<tr>
<td>Neocallimastix</td>
<td>Wheat straw</td>
<td>Batch (100 mL)</td>
<td>160</td>
<td>30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>sp. R1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neocallimastix</td>
<td>Wheat straw</td>
<td>Batch</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>hurleyensis</td>
<td></td>
<td>Continuous (100mL)</td>
<td>1000</td>
<td>280</td>
</tr>
<tr>
<td>Neocallimastix</td>
<td>Wheat straw</td>
<td>Batch (100 mL)</td>
<td>49</td>
<td>12</td>
</tr>
<tr>
<td>hurleyensis</td>
<td></td>
<td>Continuous (100 mL) :</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>low&lt;sup&gt;c&lt;/sup&gt;</td>
<td>177</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>medium</td>
<td>166</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>high</td>
<td>112</td>
<td>28</td>
</tr>
</tbody>
</table>

<sup>a</sup> Measured as cellobiase  
<sup>b</sup> Not determined  
<sup>c</sup> Values refer to low (0.04), medium (0.08) and high (0.12) dilution rates /h for continuous-flow cultures
two studies and the continuous-flow cultures in this particular study did not produce greater quantities of cell-wall degrading enzymes when compared to the earlier batch study performed by Lowe et al. (1987c).

There are relatively few reports that investigate the nature of polycentric fungi, particularly in regard to their ability to produce cellulolytic enzymes. Table 1.5 compares the cellulolytic and xylanolytic activities of some monocentric and polycentric fungi in various batch and continuous-flow systems. Borneman et al. (1989) were the first to report on the production of cellulase and xylanase using an Anaeromyces sp. PC-1 and two Orpinomyces spp. PC-2 and PC-3 grown on Coastal Bermuda grass (CBG). These polycentric isolates compared favourably with the monocentric isolates Piromyces sp. MC-1 and Neocallimastix sp. MC-2. Yanke et al. (1996) investigated the production of extracellular fibre-degrading enzymes of a number of monocentric and polycentric fungi when grown on glucose, xylan and the cellulosic substrates, filter paper and Avicel. All fungi produced endoglucanase and xylanase activity. However, Piromyces communis strain 22 and Neocallimastix patriciarum strain 27 produced substantially greater levels of fibre-degrading enzymes than Orpinomyces joyonii strain 19-2 or Neocallimastix frontalis strain RE1. In examining the effects of increased dilution rates in continuous-flow cultures of an Orpinomyces sp. grown on wheat straw, Zhu et al. (1997) found that greater quantities (up to 30 times) of cell-wall degrading enzymes (CM-cellulase, xylanase, β-glucosidase and β-xylosidase) were produced in continuous-flow cultures than in the corresponding batch cultures. Whilst these enzyme quantities were larger when compared to batch cultures in the same study, they were lower than those reported by Borneman et al. (1989). The differences demonstrated in the extracellular enzyme activities between species belonging to the same genus suggests that the enzyme systems of each fungal species are unique.
Table 1.5  
Production of cellulolytic and xylanolytic enzymes by some monocentric (Neocallichasmix and Piromyces) and polycentric (Anaeromyces and Orpinomyces) fungi in batch and continuous-flow systems

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Substrate</th>
<th>Culture system</th>
<th>Enzyme Activity (mIU/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Avicelase</td>
<td>Endoglucanase</td>
</tr>
<tr>
<td>Piromyces sp. MC-1</td>
<td>CBG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Batch</td>
<td>16</td>
<td>240&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Neocallichasmix sp. MC-2</td>
<td></td>
<td></td>
<td>19</td>
<td>320&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anaeromyces sp. PC-1</td>
<td></td>
<td></td>
<td>16</td>
<td>320&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Orpinomyces sp. PC-2</td>
<td></td>
<td></td>
<td>10</td>
<td>240&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Orpinomyces sp. PC-3</td>
<td></td>
<td></td>
<td>10</td>
<td>240&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>N.</em> frontalis</td>
<td>Glucose</td>
<td>Batch</td>
<td>N.D.</td>
<td>22</td>
</tr>
<tr>
<td><em>N.</em> patriciarum</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>25</td>
</tr>
<tr>
<td><em>O.</em> joyonii</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>15</td>
</tr>
<tr>
<td><em>P.</em> communis</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>52</td>
</tr>
<tr>
<td><em>N.</em> frontalis</td>
<td>Xylan</td>
<td></td>
<td>N.D.</td>
<td>15</td>
</tr>
<tr>
<td><em>N.</em> patriciarum</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>37</td>
</tr>
<tr>
<td><em>O.</em> joyonii</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>20</td>
</tr>
<tr>
<td><em>P.</em> communis</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>47</td>
</tr>
<tr>
<td><em>N.</em> frontalis</td>
<td>Avicel</td>
<td></td>
<td>N.D.</td>
<td>52</td>
</tr>
<tr>
<td><em>N.</em> patriciarum</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>165</td>
</tr>
<tr>
<td><em>O.</em> joyonii</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>24</td>
</tr>
<tr>
<td><em>P.</em> communis</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>166</td>
</tr>
<tr>
<td><em>N.</em> frontalis</td>
<td>Filter paper</td>
<td></td>
<td>N.D.</td>
<td>44</td>
</tr>
<tr>
<td><em>N.</em> patriciarum</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>49</td>
</tr>
<tr>
<td><em>O.</em> joyonii</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>25</td>
</tr>
<tr>
<td><em>P.</em> communis</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>88</td>
</tr>
</tbody>
</table>

| Orpinomyces sp.        | Wheat straw | Batch (100 mL) | N.D.     | 34<sup>b</sup> | 6           | 27       | < 1          | Zhu et al. (1997) |
| Continuous (100mL):    | low<sup>d</sup>   |                | N.D.     | 156<sup>b</sup> | 18          | 825      | 2            |            |
|                        | medium        |                | N.D.     | 95<sup>b</sup>  | 14          | 671      | 2            |            |
|                        | high          |                | N.D.     | 99<sup>b</sup>  | 14          | 772      | 2            |            |

<sup>a</sup> Coastal Bermuda Grass  
<sup>b</sup> Measured as CM-cellulase  
<sup>c</sup> Not determined  
<sup>d</sup> Values refer to low (0.04), medium (0.08), and high (0.12) dilution rates/h for continuous-flow cultures
1.2.2 Selection strategy for superior cellulase-producing organisms

The studies outlined in the previous section have been limited by the number of isolates under investigation. Although there have been many studies where isolations have been performed from a range of mammalian herbivores (see Section 1.2.1.4), no isolation studies have been done which specifically target anaerobic fungi with superior cellulase-producing capabilities. All anaerobic fungi are capable of producing extracellular cellulase, however, they vary in the extent of this ability (Li and Heath, 1994).

In this project an isolation programme was devised for the purpose of isolating strains of anaerobic fungi that have the potential to produce high levels of extracellular enzymes. Following this, a number of screening tests were performed on the isolates to select for the best cellulase producers. A combination of qualitative and quantitative screening strategies were employed and since there have been no previous reports on the screening of a large number of anaerobic fungal isolates for cellulolytic capability, established techniques formed the basis for the screening procedures used in this study. In this context, screening defines the use of selective procedures for detecting chemicals, enzymes, or any other biological product or activities produced by organisms and are divided into primary and secondary categories.

1.2.2.1 Primary screening

Methods for the primary screening of cellulase-producing microorganisms are based on cellulose as the growth substrate. Selection can be made on the basis of either the size of the zone of clearing around a colony due to the hydrolysis of cellulose by hydrolytic enzymes (Rautela and Cowling, 1966) or the amount of dye release from dyed cellulose-azure (C-A) (Poincelot and Day, 1972; Smith, 1977; Cresswell et al., 1988; Plant et al., 1988). Mann (1968) describes a method for determining cellulolytic activity in anaerobic rumen bacteria using the disintegration of filter paper strips to denote cellulolytic activity. Recently,
microbial activity of anaerobic fungi has been determined by following the rate of increase of head space gas pressure in axenic batch cultures using a pressure transducer (Theodorou et al., 1995).

The C-A method is based on the principle that dissolution of cellulose is an essential aspect of its degradation and was identified as a possible screening procedure that could be used to determine the relative cellulolytic activity of anaerobic fungi isolated in this study. The techniques reviewed concerning the use of C-A employed aerobic organisms only. Modifications that translate these aerobic techniques to techniques that cater for anaerobes must therefore be made. In order to achieve anaerobiosis the medium must be changed by excluding air and including a reductant and redox indicator.

**1.2.2.2 Secondary screening**

Primary screens are only qualitative as the nature of these screens precludes any quantitative comparisons of enzymatic activities. The secondary screen is both a qualitative and quantitative endeavour in that its objective is to determine the precise celluolytic activity of the organisms and to verify the production of these enzymes by the organisms selected from the results of the primary screen. (Mullings, 1985). Whereas the objective of the primary screen in this project was to identify celluolytic isolates, the secondary screen aimed to identify those isolates with the best potential for producing high cellulase levels.

**1.2.2.2.1 Quantitation of cellulase**

Cellulase assays are complicated by the multiplicity of enzymes and substrates. Furthermore, the complex structure of cellulose and cellulosic materials introduces considerable problems when attempting to measure their biodegradation. Quantitative assays can be divided into three types: alteration of some physical property of the substrate, reduction in substrate quantity, or accumulation of a measurable product of the degradative process. (Mullings,
1985). In the last and biggest category some methods enable a distinction to be made between the components of the cellulase complex. A number of quantitative assay methods are available to evaluate the cellulase components and their complex interactions that result in the solubilisation of cellulose (Table 1.6). Many modifications of these assays have been reported and although fine details of enzyme assays may differ between laboratories, comparisons can be made between data for the same genus of a particular organism.

1.2.3 Immobilised microbial cells

The use of immobilised anaerobic fungal rhizomycelia in repeat-batch culture for the production of cellulase was the second objective of this study. A review of the current state of enzyme yields has shown that repeat-batch culture is a novel approach to cellulase production in anaerobic fungi (see Section 1.2.1.7). Likewise, there have been no published reports on the immobilisation of these organisms for the production of cellulase. Thus, the purpose of this part of the study was to devise a system for the repeat-batch culture of immobilised anaerobic fungi. Characteristically, this system should include immobilised mycelia that did not overgrow bead surfaces or give rise to free rhizomycelial growth and the ability to repeat-batch for the production of cellulase. A number of related issues are important in achieving this working system and will be reviewed. These include the choice of propagules for the production of immobilised mycelia, the current situation with containment of mycelial growth to the subsurface and the types of strategies that are available in the culture of immobilised cells to optimise enzyme synthesis.
Table 1.6  *Methods of measuring cellulase activities (Wood and Bhat, 1988)*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete cellulase</td>
<td>Cotton</td>
<td>Solubilisation:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Estimation of cellulose in residue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reducing sugars released</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weight loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Loss in tensile strength</td>
</tr>
<tr>
<td>Filter paper</td>
<td>Sustainable:</td>
<td>Release of reducing sugars</td>
</tr>
<tr>
<td>Hydrocellulose</td>
<td></td>
<td>Release of reducing sugars</td>
</tr>
<tr>
<td>Avicel</td>
<td></td>
<td>Release of dyed cellulose</td>
</tr>
<tr>
<td>Solka Floc</td>
<td></td>
<td>Release of dyed cellulose</td>
</tr>
<tr>
<td>Dyed Avicel</td>
<td></td>
<td>Release of dyed soluble fragments</td>
</tr>
<tr>
<td>Cellobiohydrolase (EC 3.2.1.91.)</td>
<td>Avicel</td>
<td>Solubilisation:</td>
</tr>
<tr>
<td>(exocellobiohydrolase, exoglucanase, exocellulase, Avicelase)</td>
<td>Hydrocellulose</td>
<td>Release of reducing sugars</td>
</tr>
<tr>
<td></td>
<td>Dyed Avicel</td>
<td>Release of dyed cellulose</td>
</tr>
<tr>
<td></td>
<td>Amorphous cellulose</td>
<td>Release of reducing sugars or decrease in turbidity</td>
</tr>
<tr>
<td></td>
<td>Celluloligosaccharides</td>
<td>Increase in reducing power or product analysis by HPLC</td>
</tr>
<tr>
<td>Endo-1,4-β-D-glucanase (EC 3.2.1.4)</td>
<td>Carboxymethylcellulose</td>
<td>Release of reducing sugars</td>
</tr>
<tr>
<td>(CM-cellulase, endoglucanase, endocellulase)</td>
<td>Hydroxyethylcellulose</td>
<td>Decrease in viscosity</td>
</tr>
<tr>
<td></td>
<td>Cellooligosaccharides</td>
<td>Increase in reducing power or product analysis by HPLC</td>
</tr>
<tr>
<td></td>
<td>Cotton</td>
<td>Swelling in alkali</td>
</tr>
<tr>
<td></td>
<td>Amorphous cellulose</td>
<td>Solubilisation:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Release of reducing sugars</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decrease in turbidity</td>
</tr>
<tr>
<td>β-Glucosidase (EC 3.2.1.21)</td>
<td>o- or p-Nitrophenyl-β-D-glucosides</td>
<td>Release of o- or p-nitrophenol</td>
</tr>
<tr>
<td></td>
<td>Salcin glycosides</td>
<td>Release of glucose</td>
</tr>
<tr>
<td></td>
<td>Esculin glycosides</td>
<td>Release of glucose</td>
</tr>
<tr>
<td></td>
<td>Cellobiose</td>
<td>Release of glucose</td>
</tr>
<tr>
<td></td>
<td>Celluloligosaccharides</td>
<td>Increase in reducing power</td>
</tr>
</tbody>
</table>
1.2.3.1 Production of immobilised mycelia

1.2.3.1.1 Types of immobilisation

Numerous methods have been developed for the immobilisation of living microorganisms. The various methods for immobilisation of microorganisms can be grouped into two categories, that is, carrier binding and cross-linking without support material (Chibata et al., 1983), and entrapment (Rosevear, 1984). The last method is mainly used for the immobilisation of living cells. Entrapment of living cells is achieved by using polymer gels, microcapsules, liposomes, hollow fibres, and ultrafiltration membranes. In particular, entrapment in polymer matrices (lattice type) seems to be most suitable for the preparation of immobilised growing cells and was therefore the method of choice for the project.

1.2.3.1.2 Matrices for immobilisation by entrapment

Entrapment of living cells with natural polymers, such as agar, agarose, alginate, and κ-carrageenan, is principally carried out by ionotropic or thermal gelation (Kierstan and Coughlan, 1985). The formation of ionic networks is performed by dripping a cell/polymer suspension into a solution containing a multivalent cation, and thermal gelation is achieved by cooling a heated cell/polymer suspension. These polysaccharides, which are all isolated from seaweed, are by far the least toxic and simplest to gel. The disadvantage of using agar solutions is the effect of heat on sensitive microorganisms. The equipment for gel preparation is necessarily more complicated because of the need to keep agar in a molten state until gelation. The manufacture of molten beads has the disadvantage of requiring the use of hydrophobic liquids. The preparation of gel beads is very simple with κ-carrageenan and alginate, as only aqueous gelling solutions are required. However, alginate is a more practical proposition because κ-carrageenan is very expensive and requires heat for solvation. Alginate was thus the polymer chosen for immobilisation in this project.
1.2.3.1.2.1 Calcium alginate gel

Alginic acid is a heteropolysaccharide of L-guluronic acid and D-mannuronic acid extracted from various species of marine algae. Depending on the source, the composition and the sequence in L-guluronic and D-mannuronic acid varies widely. The monomers are arranged in a pattern of blocks along the chain, with homopolymeric regions interspersed with regions of alternating structure (McDowell, 1977).

Sodium alginate in solution is used to prepare alginate gels. The transition from sol to gel state occurs when sodium alginate is placed in contact with a solution of dibasic or polybasic cations (except magnesium). When Ca\(^{2+}\) is used (supplied as calcium chloride), gelatinous calcium alginate is the result of displacement of Na\(^+\) by Ca\(^{2+}\) (McDowell, 1977). As ionotropic gelation is very rapid, gel in the form of spheroidal beads is easily produced by extruding sodium alginate dropwise into calcium chloride.

The major disadvantages of alginate gels is that the gelling cations may be removed by the presence of chelating agents such as phosphate, lactate and citrate and displaced by cations such as Mg\(^{2+}\) and K\(^+\) (Cheetham et al., 1979; Smidsrød and Skjåk-Bræk, 1990). This means that any media used for alginate immobilised microorganisms have to include the gelling cations in the formulation and that phosphate, lactate, citrate, Mg\(^{2+}\) and K\(^+\) have to be used judiciously.

1.2.3.1.3 Propagules for the production of immobilised anaerobic fungi

A major issue in the development of an immobilisation procedure is the choice of a suitable propagule. Both homogenised hyphae (for example, Kopp and Rehm, 1983) and spores (for example, Deo and Gaucher, 1983) have been immobilised and subsequently cultured to produce immobilised mycelia. Spores are better propagules than homogenised hyphae for three reasons:
a) Homogenisation results in mechanical damage to cells and loss of viability.
b) The risk of microbial contamination is increased during homogenisation.
c) Spores can be quantified more accurately and reproducibly (by either viable or direct counts) than homogenised hyphae or mycelia (by wet weight).

The life cycle of monocentric and polycentric anaerobic fungi differ by the ability of the former to readily produce zoospores in culture (see Section 1.2.1.2). Zoospores would therefore be the propagule of choice when attempting the immobilisation of monocentric fungi. On the other hand, due to their filamentous nature and inability to produce zoospores frequently, homogenisation of rhizomycelium of polycentric fungi is perhaps a suitable method for the preparation of inoculum for the immobilisation of this type of anaerobic fungus.

1.2.3.2 Containment of immobilised mycelial growth

Bacteria and yeasts are unicellular cells that multiply by the process of binary fission or budding which allows them to form either micro colonies inside the matrix or a thin layer of cells at the periphery. The growth of filamentous fungi on the other hand occurs by the linear elongation of mycelial filaments. Consequently, the potential for the use of immobilised fungi in bioreactors is most likely to be limited by the growth of mycelia at the microbial aggregate-medium interface due to their filamentous nature (Kuek, 1986). This phenomenon has often been reported (Deo et al., 1983; Baklashova et al., 1984; Borglum and Marshall, 1984; Eikmeier et al., 1984; Kopp and Rehm, 1984; Horitsu et al., 1985; El-Sayed and Rehm 1986; Gosmann and Rehm 1986; Barbotin et al., 1990; Lohmeyer et al., 1990). Overcoming this problem is an essential prerequisite for the wide use of immobilised fungi in the fermentation industry.

Various investigations concerning morphological features of immobilised fungi mycelia have been published. The influence of various carbon substrates and nutrient limitations were investigated by Tsay and To (1987). Pertot et al. (1988) investigated the morphological differentiation of immobilised *Claviceps paspali*
mycelium during long-term semi-continuous cultivation while Chen and Huang, (1988) studied the effects of the growth of *Trichosporon cutaneum* in calcium alginate beads upon bead structure and oxygen transfer characteristics. In general, these studies reported cell leakage from gels if the cultural conditions were incorrect. It has been demonstrated that it is possible to grow *Aspergillus phoenicus* within a calcium alginate gel matrix and subsequently use it repeatedly for the production of glucoamylase (the longest period of use was six weeks) while the immobilised mycelia remained completely confined to the surface (Kuek and Armitage, 1985; Kuek, 1991). No release of biomass from the carrier into the medium during the fermentation process was observed using *T. reesei* entrapped in polyurethane foam (Linko et al., 1996). These findings encourage optimism that it may be possible to successfully produce immobilised anaerobic fungi without mycelial outgrowth.

1.2.3.3 Culture strategies to optimise cellulase synthesis by immobilised aerobic fungi

Although immobilised cell systems have been widely studied, and some are applied commercially, relatively few examples of immobilised cell systems involving filamentous fungi have been published, and even fewer examples have involved enzyme production (Table 1.7).

A first attempt at lowering the cost of cellulase production of *Trichoderma reesei* was performed by employing a *T. reesei* mutant RUT-C30 immobilised on 4% κ-carrageenan beads in continuous culture (Frein et al., 1982). In this initial study, the ability of immobilised cells to produce significant amounts of extracellular enzyme on a long-term basis (13 days) was demonstrated. Substrate requirements were dramatically lowered and carbon and nitrogen requirements were reduced to one-quarter to one-half of those of conventional continuous culture. Presumably this improvement was related to the low growth rate needed to maintain a stable immobilised cell population in continuous culture. This advantage is further illustrated in an investigation conducted by Duff (1988) who utilised the same organism in fed-batch fermentations while immobilised on
Table 1.7  Production of hydrolytic enzymes by immobilised microbial cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microorganism</th>
<th>Support material</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase</td>
<td><em>Trichoderma reesei</em></td>
<td>κ-carrageenan</td>
<td>Frein <em>et al.</em> (1982)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nylon mesh</td>
<td>Taniguchi <em>et al.</em> (1983)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polymerised 2,6-dimethylphenol (Sorfix)</td>
<td>Jirku <em>et al.</em> (1984); Jirku (1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly (2-hydroxyethyl methacrylate)</td>
<td>Kumakura and Kaetsu (1983);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kumakura <em>et al.</em> (1984a; 1984b; 1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyeurethane foam</td>
<td>Türkcr and Mavituna (1987)</td>
</tr>
<tr>
<td></td>
<td><em>Trichoderma viride</em></td>
<td>Needle-punched polyester</td>
<td>Duff (1988)</td>
</tr>
<tr>
<td></td>
<td><em>Sporotrichum cellulophilum</em></td>
<td>Stainless steel mesh</td>
<td>Sachse <em>et al.</em> (1990)</td>
</tr>
<tr>
<td></td>
<td><em>Talaromyces emersonii</em></td>
<td>Poly (2-hydroxyethyl acrylate)</td>
<td>Xin and Kumakura (1993)</td>
</tr>
<tr>
<td>Endoglucanase and xylanase</td>
<td><em>Trichoderma reesei</em></td>
<td>Stainless steel mesh</td>
<td>Webb <em>et al.</em> (1986)</td>
</tr>
<tr>
<td>Lignocellulase</td>
<td><em>Streptomyces spp.</em></td>
<td>Nonwoven materials</td>
<td>Tamada <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td><em>Aspergillus niger</em></td>
<td>Ca- alginate</td>
<td>McHale (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nylon web</td>
<td>Haapala <em>et al.</em> (1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sintered glass, Pumice stone</td>
<td>Haapala <em>et al.</em> (1996)</td>
</tr>
<tr>
<td></td>
<td><em>Aspergillus phoenicus</em></td>
<td>Ca-alginate</td>
<td>Chatel <em>et al.</em> (1990)</td>
</tr>
<tr>
<td></td>
<td><em>Aureobasidium pullulans</em></td>
<td>Ca-alginate</td>
<td>Li <em>et al.</em> (1984)</td>
</tr>
<tr>
<td>Lipase</td>
<td><em>Sporotrichum thermophile</em></td>
<td>Ca-alginate</td>
<td>Fiedurek and Lobarzewski (1990)</td>
</tr>
<tr>
<td>Protease</td>
<td><em>Myxococcus xanthus</em></td>
<td>Ca-alginate</td>
<td>Kuek (1991)</td>
</tr>
<tr>
<td>Liposomal enzymes</td>
<td><em>Penicillium chrysogenum</em></td>
<td>Ca-alginate</td>
<td>Gallo Federici <em>et al.</em> (1990); Federici <em>et al.</em> (1990)</td>
</tr>
<tr>
<td>Lignin peroxidase</td>
<td><em>Tetrhyema thermophila</em></td>
<td>Ca-alginate</td>
<td>Johri <em>et al.</em> (1990)</td>
</tr>
<tr>
<td></td>
<td><em>Phanerochaete chrysosporiun</em></td>
<td>Nylon-web</td>
<td>Fortin and Vuillemand (1990)</td>
</tr>
<tr>
<td></td>
<td><em>Phanerochaete chrysosporiun</em></td>
<td>Porous alumina</td>
<td>El-Aassar <em>et al.</em> (1990)</td>
</tr>
<tr>
<td></td>
<td><em>Phanerochaete chrysosporiun</em></td>
<td>Polyeurethane foam</td>
<td>Kily and Tiedtke (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Linko (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cornwell <em>et al.</em> (1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chen <em>et al.</em> (1991); Sanromán <em>et al.</em> (1996)</td>
</tr>
</tbody>
</table>
needle-punch polyester. This immobilised *Trichoderma* was very stable and resulted in a reduced rate of biomass generation as compared to cultures inoculated with free mycelium. In batch fermentations, the immobilised mycelium produced the same extracellular cellulase activity as free mycelium (1.55 IU/mL), while in fed-batch fermentations, the cellulase activity produced was about 20% lower than that produced by free mycelium. The improved stability associated with the immobilised system, as well as the low biomass levels in the crude enzyme broth produced provided the basis of improvement over free mycelia systems.

The physicochemical requirements of an immobilised culture can be met by transferring the cells to a medium that is optimum for product formation. This advantage is exemplified by the findings of Webb *et al.* (1986). In this investigation enhanced cellulase yield was demonstrated using *T. viride* QM9123 immobilised in stainless steel mesh biomass support particles. Data concerning the continuous production of cellulase by freely suspended and immobilised cells showed that both the bioreactor productivity and the enzyme yield of the immobilised cells were substantially higher. At a dilution rate of 0.15h\(^{-1}\) (nominal washout rate for freely suspended cells is 0.012h\(^{-1}\)), the yield of cellulase on glucose was 31% higher than that measured during batch operation while the productivity (31.5 FPA (Filter Paper Activity) U/L-h) was 35% greater than in the freely suspended batch system. The specific cellulase productivity of the immobilised cells (4.16 FPA U/(g(cell)-h) was more than 3 times that of freely suspended cells. A possible explanation for this was the low substrate concentration within the biomass support particle, which resulted from the existence of a concentration gradient, giving rise to an extremely low growth rate, which in turn stimulates cellulase production. The low cell growth rate also led to less of the substrate being used for biomass production and a consequent increase in cellulase yield. This represents a particular advantage of cell immobilisation for secondary metabolite production since, in freely suspended cell systems, a considerable proportion of the substrate is required for biomass production, either during the growth phase of a batch culture or continuously during continuous
culture. The same organism was immobilised within the open porous network of reticulated polyurethane foam matrices (Türker and Mavituna, 1987). In this investigation the absence of nitrogen source in the production medium was found to increase enzyme production. Similar results were obtained with both freely suspended and immobilised cells. A nitrogen-free production medium gave the highest enzyme titres of $1.5 \times 10^3$ FPA U/L that were about 4 times higher than that obtained with growth medium containing nitrogen. After the cessation of growth, higher enzyme yields could be achieved by simply replacing the growth medium with the production medium if the cells could be retained in the bioreactor.

The thermophilic fungus *Talaromyces emersonii* CBS 814.70 was co-immobilised with cellulose in calcium alginate beads for the production of cellulase (McHale, 1988). The immobilised system continued to produce enzyme in a fed-batch reactor system while the non-immobilised biomass had ceased production. The highest enzyme activity produced by the free-cell system in fed-batch culture was 5.5 U (activity expressed as milliequivalents of glucose produced per mL of culture filtrate per 2 h) while the highest activity recorded by the immobilised system was 10 U, almost twice the maximum produced by the non-immobilised system.

Xin and Kumakura (1993) examined the production of cellulase by *T. reesei* QM9414 cells immobilised by adhesion on paper carriers which were covered by hydrophilic and hydrophobic copolymers obtained by irradiation of monomers. The relationship between cellulase activity (FPA) and surface property of the carrier was investigated. Hydroxypropyl methacrylate (HPMA) showed the highest FPA (2.6 IU/mL); this was 1.5 times higher than that of free cells. The cells could be immobilised onto the paper that was covered with various other polymers, and could multiply normally, however, the amount of cells that were immobilised varied with the type of polymer. This study suggested that relatively mild hydrophilic polymers such as HPMA enhance the immobilisation of cells that grow actively to produce high levels of cellulase.
The production of endo-1,4-β-glucanase and xylanase using \textit{T. reesei} supported on polyurethane foam and nylon-web has been investigated by Haapala \textit{et al.} (1994; 1996, respectively). \textit{T. reesei} supported on both types of matrices resulted in higher enzyme activities in comparison to the free mycelium, and the immobilisation made repeat-batch fermentations possible. Freely suspended \textit{T. reesei} cells in bioreactors produced higher concentrations of protein that resulted in lower specific enzyme activities. The nitrogen source in the production medium used for \textit{T. reesei} cells immobilised on polyurethane foam had a marked effect on culture pH during the course of the fermentation and consequently xylanase activity. In this study one unit (U) was defined as nanomoles of reducing sugar liberated per second. An increase in lactose concentration from 7 to 27 g/L resulted in an increase in endoglucanase activity (maximum 730 U/mL), xylanase activity (maximum 3350 U/mL) and filter paper activity (maximum 3.0 FPU (Filter Paper Units) /mL). Similarly, the nitrogen source had a marked effect on endoglucanase and xylanase production on lactose, cellulose and l-sorbose-based medium with nylon-web immobilised \textit{T. reesei}. Maximum enzyme activities were obtained using synthetic cellulose-based medium and were 840 U/mL, 4790 U/mL and 3.0 FPU/mL for endoglucanase, xylanase and filter paper activity respectively. However, the simplified lactose containing industrial by-product-based medium proved to be a good alternative to the costly cellulose containing synthetic medium.

1.2.3.3.1 Culture strategies for the production of cellulase using immobilised anaerobic fungi

The immobilised cells discussed in the previous section each have a particular advantage pertaining to the physiology of the immobilised fungus. At the commencement of this project there were no reports into the physiology of cellulase production by anaerobic fungi. The growth-dissociated production of cellulase by \textit{T. reesei} provides confidence that anaerobic fungi may also produce cellulases in this way. In investigating the potential of immobilisation as a novel and possibly advantageous approach to cellulase production in the anaerobic fungi
an understanding of the relationship between rhizomycelial growth and the synthesis of cellulase can be gained.

Immobilisation not only allows for the exploitation of organisms that exhibit growth-dissociated synthesis of enzymes, it also has the operational advantage of using microbial cells repeatedly and continuously. Therefore, if the physiology of cellulase synthesis in anaerobic fungi is found to be growth-associated there is the alternate operational advantage of re-using the same population of cells. Repeat-batch culture was nominated as the operational mode in fermentations involving immobilised cells in this project and will allow for the incubation of cells over an extended period. Immobilisation makes repeat-batch fermentation possible because the technique enables easy recovery of biomass for re-use.
CHAPTER TWO

Relative cellulolytic activity of anaerobic fungi isolated from ruminant and non-ruminant sources during cultivation on cellulose-azure

2.1 Introduction

Anaerobic fungi are normal members of the rumen microflora (Mountfort, 1987) and are especially abundant in ruminants receiving highly fibrous diets (Bauchope, 1979). They have been isolated from many sites along the digestive tract of ruminants (Trinci et al., 1994). As well as being present in the more important species of domesticated ruminants (sheep, goats, cattle and water buffalo), anaerobic fungi occur widely among many different species of herbivorous mammals, including ruminant-like (such as camels and llamas) and other foregut fermenting non-ruminant animals (such as kangaroos) as well as hindgut fermenting animals (such as horses and elephants) (Orpin and Joblin, 1988).

As mentioned in the previous chapter, one of the major characteristics of all anaerobic fungi is their production and secretion of a range of polysaccharide-degrading enzymes. Consequently, the interest in the industrial application of anaerobic fungi lies in their use in the conversion of cellulosic biomass to valuable products. For purposes such as these, the fungi should show a high activity toward cellulose breakdown. The first stage in a selection strategy is the primary screen. This is predominantly a qualitative endeavour in which a large number of organisms are screened either directly or indirectly for their ability to degrade cellulose. Most screening procedures devised for the detection of cellulolytic microorganisms involve the clearing of opaque culture tubes or plates of cellulose-containing agar media (Rautela and Cowling, 1966; Tansey, 1971; Walsh and Stewart, 1969). These cleared zones result from the action of cellulolytic enzymes.
on the suspended cellulose particles. In these procedures the results are often difficult to interpret because the zones of clearing are not always easy to distinguish from the unaffected medium. In order to overcome this problem Smith (1977) devised a method involving the use of a dyed cellulose substrate for the detection of cellulolytic fungi.

The first stage of primary screening for microorganisms of potential industrial application is their isolation. In this present study the isolation procedure was designed in such a way that cellulolytic organisms were recognised at the isolation stage. This was done by isolating anaerobic fungi from a variety of ruminant and non-ruminant herbivores using a range of cellulosic substrates to increase the selection of highly cellulolytic strains. Following their isolation a preliminary assessment of the organisms’ ability to produce cellulase was performed by comparing the relative cellulolytic activity of these fungal isolates using cellulose-azure (C-A). A test-tube method, modified for use with anaerobic microorganisms and based on the work of Smith (1977), is described which involves layering agar medium containing C-A onto a basal medium. The cellulolytic activity of fungi inoculated into the upper layer breaks down the C-A, releasing the azure which diffuses into the previously clear lower layer. The amount of dye released is a measure of the cellulolytic activity of the fungi. This simple dye release method was modified in this present study, to give a semi-quantitative measure of cellulolytic activity by comparing the extent of dye release with a prepared set of standard C-A tubes.
2.2 Materials and methods

2.2.1 Media

2.2.1.1 Preparation of substrates

The following substrates were used in the isolation of anaerobic fungi:

- Filter paper (Whatman No.1)
- Oaten straw
- Newsprint (ink-free margins)

All substrates were subjected to the physical treatment of ball milling and, in the case of straw and newsprint, further treatment was applied using the alkaline hydrogen peroxide method of Gould and Freer (1984) prior to ball milling.

The filter paper and newsprint were first cut into small pieces. The straw was milled in a hammer mill 3100 (Falling Number ABS 12611 Stockholm, Sweden) to pass through a sieve with 1 mm pores. A 2% suspension was made by adding 6 g of cellulosic material to 300 mL of GDW. All substrates were ball-milled in a 5 L porcelain jar containing a combination of small glass marbles, weighing a total of 1733 g, and large glass marbles, weighing a total of 181 g. The jar was rolled at about 40 rpm in a cold room for 48 hours. The slurry was decanted and autoclaved at 110°C for 25 minutes. All sterile ball-milled substrates were stored at 4°C.

The milled straw or cut newsprint (100 g) was placed in 5 L of GDW containing 1% (v/v) H₂O₂ for chemical pre-treatment. The suspension was adjusted to pH 11.5 with 10 M NaOH and allowed to stir gently at room temperature (25°C) overnight (usually 18-24 hours). No further adjustments in pH were made during the course of the reaction. The insoluble residue was collected by vacuum filtration through two layers of muslin (pore size 150 µm) and washed with GDW.
until the pH of the filtrate was neutral. Slurries of treated material were shell frozen in a dry ice/ethanol bath in 500 mL Dynavac flasks before being freeze dried on a Dynavac freeze drier and finally ball-milled.

2.2.1.2 Isolation medium

Basal medium for the growth of anaerobic fungi was that described by Phillips and Gordon (1989) as a modification of medium 10 (M10) of Caldwell and Bryant, (1966) without added carbohydrates. This basal medium was supplemented with the various ball-milled substrates to give a total concentration of 0.2% (w/v) in the isolation medium. The composition of basal medium 10 is shown in Table 2.1. All ingredients, except the reducing agent, were dissolved in distilled water and boiled for 5 minutes. The reductant was made up just before use by dissolving 0.625 g cysteine.HCl in 24 mL distilled water. The pH was adjusted to 11 with 10 M NaOH; 0.625 g sodium sulphide was added and dissolved by mixing. A boiling solution of reducing agent was added at a rate of 1 mL/100 mL to the boiling medium which was bubbled with CO₂ on ice until cold (about 15 minutes for 100 mL media).

2.2.1.3 Cellulose-azure (C-A) medium

A number of dyed cellulose powders are available. Sigma Type II cellulose-azure was found unsuitable for this agar-based method because dye diffused into the lower layer in uninoculated tubes (controls). Sigma Type I and Calbiochem No. 219481 are two cellulose-azure powders which do not release dye prior to inoculation. They are both lightly dyed and fibrous, whereas the Sigma Type II is a heavily dyed powder. Therefore, on the basis of suitability and availability, cellulose-azure (Sigma Type I) was employed in this subsequent test.

Due to the fibrous nature of the cellulose-azure it was necessary to first ball-mill the product. Ball-milling was performed using a 500 mL glass bottle (Schott, Weisbaden, Federal Republic of Germany) which contained glass marbles.
<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity/100 mL medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral solution I K₂HPO₄</td>
<td>(3.0 g/L) 15 mL.</td>
</tr>
<tr>
<td>Mineral solution II KH₂PO₄</td>
<td>(3.0 g/L) 15 mL.</td>
</tr>
<tr>
<td>NaCl</td>
<td>(6.0 g/L)</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>(6.0 g/L)</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>(0.08 g/L)</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>(0.13 g/L)</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>(8 %) 7 mL.</td>
</tr>
<tr>
<td>Haemin</td>
<td>(0.5 %) 0.2 mL.</td>
</tr>
<tr>
<td>Volatile fatty acids</td>
<td></td>
</tr>
<tr>
<td>Acetic</td>
<td>(17 mL.)</td>
</tr>
<tr>
<td>Propionic</td>
<td>(6 mL.)</td>
</tr>
<tr>
<td>n-butyric</td>
<td>(4 mL.)</td>
</tr>
<tr>
<td>i-butyric</td>
<td>(1 mL.)</td>
</tr>
<tr>
<td>n-valeric</td>
<td>(1 mL.)</td>
</tr>
<tr>
<td>i-valeric</td>
<td>(1 mL.)</td>
</tr>
<tr>
<td>DL-2-methylbutyric</td>
<td>(1 mL.)</td>
</tr>
<tr>
<td>Resazurin</td>
<td>(0.1%) 0.1 mL.</td>
</tr>
<tr>
<td>Trypticase peptone (BBI, Microbiology Systems, Cockysville, Md.)</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Yeast extract (Oxoid, Hampshire, England)</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Glucose¹</td>
<td>(BDH, Kilsyth, Australia) 0.033 g</td>
</tr>
<tr>
<td>Cellobiose¹</td>
<td>(Sigma Chemical Co., St Louis, Mo) 0.033 g</td>
</tr>
<tr>
<td>Starch¹</td>
<td>(Sigma)               0.067 g</td>
</tr>
<tr>
<td>Xylan¹</td>
<td>(larchwood) (Sigma)   0.067 g</td>
</tr>
</tbody>
</table>

¹ Carbohydrates added for complete medium M10X
weighing a total of 250 g), 90 mL GDW and 1.8 g C-A. The bottle was rolled at about 96 rpm at room temperature for 36 hours. The suspension was decanted, washed and centrifuged. A 6% (w/v) suspension was prepared in GDW, gassed under N₂ and autoclaved at 115°C for 10 minutes.

Basal medium (M10) was prepared without carbon source, using agar (Oxoid) at a concentration of 7.5 g/L and the medium was autoclaved at 121°C for 15 minutes. The sterilised basal medium was then dispensed as 2.0 mL aliquots into pre-sterilised Hungate tubes (16 by 125 mm) (Bellco glass, Inc., Vineland NJ) in an anaerobic chamber (Coy Laboratory Products Inc. Ann Arbor, MI) which contained an atmosphere of 95% CO₂ and 5% H₂ (v/v). The tubes were placed vertically to allow the agar to solidify as deeps.

The 6% ball-milled C-A suspension was mixed with pre-sterilised basal medium (M10) when still molten to give a final C-A concentration of 2.0%. The mixture was layered over the 2 mL basal medium (0.5 mL/tube) and left to solidify in the anaerobic chamber. The prepared tubes were stored at 4°C.

2.2.2 Isolation procedures

2.2.2.1 Source of organisms

Anaerobic fungi were isolated from rumen and faecal samples from a range of domesticated and exotic herbivores listed in Table 2.4 (see Results).

Rumen fluid was collected from three fistulated sheep (adult wether Merino/Border Leicester cross) housed at the Commonwealth Scientific and Industrial Research Organisation (C.S.I.R.O.), Division of Animal Production, Prospect (New South Wales, Australia). Three different diets were fed to the sheep during the course of isolations. The first was a milled and pelleted diet (600 g) comprising 3 parts lucerne and 2 parts oats (60/40). The sheep were fed once daily at 10.00 am and sampled at 2.00 pm. After isolating a number of fungi,
the diet was changed to a roughage feed consisting of equal parts milled lucerne and oaten hay (R9) (800 g). The sheep remained on this diet for a period of 3 weeks before attempting any isolations. In addition, rumen fluid was taken from two sheep which were fed this roughage diet which incorporated newsprint. These sheep remained on this diet for two weeks before sampling. The untreated, inkfree newsprint (unprinted margins) was first shredded then ground through a hammer mill with a 3 mm inch slot screen. The milled newsprint was fed at a ratio of 50 g newsprint to 700 g R9 hay. Digesta was also obtained from a fistulated cow (breed unknown) fed on a diet of lucerne cubes housed at C.S.I.R.O. Long Pocket (Queensland, Australia). Fresh faeces from the exotic herbivores were collected in plastic bags from the ground of animal enclosures at Western Plains Zoo, Dubbo (New South Wales, Australia).

2.2.2.2 Roll tube method for the isolation of anaerobic fungi

Anaerobic fungi were isolated using a roll tube method (Joblin, 1981). Roll tube medium consisted of basal medium which was supplemented with the various ball-milled substrates (see Section 2.2.1.2). Complete medium M10X which contained carbohydrates (Table 2.1) was also used in isolations. The medium was dispensed (2.8 mL) under CO₂ into screw-cap Hungate tubes containing 60 mg agar, sealed with black butyl rubber septa and screw caps and autoclaved at 121°C for 15 minutes (110°C for 25 minutes for media containing ball-milled substrates). Prior to inoculation, 0.3 mL filtered (0.22 μm pore size) anaerobic antibiotic solution containing 12 mg sodium benzyl penicillin and 2 mg streptomycin sulphate per mL (both from Sigma) was added aseptically to each roll tube.

Dilutions for the isolation of fungi were made in anaerobic dilution solution (ADS) (9 mL per tube) which was prepared in the same way as basal medium M10, with the deletion of growth factors, antibiotics, carbon sources and volatile fatty acids (Table 2.2).
Table 2.2  Anaerobic dilution solution (ADS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity/100 mL medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral solution I</td>
<td>15.0 mL</td>
</tr>
<tr>
<td>Mineral Solution II</td>
<td>15.0 mL</td>
</tr>
<tr>
<td>Na₂CO₃ (8%)</td>
<td>3.8 mL</td>
</tr>
<tr>
<td>Resazurin (0.1%)</td>
<td>0.1 mL</td>
</tr>
</tbody>
</table>

2.2.2.3 Isolation from rumen fluid

Rumen fluid obtained from fistulated sheep was filtered through a layer of nylon cloth (average pore size, 160 μm), and collected under a CO₂ atmosphere. The rumen fluid was serially diluted 10-fold in an anaerobic dilution solution contained in Hungate-type anaerobic culture tubes fitted with butyl rubber septa using 1 mL transfers. All procedures were performed with sterile anaerobic techniques using 1 mL syringes and 23 gauge needles (Terumo Medical Corp., Elkton, MD). Subsequently, 0.2 mL of each dilution was inoculated in triplicate into Hungate tubes containing molten isolation medium at 50°C and immediately rolled on ice. After 4-5 days incubation at 39°C, colonies were examined using a dissecting microscope at a magnification of x16. Individual colonies were counted and representatives from each animal were transferred with a sterile loop under CO₂ gassing into fresh broths containing 5 mL M10X. This procedure was repeated until cultures were pure and free of bacteria. Methanogenic bacteria and anaerobic mycoplasmas, which were not eliminated by the first antibiotic treatments (penicillin and streptomycin), were subsequently eliminated by treatment with chloramphenicol (1 mg/mL) and lincomycin (0.425 mg/mL) (both from Sigma) respectively (Phillips and Gordon, 1995b).
2.2.2.4 Isolation from faeces

Moist faeces from zoo animals of unknown age (ca. 1.0-2.0 g fresh faeces per bottle) were collected from Dubbo Zoo and used to inoculate 100 mL serum bottles containing 50 mL basal medium M10. The enrichment medium contained the antibiotics penicillin (60 mg), streptomycin (10 mg) and chloramphenicol (2.2 mg) and either one of the following cellulosic substrates present at 0.5% (w/v):

- Fibrous cellulose (Grade CF-11 Whatman Chemical Separation Ltd., England.)
- Hammer milled oat straw
- Hammer milled newsprint (ink free margins)

Media were dispensed into 100 mL serum bottles fitted with butyl rubber septa and metal crimp seals (Miller and Wolin, 1974) and then sterilised at 121°C for 15 minutes. An interval of up to 10 hours occurred between the collection at the zoo and its incubation at the laboratory. The ambient temperature during transit was about 25°C. Additional faecal samples were collected into gassed Schott bottles as source inoculum for M10X agar plates containing antibiotics for the isolation of polycentric strains. This was performed on arrival at the laboratory approximately 9 hours after collection and was done according to the method of Phillips (1989). A plug of agar was cut out and a spoonful of faecal matter was embedded. After 5 days at 39°C, polycentric fungi arising from the faecal sample gave spreading colonies that covered the plate.

The enrichment cultures were incubated for 4 days at 39°C and subsequently used to inoculate roll tubes containing the corresponding substrate as the enrichment culture, from which monocentric anaerobic fungi were isolated as described previously for rumen fluid.
2.2.2.5 Culture maintenance

All monocentric cultures were maintained at 39°C by subculture every 4-5 days in basal medium containing hammer milled oat straw (0.5% w/v) and cellobiose (0.025% w/v). Polycentric cultures were subcultured at appropriate intervals, usually every 5 to 7 days, depending on rate of growth by removing small sections (5 mm x 5 mm) from the margin of the fungal colony which developed on the agar and transferring to petri dishes containing medium M10X. Stock cultures of all strains were also preserved anaerobically at -196°C in ADS with 5% (v/v) dimethylsulphoxide (DMSO) as cryoprotectant using the method of Phillips and Gordon (1988). Monocentric cultures for freezing were first grown in oat straw medium for 4-5 days. Polycentric fungi were similarly cultured on M10X agar in petri dishes containing a section of straw embedded in the agar. A loopful of straw containing the viable fungal culture was placed into sterilised 750 µL skirted freezing vials (Sigma) containing 0.5 mL solution of DMSO in ADS. A cooling rate of about 1.5°C per min was employed using dry ice before the vials were immersed into liquid nitrogen. The frozen cultures were resuscitated by rapid thawing at 39°C and inoculated into fresh maintenance medium.

2.2.3 Semi-quantitative measurement of cellulolytic activity using cellulose-azure

2.2.3.1 Preparation of pre-dyed standard deeps

A set of 12 tubes were prepared as standards for the semi-quantitative measurement of cellulolytic activity using cellulose-azure. Blue dye for these standards was prepared by digesting a 6% suspension of ball-milled C-A (3.0 mL) with 4 units (7.2 mg) of cellulase (Sigma, C-0898; 1,4-[1,3;1,4]-β-D-Glucan 4 glucano hydrolase; EC 3.2.1.4 from Trichoderma viride Type VI) in 7.0 mL of 50 mM MES (2-[N-Morpholino]ethanesulfonic acid) adjusted to pH 6.0
with 10 M NaOH. The suspension was incubated at 50°C for 4 days. The 12 standard tubes were prepared by adding varying dilutions of blue dye (1.5 mL) to 1.0 mL of basal medium M10 containing agar (7.5 g/L) according to the following protocol (Table 2.3).

Table 2.3  Protocol for the preparation of C-A standard tubes

<table>
<thead>
<tr>
<th></th>
<th>Tube number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10 11 12</td>
</tr>
<tr>
<td>H₂O (mL)</td>
<td>1.5 1.35 1.2 1.05 0.9 0.75 0.675 0.6 0.45 0.3 0.15 0</td>
</tr>
<tr>
<td>Dye (mL)</td>
<td>0 0.15 0.3 0.45 0.6 0.75 0.825 0.9 1.05 1.2 1.35 1.5</td>
</tr>
</tbody>
</table>

2.2.3.2 Inoculation of C-A medium

Ball-milled C-A medium was inoculated using 0.25 mL from an active growing monocentric culture (4 day old). When testing polycentric fungi the inoculum was a small block of agar (5 mm x 5 mm) from the growing margin of a culture. All test strains were incubated at 39°C and scored daily for extent of dye release with reference to the prepared set of standards. Uninoculated controls were also used to verify that dye release was due only to cellulolytic activity and not to other factors.
2.3 Results

2.3.1 Isolation of anaerobic fungi from rumen fluid and faeces

Anaerobic fungi were isolated from faeces of 7 of the 11 species of zoo animals surveyed (Table 2.4). The source animals for these isolations were banteng cattle, eland, red kangaroo, camel, fallow deer, water buffalo and southern white rhinoceros. Successful isolations of polycentric fungi were possible using faeces from banteng cattle, camel, water buffalo and southern white rhinoceros. These faeces were stored for up to 10 hours in gassed Schott bottles at room temperature.

The fungal isolates were classified according to colonial morphology, the size of the fungal rhizoid, and the appearance of zoospores as viewed by phase contrast light microscopy. The monocentric cultures in this study were assigned to the genera of anaerobic fungi on the basis of production of multiflagellate zoospores (Neocallimastix: Orpin, 1975; Heath et al., 1983; Orpin and Munn, 1986) or monoflagellate zoospores with a filamentous branching rhizoid or a globular rhizoid (Piromyces sp. and Caecomyces sp. respectively; Gold et al., 1988). The polycentric fungal isolates which formed large, spreading colonies on the agar plates were examined for the morphology of their rhizoids and zoospores which led to their classification as species of either Orpinomyces sp. (Barr et al., 1989; Li et al., 1991) or Anaeromyces sp. (Breton et al., 1990). Figures 2.1, 2.2 and 2.3 illustrate the morphological characteristics of some representative isolates.

With reference to Table 2.4 it appears that monocentric genera are not restricted to any particular animal species or digestive anatomy. The majority of fungi isolated from the rumen fluid of sheep were of the genus Neocallimastix. A similar result was obtained for the eland and fallow deer whereas only Piromyces sp. were isolated from cattle and kangaroo. Only one Caecomyces sp. was isolated from the 13 animals tested. Banteng cattle and rhinoceros showed the most diversity with respect to the number of different species of anaerobic fungi isolated.
A total of 46 strains of anaerobic fungi were isolated from 13 animals, 42 of which were of the monocentric type. Tables 2.5 and 2.6 expand on this isolation data and tabulate the source and strain number of the isolate, the genus, the isolation substrate, and the diet of the animal from which the strain was isolated. All sheep fungal strains were isolated on cellulosic substrates. About 60% of fungal strains isolated from the zoo animals were isolated on complete medium M10X.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Common name</th>
<th>Specific name</th>
<th>Neocallimastix</th>
<th>Piromycetes</th>
<th>Caecomyces</th>
<th>Orpinomyces</th>
<th>Anaeromyces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>Bos taurus</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>Ovis aries</td>
<td></td>
<td>13</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African elephant</td>
<td>Loxodonta africana</td>
<td></td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Banteng cattle</td>
<td>Bos javanicus</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Bison</td>
<td>Bison bison</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camel</td>
<td>Camelus dromedarius</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eland</td>
<td>Taurotragus oryx</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fallow deer</td>
<td>Cervus dama</td>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gaunacoë</td>
<td>Lama glama gaunicoe</td>
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<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red kangaroo</td>
<td>Macropus rufus</td>
<td></td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scimitar horned oryx</td>
<td>Oryx dammah</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southern white rhinoceros</td>
<td>Ceratotherium simum</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Water buffalo</td>
<td>Bubalus bubalis</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total number of fungal isolates = 46

---

1 Hindgut fermenters  
2 Foregut fermenter; all other animals are ruminants  
3 Fungal isolations unsuccessful in these herbivores
Figure 2.1  Morphology of vegetative growth and zoospores of some fungal isolates by phase-contrast microscopy:

(A) Multiflagellate zoospore of Neocallimastix sp. DLX2 from deer; (B) Monoflagellate zoospore of Piromyces sp. K5X1 from kangaroo; (C) Fungal plant of Piromyces sp. SS2 from sheep; (D) Small fungal plant of Neocallimastix sp. DLS3 from deer (E) Fungal plant of Neocallimastix sp. LS11 from sheep
Bars: 10 μm (A and B), 100 μm (C), 200 μm (D and E)
Figure 2.2 Morphology of Neocallimasix sp. DLX2 by phase contrast microscopy. Sporangia (sp) and rhizomycelia (rh) are indicated. Bar = 20 μm

Figure 2.3 A monocentric isolate, Neocallimasix sp. DL3, grown on ball-milled cellulose in an agar roll tube. Note the zone of clearing. Bar = 3 mm
Table 2.5  
Isolation details of anaerobic fungi isolated from rumen fluid

Substrate abbreviations:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMN-AHP</td>
<td>ball-milled newsprint; alkaline hydrogen peroxide treated</td>
</tr>
<tr>
<td>BMS-AHP</td>
<td>ball-milled straw; alkaline hydrogen peroxide treated</td>
</tr>
<tr>
<td>BMC</td>
<td>ball-milled cellulose</td>
</tr>
<tr>
<td>M10X</td>
<td>complete medium M10X</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain identification and source</th>
<th>Genus</th>
<th>Isolation substrate</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine rumen fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SN1</td>
<td><em>Piromyces</em></td>
<td>BMN-AHP</td>
<td>Lucerne cubes</td>
</tr>
<tr>
<td>SN2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>SS2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>SX1</td>
<td>&quot;</td>
<td>M10X</td>
<td>&quot;</td>
</tr>
<tr>
<td>Ovine rumen fluid</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LN1</td>
<td><em>Neocallimastix</em></td>
<td>BMN-AHP</td>
<td>60/40</td>
</tr>
<tr>
<td>LN3</td>
<td>&quot;</td>
<td>&quot;</td>
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<td>R9</td>
</tr>
<tr>
<td>LN8</td>
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<td>&quot;</td>
<td>R9/NP</td>
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<td>&quot;</td>
<td>R9/NP</td>
</tr>
<tr>
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<td>&quot;</td>
<td>R9</td>
</tr>
<tr>
<td>LS4</td>
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<td>BMN-AHP</td>
<td>60/40</td>
</tr>
<tr>
<td>LS8</td>
<td>&quot;</td>
<td>&quot;</td>
<td>60/40</td>
</tr>
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<td>&quot;</td>
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<td>&quot;</td>
<td>&quot;</td>
<td>R9</td>
</tr>
<tr>
<td>SS1</td>
<td><em>Piromyces</em></td>
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<td>R9/NP</td>
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</table>
Table 2.6  Isolation details of anaerobic fungi isolated from faeces

<table>
<thead>
<tr>
<th>Strain identification and source</th>
<th>Genus</th>
<th>Isolation substrate</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banteng cattle faeces</td>
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<td></td>
</tr>
<tr>
<td>BLN1</td>
<td>Neocallimastix</td>
<td>BMN-AHP</td>
<td>Mixall&lt;sup&gt;1&lt;/sup&gt; calcium/molasses blocks</td>
</tr>
<tr>
<td>BLN2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>lucerne hay</td>
</tr>
<tr>
<td>BLX1</td>
<td>&quot;</td>
<td>M10X</td>
<td>graze available on exhibit</td>
</tr>
<tr>
<td>BSX1</td>
<td>Piromyces</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>BCR1</td>
<td>Orpinomyces</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>Buffalo faeces</td>
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</tr>
<tr>
<td>WLX1</td>
<td>Neocallimastix</td>
<td>M10X</td>
<td>lucerne hay oaten chaff graze available</td>
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<tr>
<td>WBR1</td>
<td>Orpinomyces</td>
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<td></td>
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<tr>
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<tr>
<td>SX2</td>
<td>Piromyces</td>
<td>M10X</td>
<td>Mixall graze available</td>
</tr>
<tr>
<td>CAR1</td>
<td>Orpinomyces</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>Deer faeces</td>
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<td></td>
</tr>
<tr>
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<td>Neocallimastix</td>
<td>BMS-AHP</td>
<td>Mixall oats graze available on exhibit</td>
</tr>
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<td>DLS2</td>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>DLS3</td>
<td>&quot;</td>
<td>BMC</td>
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</tr>
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<td>DLF1</td>
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<td>M10X</td>
<td></td>
</tr>
<tr>
<td>DLX1</td>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>DLX2</td>
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<td>&quot;</td>
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</tr>
<tr>
<td>DLX3</td>
<td>&quot;</td>
<td>&quot;</td>
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<td>Eland faeces</td>
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<tr>
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<td>Neocallimastix</td>
<td>M10X</td>
<td>Mixall and oats lucerne hay Graze on exhibit</td>
</tr>
<tr>
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<td>&quot;</td>
<td>copper blocks</td>
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<td>Kangaroo faeces</td>
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</tr>
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<td>Piromyces</td>
<td>BMS-AHP</td>
<td>Ad lib. kangaroo pellets</td>
</tr>
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<td>KSS2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Ad lib. meadow hay</td>
</tr>
<tr>
<td>KSF1</td>
<td>&quot;</td>
<td>BMC</td>
<td>eucalypt leaves as browse</td>
</tr>
<tr>
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<td>&quot;</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>KSF3</td>
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<td>&quot;</td>
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</tr>
<tr>
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</tr>
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<td>Piromyces</td>
<td>M10X</td>
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<td></td>
</tr>
<tr>
<td>RNX1</td>
<td>Caecomyces</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>WRP1</td>
<td>Anaeromyces</td>
<td>&quot;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Prepared on zoo site and consists of 2 x bales lucerne hay, 6 x bales oaten hay and 75 kg oats
2.3.2 Cellulose-azure as a semi-quantitative assay for the detection of cellulolytic activity

All anaerobic fungi uncoupled blue dye from the bound cellulose powder during incubation, and the free dye diffused into the basal layer of medium. However, not all fungi degraded C-A to the same end point. Figures 2.4 and 2.5 illustrate the set of standards against which the cultures were scored and the appearance of selected cultures after incubation for 8 days respectively.

The performance of 46 isolates when compared to a set of prepared standards over the 8 day incubation period is given in Table 2.7. This table ranks the strains according to the maximum rate of colour release calculated as change in colour score per day. The distribution of rates of colour release indicate that 54% of strains scored a rate of 1; 37% scored a rate of 2 while 9% of isolates scored the highest rate of 3. This 9% represented the three strains WBR1, CAR1, ELX1 and DLX1 all of which scored a ranking of 10 or higher with reference to the prepared set of standards. The intensity of colour was estimated on a scale of 1 to 12, 1 representing no colouration and 12 representing intense colour. The scores given for the extent of dye release at the end of incubation ranged from 11 to 2. A highly active strain toward C-A which scored 11 was an *Orpinomyces* sp., strain WBR1 isolated from a water buffalo, and produced the first detectable effect within 2 days, with the colour intensity of the basal layer increasing during subsequent incubation. Two *Piromyces* sp., SS1 and SX2, from sheep and camel respectively, shared the lowest score of 2 and also produced a rate of colour release rank of 1. The distribution of scores amongst *Neocallimastix* sp. and *Piromyces* sp. shows that they are similarly effective on C-A. However, it is apparent that the three *Orpinomyces* sp. did perform better than the single *Anaeromyces* sp. with respect to colour score. Fungal strains from the domestic animals, sheep and cattle, gained scores of 6 and lower whilst the zoo strains were evenly distributed over a range of scores between 11 and 2.
Figure 2.4  Prepared set of standards representing various quantities of digested cellulose from which growing cultures were scored. 1 = no colour; 12 = maximum colour

Figure 2.5  Appearance of selected cultures after 8 days incubation

An un inoculated control tube = 1; and tubes inoculated with various fungal strains demonstrating diffusion of released dye: SX2=2, ELX1=3, WBR1=4, BCR1=5 and DLS3=6. Numbers in brackets indicate score

Upper layer containing cellulose-azure (A) overlying a clear layer of basal medium M10 (B)
Table 2.7  *Relative performance of fungal isolates during incubation on cellulose-azure*

**EXTENT OF DIFFUSION KEY:**
Numbers refer to the intensity of blue colouration in a prepared set of standards:
1 = no colour; 12 = maximum colour.

<table>
<thead>
<tr>
<th>Strain identification</th>
<th>Source</th>
<th>Genus</th>
<th>Incubation Period (Days)</th>
<th>Max. rate of colour release $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBR1</td>
<td>buffalo</td>
<td><em>Orpinomyces</em></td>
<td>8  9  10  11  11  11</td>
<td>3</td>
</tr>
<tr>
<td>CAR1</td>
<td>camel</td>
<td><em>Orpinomyces</em></td>
<td>4  5  8  9  9  10</td>
<td>3</td>
</tr>
<tr>
<td>ELX1</td>
<td>eland</td>
<td><em>Neocallimastix</em></td>
<td>3  3  6  7  9  10</td>
<td>3</td>
</tr>
<tr>
<td>DLX1</td>
<td>deer</td>
<td><em>Neocallimastix</em></td>
<td>1  3  6  8  10  10</td>
<td>3</td>
</tr>
<tr>
<td>KXS1</td>
<td>kangaroo</td>
<td><em>Piromyces</em></td>
<td>1  2  2  4  6  7  2</td>
<td></td>
</tr>
<tr>
<td>RSX2</td>
<td>rhinoceros</td>
<td><em>Piromyces</em></td>
<td>3  3  5  5  6  7  2</td>
<td></td>
</tr>
<tr>
<td>KSF2</td>
<td>kangaroo</td>
<td><em>Piromyces</em></td>
<td>2  3  5  6  6  6  2</td>
<td></td>
</tr>
<tr>
<td>DLS1</td>
<td>deer</td>
<td><em>Neocallimastix</em></td>
<td>2  3  5  6  6  6  2</td>
<td></td>
</tr>
<tr>
<td>DLF1</td>
<td>deer</td>
<td><em>Neocallimastix</em></td>
<td>2  3  5  6  6  6  2</td>
<td></td>
</tr>
<tr>
<td>DLS3</td>
<td>deer</td>
<td><em>Neocallimastix</em></td>
<td>1  1  1  3  4  6  2</td>
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</tr>
<tr>
<td>LS4</td>
<td>sheep</td>
<td><em>Neocallimastix</em></td>
<td>1  2  4  4  5  6  2</td>
<td></td>
</tr>
<tr>
<td>LS10</td>
<td>sheep</td>
<td><em>Neocallimastix</em></td>
<td>1  2  4  5  5  6  2</td>
<td></td>
</tr>
<tr>
<td>DLS2</td>
<td>deer</td>
<td><em>Neocallimastix</em></td>
<td>1  1  2  3  3  5  2</td>
<td></td>
</tr>
<tr>
<td>DLX2</td>
<td>deer</td>
<td><em>Neocallimastix</em></td>
<td>1  2  4  4  5  5  2</td>
<td></td>
</tr>
<tr>
<td>BLN2</td>
<td>banteng</td>
<td><em>Neocallimastix</em></td>
<td>1  2  3  5  5  5  2</td>
<td></td>
</tr>
<tr>
<td>LS9</td>
<td>sheep</td>
<td><em>Neocallimastix</em></td>
<td>1  3  4  5  5  5  2</td>
<td></td>
</tr>
<tr>
<td>LS13</td>
<td>sheep</td>
<td><em>Neocallimastix</em></td>
<td>1  3  4  5  5  5  2</td>
<td></td>
</tr>
<tr>
<td>LN3</td>
<td>sheep</td>
<td><em>Neocallimastix</em></td>
<td>2  2  4  4  4  4  2</td>
<td></td>
</tr>
<tr>
<td>BLX1</td>
<td>banteng</td>
<td><em>Neocallimastix</em></td>
<td>1  1  1  1  1  3  2</td>
<td></td>
</tr>
<tr>
<td>ELX2</td>
<td>eland</td>
<td><em>Neocallimastix</em></td>
<td>1  1  1  1  1  3  2</td>
<td></td>
</tr>
<tr>
<td>LN10</td>
<td>sheep</td>
<td><em>Neocallimastix</em></td>
<td>1  1  1  1  1  3  2</td>
<td></td>
</tr>
<tr>
<td>BCR1</td>
<td>banteng</td>
<td><em>Orpinomyces</em></td>
<td>3  3  4  4  4  5  1</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Maximum rate of colour release is calculated as colour score per day.
Table 2.7  *Relative performance of fungal isolates during incubation on cellulose-azure* (Cont.)

<table>
<thead>
<tr>
<th>Strain identification</th>
<th>Source</th>
<th>Genus</th>
<th>Incubation Period (Days)</th>
<th>Max. rate of colour release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>SN1</td>
<td>cattle</td>
<td>Piromyces</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>LS8</td>
<td>sheep</td>
<td>Neocallimastix</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>LS11</td>
<td>sheep</td>
<td>Neocallimastix</td>
<td>1</td>
<td>1</td>
</tr>
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<td>sheep</td>
<td>Neocallimastix</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>WLX1</td>
<td>buffalo</td>
<td>Neocallimastix</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>BLN1</td>
<td>banteng</td>
<td>Neocallimastix</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>KSF3</td>
<td>kangaroo</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>KSS2</td>
<td>kangaroo</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RSX1</td>
<td>rhinoceros</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RNX1</td>
<td>rhinoceros</td>
<td>Caecomyces</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>WRP1</td>
<td>buffalo</td>
<td>Anaeromyces</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>LN1</td>
<td>sheep</td>
<td>Neocallimastix</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LS12</td>
<td>sheep</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
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<td>KSF1</td>
<td>kangaroo</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>KSS1</td>
<td>kangaroo</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BSX1</td>
<td>banteng</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DLX3</td>
<td>deer</td>
<td>Neocallimastix</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SN2</td>
<td>cattle</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SS2</td>
<td>cattle</td>
<td>Piromyces</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>SX1</td>
<td>cattle</td>
<td>Piromyces</td>
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<td>2</td>
</tr>
<tr>
<td>LN6</td>
<td>sheep</td>
<td>Neocallimastix</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LN9</td>
<td>sheep</td>
<td>Neocallimastix</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SX2</td>
<td>camel</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SS1</td>
<td>sheep</td>
<td>Piromyces</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

---

¹ Maximum rate of colour release is calculated as colour score per day
2.4 Discussion

In this study ruminant and non-ruminant herbivores were used as source material for the isolation of anaerobic fungi from a total of 13 animals. Failure to isolate anaerobic fungi from 4 of the 11 zoo animals surveyed may be due to technique problems, that is, the faeces may have been too old which does not necessarily mean that these animals do not have fungi in their digestive systems. Milne et al., (1989) showed that fungi could not be isolated from sheep faecal pellets after storage for 1 day at 20°C or 39°C. In this regard, it is possible to believe that gut fungi are likely to be present in all ruminant and non-ruminant herbivores given the fastidious cultural requirements of the anaerobic fungi and the few attempts to isolate them on a restricted range of media in this study.

Cellulosic substrates were included in the isolation medium to increase the likelihood of selecting highly cellulytic fungi. This, coupled with the use of enrichment culture makes the isolation procedure a preliminary screen for cellulytic fungi. The physical and chemical pre-treatment methods of the cellulosic substrates were used to increase the possibility of isolating anaerobic fungi by making the substrate more accessible to them. Physical and chemical pre-treatments serve to separate cellulose from its protective sheath of lignin and increase the surface area of the cellulose crystallite by size reduction and swelling. Treatments to increase the effective surface area accessible to enzymes will increase hydrolysis. Cutting and grinding generally increase the rate of hydrolysis and ball-milling seems to be the most effective treatment of this type (Mandels et al., 1974). Alkaline hydrogen peroxide treatment enhances susceptibility of the cellulose polymer to hydrolysis as a result of partial lignin removal by hydrolysis and oxidation (Lewis et al., 1987).

The numbers of fungal isolates obtained from zoo faecal samples on cellulosic substrates were relatively low. Therefore, in an effort to increase the number of fungi, isolations were also performed on complete medium M10X. No fungi from eland, camel, buffalo and rhinoceros faeces were isolated on cellulosic substrates.
Fungal isolates from these herbivores were all isolated on M10X. Out of a total of 46 strains only one *Caecomyces* sp. was isolated. This strain was isolated using rhinoceros faeces as the source material and M10X as the isolation medium. Cellulose is used by most genera of anaerobic fungi although *Caecomyces* sp. apparently do not utilise this polymer in purified form (Hébraud and Fèvre, 1988; Phillips and Gordon, 1988). However, straw also contains hemicellulose (xylan) which *Caecomyces* can degrade and Gordon and Phillips (1989) have observed both growth and degradation of straw by the *Caecomyces* sp. NM1.

It has been shown that the same fungi can be isolated from different parts of the digestive tract of sheep (Wong et al., 1995). In this current study 91% of the isolates were of the monocentric type. The majority of fungi isolated from the rumen fluid of sheep belonged to the genus *Neocallimastix*. A similar result was obtained by Milne et al., (1989). *Neocallimastix* sp. isolates were also isolated from eland and fallow deer whereas only *Piromyces* sp. were isolated from cattle and kangaroo. Polycentric fungi were isolated in low frequency in this study with a total of 4 strains obtained from banteng cattle, camel, water buffalo and rhinoceros. Polycentric fungi are yet to be isolated from sheep in this country and their isolation appears to be confined to the larger herbivores (Phillips, 1989; Phillips and Gordon, 1995b).

Sheep were fed a roughage diet which incorporated newsprint to increase the number of fungi isolated on cellulosic substrates. It has been shown that diets containing a higher fibre content increase the population densities of anaerobic fungi in the rumen and feeding concentrates decreases densities (Grenet et al., 1989). All sheep fungal strains were isolated on cellulosic substrates. Over half of the zoo fungal strains were isolated on complete medium M10X, a result which may be a reflection on the animals’ diets (refer to Table 2.6). The diets of the zoo animals are not specifically high in fibre and appear supplemented with readily digestible concentrates.
Having isolated a variety of anaerobic fungi from different herbivores their relative cellulolytic ability was assessed using cellulose-azure. This method in its present form was designed as a qualitative test, that is, the fungal culture either utilised the C-A or it did not. However, since all fungal strains were capable of utilising the cellulose-azure it was used in this screening procedure to estimate the relative cellulolytic activity of the strains. This was performed by comparing the test cultures with a set of standards, thereby producing a semi-quantitative result from the screening test. The isolates were ranked according to the maximum rate of colour release calculated as colour score per day. In most cases there was good correlation between the colour score at the end of the 8-day incubation period and the rate of colour release.

Those fungal strains isolated on cellulosic substrates appeared to be no more effective on C-A than those fungi isolated on complete medium M10X. Similarly, there did not appear to be any correlation between the diets of the source animal and the performance of the strain on cellulose-azure. For example, those strains isolated from sheep fed newsprint did not utilise C-A any better than those sheep not fed a specialist diet. However, these results must be considered knowing that the cellulose-azure method provided an end-point for cellulolysis only and lacked the sensitivity necessary for detecting cultures with only slightly different cellulolytic capabilities.

There were other limitations to this mode of measurement. Scoring was found to be subjective because the colour of the dye diffused by the growing culture was a slightly different shade of blue in comparison to the prepared set of standard tubes. This made discerning differences in dye intensity difficult at times. Quantitative measurement of the degree of colour was attempted with the use of a spectrophotometer but with limited success and as such the method used in this study can be used only for the primary ranking of cellulolytic fungal strains. The change of blue colour of the dye to other hues may be due to the presence of resazurin in the medium or the reductive alteration of the dye molecule. The colorimetric method may have been limited because of this change in colour.
However, this method has been quantified so that cellulolytic isolates can be ranked in order of their activities and also the actual cellulase activity can be defined (Plant et al., 1988). The method involves scanning the tubes from top to bottom, in a gel scanner, and obtaining an absorption spectrum as a measure of cellulolytic activity relative to the distance the dye has diffused in the agar. Possibly, the quantification of this method would have defined the cellulolytic activity of the isolates in this present study more precisely. However, the C-A method was based on visual identification of the isolates, a characteristic of many primary screens.

Cellulose-azure is an acid-swollen cellulose preparation dyed with Remazol Brilliant Blue R, and solubilisation of this cellulose does not measure ability to degrade insoluble, crystalline celluloses encountered in nature. Because of its lack of crystallinity, cellulose-azure is not a suitable substrate for determining the ability of anaerobic fungi to produce the total cellulolytic enzyme complex. However, the cellulose-azure test provides a rapid and generally reliable qualitative and semi-quantitative indicator of the cellulose degrading abilities of a large number of isolates, although influenced by the growth rate of the test strain.
CHAPTER THREE

Determination of rates of cellulolysis of anaerobic fungi as a secondary screen using U-[\(^{14}\)C]-labelled cellulose

3.1 Introduction

The previous chapter described a strategy to identify organisms with cellulolytic capabilities, by isolating anaerobic fungi from a variety of ruminants including sheep, cattle, buffalo, eland, camel and deer, from hindgut fermenters such as rhinoceros, and from non-ruminant foregut fermenters such as kangaroo. The cellulose-azure method was employed as a semi-quantitative assay that detected gross differences in cellulolytic activity between these fungal strains.

Having showed that the fungi were truly cellulolytic in the primary screen using cellulose-azure, the next step was to refine the screening programme by quantifying this cellulolytic activity. Radioactive fibre substrates provide a useful means of rapidly screening a large number of fungi for their relative abilities to degrade plant fibre (Gordon, 1987; 1990). In this study, cellulolytic activity was determined by the ability of isolates to degrade a radioactively labelled fibre substrate, namely \(^{14}\)C-cellulose obtained from *Acetobacter xylinum*. The time course of release of radioactivity from the U-[\(^{14}\)C]-labelled insoluble cellulose was followed by scintillation counts on supernatant fractions from growing cultures. The objective of the study was to assay for total cellulase activity by studying the kinetics of solubilisation of cellulose by the growing cultures. Maximum solubilisation rates were calculated over a 24 hour period and each isolate was ranked according to this rate. In addition, the ability to solubilise \(^{14}\)C-cellulose was compared with the cellulose-azure results of chapter two.
3.2 Materials and methods

3.2.1 Preparation of U-[^14]C-labelled bacterial cellulose using *Acetobacter xylinum*


*A. xylinum* (ATCC 23770; gift from Dr. R. Mackie, while in South Africa) grows as a cellulose pellicle that forms at the liquid surface and can be removed as a single sheet. Glucose-phosphate-peptone-yeast medium (Table 3.1) was used to culture *A. xylinum*.

<table>
<thead>
<tr>
<th>Table 3.1 Glucose-phosphate-peptone-yeast medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component</td>
</tr>
<tr>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
</tr>
<tr>
<td>Peptone (Difco)</td>
</tr>
<tr>
<td>KH₂PO₄</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>pH (adjusted, if necessary)</td>
</tr>
</tbody>
</table>

The medium was either dispensed into Wheaton tubes for subculture (5 mL) or larger volumes into glass bottles for later use in plastic tissue culture flasks and autoclaved at 121°C for 15 minutes.
In order to generate enough bacterial cellulose the culture was transferred at monthly intervals by removing the whole pellicle with a sterile loop and placing it in a fresh broth of glucose-phosphate-peptone-yeast medium. The tube was capped tightly and briefly shaken before removing the pellicle. The loosely capped tube was incubated at about 25°C.

Sterile glucose-phosphate-peptone-yeast medium (150 mL) was dispensed into disposable plastic tissue culture flasks (A/S Nunc, Kamstrup, Denmark). D-U-[\(^{14}\)C]-glucose (0.19 mCi, Amersham, Sydney, Australia) was sufficiently diluted in GDW to yield 1 mL of glucose solution for each flask. The solution was filtered into a sterile container using a sterile membrane with 0.2 μm pores. The filtered glucose solution (1 mL) was aseptically dispensed into each flask. After dispensing the medium and labelled glucose, each flask was immediately inoculated with a single pellicle from a 5 mL culture that had been incubated for 7-10 days. Flasks were incubated in a flat position with loosened caps for 17-18 days. After incubation, the pellicles were removed from the flask and washed with distilled water to remove adhering culture medium and bacterial cells. The pellicles were then boiled in 2 M KOH for 20 minutes and washed with GDW to remove residual alkali. The cellulose was macerated in a kitchen blender at high speed for 30 seconds and the \(^{14}\)C-cellulose was recovered after freeze drying the slurry on a Dynavac freeze drier.

### 3.2.2 Culture procedures

In order to assay all 46 fungal isolates the procedure was divided into three separate runs. Each run consisted of 16 cultures (in triplicate) and also included background and uninoculated tubes. One fungal culture, \textit{Neocallimastix} sp. DLS3 isolated from a deer, was included in each run to ensure standardisation between each run. It was chosen on the basis of its performance in a preliminary run. In all three subsequent runs it was placed in the top four.
3.2.2.1 Media

The labelled cellulose was firstly ball-milled to aid in the dispensing of the growth media. A 1% (w/v) suspension of labelled cellulose in GDW was ball-milled in a 500 mL Schott bottle containing glass marbles (weighing a total of 250 g) at about 96 rpm at room temperature. The ball-milled product was sterilised by autoclaving at 110°C for 25 minutes and stored in the refrigerator. Counts performed on this product indicated that ball-milling did not affect the specific activity of 14C-cellulose. Five millilitre volumes of basal medium M10 containing 0.2% ball-milled 14C-cellulose were dispensed into Hungate tubes and then autoclaved at 110°C for 25 minutes.

3.2.2.2 Inoculation of U-[14C]-cellulose media

Inoculation of broths containing 14C-cellulose was performed using 0.25 mL from a 4 day old monocentric culture grown in M10X media. In the case of polycentric fungi, straw embedded in M10X media from a petri dish containing the actively growing fungus was used as the source inoculum. Three replicas were performed per culture as well as an uninoculated control.

3.2.3 Assay procedure

Samples (0.25 mL) were withdrawn from each culture tube immediately after inoculation by sterile anaerobic procedures using a 1.0 mL syringe. Further 0.25 mL aliquots were removed at regular accurately timed intervals of approximately 24 hours thereafter. Residual cellulose and, if present, fungal cells, were sedimented by centrifugation at 10 000 x g for 2 minutes in an Eppendorf microcentrifuge. The 0.25 mL aliquots of the supernatants were then added to 2.5 mL volumes of scintillation cocktail (Optiphase ‘Hisafe’ 3, LKB Scintillation Products, England). After correction for background, the disintegrations per minute value for each sample was plotted against incubation time.
3.2.3.1 Liquid scintillation counting procedures

$^{14}$C measurements of the culture supernatant were by Beta liquid scintillation counting using a Minaxi Counter (Packard, USA). The radioactivity was determined as disintegrations per minute (DPM) by taking into account counting efficiency which was determined by spiking samples with an isotope of known radioactivity. The standard was $[^{14}\text{C}]$ cyclohexanone (Amersham, Sydney, Australia) having a count of 13,339 DPM/100 µL.
3.3 Results

Maximum solubilisation rates of all 46 strains are presented in Table 3.2. These were calculated over a 24-hour period which gave the maximum solubilisation rate. From the three assay runs the top twelve ranking isolates were chosen based on maximum rates. The cut-off point was determined logistically by the number of isolates that could be retained for further secondary screening. In addition, strain WBR1 and strain BCR1 were included in this list on the basis of their high solubilisation rates and the desire to include polycentric fungi in the secondary screening procedure. The twelve isolates were re-assayed and ranked according to their solubilisation rates (Table 3.3).

Monocentric type fungi appear to perform better than the polycentric type (Table 3.3). The four top ranking isolates were Neocallimastix species whilst the last two ranked strains were of the Orpinomyces genus. Table 3.3 also presents the cellulose-azure results of the strains and indicates that these results did not necessarily correspond to the maximum solubilisation rate of the particular strain. It was found that half of the top 12 ranking strains were isolated on cellullosic substrates. Three-quarters of these fungal strains were isolated from zoo animals. The strain that scored the highest $^{14}$C-cellulose solubilisation rate was Neocallimastix sp. DLS3, a deer isolate. In comparison, it scored a cellulose-azure rank of 2. The strain registering the highest rank with regard to the ability to utilise cellulose-azure was Neocallimastix sp. ELX1, an eland isolate. This fungal isolate did attain a place in the top 12 strains chosen on the basis of their ability to solubilise $^{14}$C-cellulose, ranking second.

Figure 3.1 is a linear plot of the time course of release of radioactivity from labelled bacterial cellulose by a growing culture of Neocallimastix sp. DLS3. This plot shows the typical form of a batch growth curve. The plots of the remaining eleven selected isolates are given in Appendix 1.
<table>
<thead>
<tr>
<th>Run I</th>
<th>Run II</th>
<th>Run III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Maximum</td>
<td>Strain</td>
</tr>
<tr>
<td></td>
<td>Solubilisation¹</td>
<td></td>
</tr>
<tr>
<td>LS11</td>
<td>73.78</td>
<td>LS4</td>
</tr>
<tr>
<td>DLS3</td>
<td>68.51</td>
<td>SN2</td>
</tr>
<tr>
<td>KSF2</td>
<td>64.96</td>
<td>SS2</td>
</tr>
<tr>
<td>RSX1</td>
<td>62.15</td>
<td>DLS3</td>
</tr>
<tr>
<td>SX1</td>
<td>60.47</td>
<td>KSF3</td>
</tr>
<tr>
<td>KSS1</td>
<td>57.98</td>
<td>LN8</td>
</tr>
<tr>
<td>LS13</td>
<td>51.11</td>
<td>BLX1</td>
</tr>
<tr>
<td>DLF1</td>
<td>50.81</td>
<td>SS1</td>
</tr>
<tr>
<td>LS12</td>
<td>48.35</td>
<td>LN6</td>
</tr>
<tr>
<td>KSF1</td>
<td>47.74</td>
<td>LS10</td>
</tr>
<tr>
<td>DLS2</td>
<td>43.38</td>
<td>LN1</td>
</tr>
<tr>
<td>DLS1</td>
<td>40.52</td>
<td>LN9</td>
</tr>
<tr>
<td>LN10</td>
<td>39.75</td>
<td>LS8</td>
</tr>
<tr>
<td>BLN1</td>
<td>36.63</td>
<td>LS9</td>
</tr>
<tr>
<td>BLN2</td>
<td>36.11</td>
<td>SN1</td>
</tr>
<tr>
<td>SX2</td>
<td>18.04</td>
<td>LN3</td>
</tr>
</tbody>
</table>

¹ Rate (DPM/mL x 10³/d)
² Not determined. Growth of these cultures on ¹⁴C-cellulose was very poor
Table 3.3  *Maximum solubilisation rates of top 12 ranking fungal cultures (Average DPM/mL x 10³ per 24 hours)*

<table>
<thead>
<tr>
<th>Strain identification</th>
<th>Genus</th>
<th>Isolation substrate</th>
<th>Source</th>
<th>Maximum solubilisation Rate (DPM/mL x 10³/d)</th>
<th>Rank</th>
<th>Cellulose-azure rank²</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS3</td>
<td><em>Neocallimastix</em></td>
<td>BMS-AHP</td>
<td>deer</td>
<td>66.70</td>
<td>1</td>
<td>2 (5)²</td>
</tr>
<tr>
<td>ELX1</td>
<td><em>Neocallimastix</em></td>
<td>M10X</td>
<td>eland</td>
<td>66.42</td>
<td>2</td>
<td>3 (10)</td>
</tr>
<tr>
<td>LS11</td>
<td><em>Neocallimastix</em></td>
<td>BMS-AHP</td>
<td>sheep</td>
<td>65.00</td>
<td>3</td>
<td>1 (5)</td>
</tr>
<tr>
<td>DLX2</td>
<td><em>Neocallimastix</em></td>
<td>M10X</td>
<td>deer</td>
<td>59.94</td>
<td>4</td>
<td>2 (5)</td>
</tr>
<tr>
<td>SN2</td>
<td><em>Piromyces</em></td>
<td>BMN-AHP</td>
<td>cattle</td>
<td>56.59</td>
<td>5</td>
<td>1 (3)</td>
</tr>
<tr>
<td>KSX1</td>
<td><em>Piromyces</em></td>
<td>M10X</td>
<td>kangaroo</td>
<td>55.15</td>
<td>6</td>
<td>2 (7)</td>
</tr>
<tr>
<td>RSX1</td>
<td><em>Piromyces</em></td>
<td>M10X</td>
<td>rhinoceros</td>
<td>55.00</td>
<td>7</td>
<td>1 (4)</td>
</tr>
<tr>
<td>KSF2</td>
<td><em>Piromyces</em></td>
<td>BMC</td>
<td>kangaroo</td>
<td>51.12</td>
<td>8</td>
<td>2 (6)</td>
</tr>
<tr>
<td>LS4</td>
<td><em>Neocallimastix</em></td>
<td>BMS-AHP</td>
<td>sheep</td>
<td>50.09</td>
<td>9</td>
<td>2 (6)</td>
</tr>
<tr>
<td>SS2</td>
<td><em>Piromyces</em></td>
<td>BMS-AHP</td>
<td>cattle</td>
<td>45.14</td>
<td>10</td>
<td>1 (3)</td>
</tr>
<tr>
<td>BCR1</td>
<td><em>Orpinomyces</em></td>
<td>M10X</td>
<td>banteng cattle</td>
<td>42.13</td>
<td>11</td>
<td>1 (5)</td>
</tr>
<tr>
<td>WBR1</td>
<td><em>Orpinomyces</em></td>
<td>M10X</td>
<td>water buffalo</td>
<td>41.84</td>
<td>12</td>
<td>1 (11)</td>
</tr>
</tbody>
</table>

¹ Mean rate calculated from the values of three replicates
² Rank calculated as maximum rate of colour release (from previous chapter)
³ Number in brackets represents colour score at day 8 of incubation
Figure 3.1 Solubilisation of U-[^14]C]-labelled bacterial cellulose by a growing culture of Neocallimastix sp. DLS3
Those strains that were subsequently eliminated from the screening programme based on their maximum solubilisation rates are listed in Table A.2 (Appendix 2). Appendix 2 also contains the linear plots of the time release of radioactivity from labelled bacterial cellulose by growing cultures of these 34 strains.

Included in the list of eliminated strains were the only Caecomyces and Inaeromyces strains isolated in the programme. Four isolates, including species of Neocallimastix and Piromyces, did not grow or grew very poorly on ¹⁴C-cellulose. It is possible that these strains were not able to grow on pure cellulose contained in the medium as all isolates were checked for viability at the time of inoculation.
3.4 Discussion

The cellulolytic activity of 46 strains of anaerobic fungi was determined in this study by investigating their ability to release soluble radioactivity from $^{14}$C-cellulose. These strains, which were isolated from a range of ruminant and non-ruminant sources on cellulosic substrates, were previously subjected to a primary screen that tested their ability to degrade cellulose-azure. All test strains were able to utilise cellulose-azure but only a crude ranking was achieved using this screening technique. The present method in this study was employed to rapidly screen many different isolates of anaerobic fungi and led to the selection of those which most efficiently attacked cellulose.

Twelve strains which recorded the highest maximum solubilisation rates were retained for further screening while the remaining 34 strains were eliminated from the screening procedure. Of these top 12 ranking isolates a general ranking can be applied, viz.: *Neocallimastix > Piromyces > Orpinomyces*. However, it is observed that *Neocallimastix* and *Piromyces* strains are included amongst the 34 isolates that were eliminated. Therefore, relative ranks should be considered as specific to the individual strain rather than being representative of the other isolates that are members of the same genus. The exception to this is in the case of the performance of the *Caecomyces* strain and the *Anaeromyces* strain. Both of these strains were amongst those strains that were eliminated from the screening programme. Gordon (1990) reports a similar result with a *Caecomyces* strain CN1 ranking lowest among six different fungal strains when incubated on oat cell walls labelled with $[^{14}$C] glucose. It is important to note that the synthesis of cellulases may depend on the stage of growth. It is possible that some of the eliminated strains found in Appendix 2 might produce cellulases later in the growth cycle. That is, they may display a maximum solubilisation rate after 120 hours incubation.

Discrepancies in ranks occurred when viewing the results of the two screening methods. Some strains that did perform well in the cellulose-azure test were
eliminated from further screening tests on the basis of their inferior ability to solubilise $^{14}$C-cellulose. A variation between the two screening techniques is exemplified by observing the four strains that ranked highest in cellulose-azure screen. Of these four strains only ELX1 and WBR1 achieved a place in the top 12, whereas CAR1 and DLX1 were amongst the 34 strains eliminated. However, when the results of the two screening procedures are viewed in terms of the percentage of strains that scored more than 5 in the cellulose-azure test it is found that only 6 of the 34 eliminated isolates (18%) achieved these scores. This compares with 5 of the top 12 strains (42%) that had a colour score of 6 or higher. Nevertheless, due to the subjective results of the cellulose-azure test and the quantitative nature of the $^{14}$C-cellulose method, the latter screening technique must be viewed as superior.

The differences in the polymer structure that occur in cellulose-azure and the bacterial cellulose may also be responsible for variations observed among the strains. The *Acetobacter* cellulose substrate is a native (type 1) cellulose with a degree of polymerisation of $>3000$ (Hestrin, 1963), a crystallinity index considerably higher than that of the much-used substrates filter paper and Avicel, and a high total pore volume (Grethlein, 1985). Despite its higher crystallinity, this substrate is more readily degraded by a larger section of cellulolytic microorganisms than filter paper or Avicel.

According to Highley (1988), release of dye from cellulose-azure is not a guarantee of ability to degrade native cellulose, since the cellulose used in cellulose-azure is acid-swollen and more susceptible to degradation. In 1986, du Preez and Kistner showed that total cellulase activity can be measured using U-$^{14}$C-labelled bacterial cellulose by monitoring the solubilisation of this substrate by purified exo- and endocellulase fractions isolated from *T. reesei*, separately and in combination. Consequently, by determining the rates of cellulolysis of fungal strains using bacterial $^{14}$C-cellulose, the selection of anaerobic fungi with superior cellulolytic activity can be guaranteed.
CHAPTER FOUR

Investigations into the hydrolytic potential of anaerobic fungi

4.1 Introduction

Anaerobic fungi isolated from ruminant and non-ruminant herbivores have a high digestion capacity for cellulose. They produce extracellular enzymes with exoglucanase, endoglucanase, and β-glucosidase activities in vitro, when grown on cellulose (Gordon and Phillips, 1989; Lowe et al., 1987b; Mountfort and Asher, 1985; and Williams and Orpin, 1987b). Anaerobic fungi also produce an array of hemicellulolytic enzymes that allow for complete degradation of the xylan heteropolymer. Xylanase and β-xylosidase are produced by both monocentric and polycentric anaerobic fungi (Borneman et al., 1989). Xylanase is the most active of all endo-acting polysaccharide hydrolases studied from the anaerobic fungi (Mountfort and Asher, 1989).

In the previous chapter, twelve strains of anaerobic fungi were selected from a total of 46 on the basis of their ability to solubilise bacterial cellulose. This chapter reports on a comparison of the activities of several enzymes involved in the hydrolysis of a number of cellulosic substrates. To establish the production variables favouring high enzyme activity, investigations into the enzyme activities of two fungal isolates belonging to the genera *Neocallimastix* and *Orpinomyces* were firstly carried out. The effect of production variables on enzyme productivity was determined by conducting sequential cultures where only one variable at a time was altered. The objective was to derive an optimal operation strategy to obtain hydrolytic activity. The results of these initial studies led to the examination of the cellulolytic and xylanolytic activity of the twelve strains when
grown on oat straw with the aim of identifying those strains with the best potential for producing high enzyme levels.

The complete hydrolysis of oat straw requires an assortment of enzymes because the xylan structure is more heterogenous than that of cellulose. Unlike cellulose, xylan is a complex polysaccharide comprising a backbone of xylose residues linked by \( \beta(1\rightarrow 4) \) bonds. Depending on the source, the \( \beta\text{-D-xylopyranosyl} \) units are substituted with mainly \( O\text{-acetyl} \), arabinosyl, or glucuronosyl residues (Biely, 1985). The arabinosyl substituents are sometimes esterified with ferulic and \( p\text{-coumaric} \) acids (Meuller-Harvey et al., 1986). Therefore, both cellulolytic and xylanolytic enzyme activity was measured in this study in order to assess the isolates ability to degrade oat straw. Cellulolytic enzyme production was measured by carboxymethylcellulase (endo-1,4-\( \beta\text{-D-glucanase}, \text{EC 3.2.1.4} \)) and \( \beta\text{-glucosidase} \) (cellobiase and \( \beta\text{-D-glucoside glucohydrolase}, \text{EC 3.2.1.21} \)). The enzymatic hydrolysis of the complex xylans requires the combined action of xylanase (endo 1,4-\( \beta\text{-D-xylan xylanohydrolase}, \text{EC 3.2.1.8} \)), \( \beta\text{-xylosidase} \) (\( \beta\text{-D-xyloside xylanohydrolase}, \text{EC 3.2.1.37} \)) and enzymes responsible for the cleavage of side groups (Puls, et al., 1988). Therefore, xylanolytic enzyme production was measured by xylanase and \( \beta\text{-xylosidase} \).
4.2 Materials and methods

4.2.1 Organisms

Parameters for optimum cellulase production were determined by investigating the hydrolytic activity of two strains, namely, a *Neocallimastix* sp. DLS3 isolated from a deer and an *Orpinomyces* sp. WBR1 isolated from a water buffalo. These strains are representatives of monocentric and polycentric fungi respectively. Enzyme studies on the remaining twelve isolates identified in the previous chapter were performed using a set of standard culture conditions as determined by the preliminary investigations.

4.2.2 Culture medium and growth conditions

Inocula for all enzyme studies using monocentric fungi were grown in basal medium M10 with the addition of 0.5% oat straw containing 0.025% cellulbiose. The straw medium was inoculated using 0.25 mL from an actively growing fungus cultured in M10X medium and was grown for 4 days. Polycentric fungi were cultured on M10X agar in petri dishes containing a section of straw embedded in the agar. After growth on the inoculation medium the entire amount of straw containing the monocentric culture was used to inoculate the test substrate. An equivalent amount of straw was used when testing polycentric strains.

Media used in enzyme studies using the two test strains DLS3 and WBR1 included either of the following cellulosic substrates present at the indicated concentrations (w/v). Cellulosic substrates were treated using alkaline hydrogen peroxide (AHP) as described in Section 2.2.1.1.

* Filter Paper strips
  (Whatman No. 1; strips cut into 7.0 x 0.8 cm lengths) 0.5%
* Hammer milled oat straw 1.0%
* Hammer-milled oat straw, AHP-treated 0.5%, 1.0%
* Hammer milled newsprint, untreated, ink-free margins 1.0%
* Hammer-milled newsprint, AHP-treated 0.5%, 1.0%

The substrates were added at the designated concentrations to 50 mL of basal medium 10. Media were dispensed into 100 mL serum bottles fitted with butyl rubber septa and metal crimp seals and then sterilised at 121°C for 15 minutes.

All experimental cultures were incubated at 39°C either still or with shaking, in a reciprocating shaking water bath (Paton Industries, South Australia) at 30 oscillations per minute.

4.2.3 Sampling and enzyme preparation

Samples of growth medium (2.0 mL) were removed from the culture vessels at regular intervals by sterile anaerobic procedures with 2.5 mL syringes, transferred to 2.0 mL plastic eppendorf tubes and centrifuged at 8000 x g for 10 minutes (Micro Spin 245, Sorvall Instruments, Dupont, Wilmington, DE). Supernatants were carefully removed with a pasteur pipette and placed into eppendorf tubes awaiting dialysis.

4.2.3.1 Dialysis of culture liquor

Culture liquors were dialysed to remove sugars remaining from the growth medium and soluble products of cellulolytic action formed during growth, both of which may interfere with enzyme assays. An appropriate amount of dialysis tubing (1.0 cm flat width, Selby Scientific and Medical) which had been stored in the refrigerator in GDW containing 0.1 mM NaN₃, was cut and then rinsed with water. The lengths were clamped at the bottom and 2.0 mL of cell free culture liquor was carefully transferred into the tubing. Any air bubbles were expelled and the tube was clamped at the top. The liquors were dialysed for 18 to 20 hours at 4°C against 10 mM MES-NaOH buffer (pH 6.0) containing 0.1 mM NaN₃ with
gentle stirring. For each 2 mL of sample 100 mL of buffer was used. After dialysis the culture liquors were stored at -20°C until required for enzyme assays.

4.2.4 Enzyme assay procedures

Enzyme assays with culture fluid were performed in duplicate at 39°C in MES-NaOH buffer (pH 6.0). All enzyme reactions were linear over the period of assays. Blanks of enzyme without substrate and substrate without enzyme were included in all enzyme assays and sample values were corrected for any blank value. Enzyme production was expressed in International Units (IU) where one IU is defined as one micromole of product produced per minute.

4.2.4.1 Determination of reducing sugars

Reducing sugar estimations were made by the tetrazolium blue (TZ) procedure (Jue and Lipke, 1985). The reagent was prepared by adding 1 g of tetrazolium blue (Sigma) to 500 mL of 0.1 M NaOH. The mixture was heated to 60°C with vigorous stirring, resulting in a clear yellow solution. Sodium potassium tartrate (Sigma) was dissolved in GDW to a concentration of 1.0 M. The two solutions were filtered through a 0.2 μm nylon membrane (Alltech) and mixed in equal volumes. The mixed reagent was stored in the dark at 4°C.

4.2.4.2 Carboxymethyl cellulase

Carboxymethyl cellulase (CM-cellulase) assays were based on the method of Miller et al. (1960). CM-cellulose (sodium salt, low viscosity, C-8758) was purchased from Sigma and was dissolved in 50 mM MES-NaOH buffer (pH 6.0) at a concentration of 2.0 % (w/v). Between 2 and 50 μL of culture liquor was added to an eppendorf tube and made up to 0.5 mL with 50 mM MES-NaOH buffer. Aliquots of the diluted enzyme mix was added to an equal volume of substrate solution in a final volume of 200 μL in a glass tube (16 by 100 mm) sealed with a teflon-lined screw cap. The reactions were stopped by the addition
of 4 mL of TZ reagent after 30 minutes of incubation and immediately heated in a boiling water bath for exactly 3 minutes. The concentration of reducing sugars was measured with D-glucose as the standard. The tubes were cooled for 3 minutes in running water, dried, and absorbance was determined at 660 nm in a Bausch and Lomb Spectronic 21 spectrophotometer.

4.2.4.3 Xylanase

Xylanase assays were based on Forsberg et al. (1981) and Pearce and Bauchop (1985). The xylan (from oat spelt, Sigma X-0376) was washed before use in enzyme studies by suspending 2% (w/v) xylan in 50 mM MES-NaOH pH 6.0 at 39°C for 1 hour. The solution was centrifuged in a plastic centrifuge tube at low speed (≈1520 x g) for 5 minutes and the upper solution was carefully removed. This solution, with a nominal concentration of 2.0%, was used as the substrate. The conditions for assaying the enzyme were identical to those used for the CM-cellulase assay. The concentration of reducing sugars was measured using the TZ method with D-xylose as the standard.

4.2.4.4 Aryl-β-glucosidase and aryl-β-xylosidase

Aryl-β-glucosidase and aryl-β-xylosidase assays were based on previously described methods (Pearce and Bauchop, 1985; Williams and Withers, 1982; Garcia-Campayo and Wood, 1993). Assays were performed in a microtitre plate. p-Nitrophenol derivatives of β-D-glucose or β-D-xylose (5 mM; Sigma) were incubated with 10 mM MES-NaOH buffer (pH 6.0) and diluted culture liquor (between 50-200 µL of culture liquor diluted to 300 µL) in a total volume of 100 µL for 30 minutes. The reactions were stopped by the addition of 100 µL of 0.2 M Na₂CO₃, and the amount of p-nitrophenol produced was determined from a standard curve by measurement of absorbance at 405 nm using a Titretek Multiskan Plus plate reader (Flow Laboratories Ltd, Australia).
4.2.4.5 Cellobiase

Cellobiase was measured using the method of Deshpande and Eriksson (1988). The reaction mixture consisted of 0.5 mL of a 0.5% cellobiose (w/v) solution in 50 mM MES-NaOH buffer (pH 6.0) and a known volume of suitably diluted enzyme solution (100-200 µL) in a total volume of 1 mL. Following incubation at 39°C for 30 minutes, the reaction was terminated in a boiling water bath for 5 minutes. Glucose estimation was performed using the method of Faichney and White (1983). The colour reagent consisted of glucose oxidase, peroxidase and di-ammonium 2,2'-azino-di- (3-ethyl-benzothiazoline-6-sulfonate) (ABTS). Five millilitres of glucose colour reagent was added to 1 mL aliquots of the reaction mixture, mixed and incubated at 20-25°C. Absorbance at 436 nm was read after 25-50 minutes in a Bausch and Lomb Spectronic 21 spectrophotometer. The amount of glucose released in the incubation sample was measured with D-glucose as the standard.
4.3 Results

4.3.1 Effect of various cellulosic substrates on enzyme production

The effects of several growth substrates on the activities of CM-cellulase, xylanase, β-glucosidase, and β-xylosidase in culture liquor of Neocalimastix sp. DLS3, and Orpinomyces sp. WBR1 were examined (Table 4.1 and 4.2, respectively). In general, cellulolytic and xylanolytic enzyme activities were highest for Neocalimastix sp. DLS3.

Oat straw, either treated or untreated, was a good inducer for both cellulolytic and xylanolytic enzymes for both strains of fungi. The cellulolytic enzymes produced by the two fungi when grown on filter paper were comparable to those activities recorded when straw was the growth substrate. In contrast, filter paper had a slightly smaller effect on xylanolytic activity. Cellulolytic and xylanolytic activities were low for both strains when the cultures were grown on newsprint. Strain WBR1 failed to produce any enzyme activity when grown on 1.0% hammer-milled newsprint while the substrate had a relatively small effect on enzyme activities when DLS3 was tested.

Shaking the culture significantly reduced enzyme activity of both strains of fungi when the cultures were grown in newsprint. The results are slightly more variable with the substrates, filter paper and straw. However, in general, static cultures of the fungi produced more cellulolytic and xylanolytic activities when grown on these substrates.

Enzyme activities of DLS3 and WBR1 grown on treated substrates at 0.5% and 1.0% are quite variable and it is difficult to gauge at what concentration treated straw or newsprint results in highest enzyme activities.
### Table 4.1

*Maximum extracellular enzyme production in dialysed culture fluids of Neocallimastix sp. DLS3 grown either statically or shaken for 7 days in media containing various cellulosic substrates*

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>β-glucosidase</th>
<th>β-xylosidase</th>
<th>CM-cellulase</th>
<th>Xylanase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>shaken</td>
<td>static</td>
<td>shaken</td>
<td>static</td>
</tr>
<tr>
<td>Filter paper (0.5%)</td>
<td>35.5</td>
<td>38.8</td>
<td>39.2</td>
<td>11.3</td>
</tr>
<tr>
<td>Hammer milled straw (1.0 %)</td>
<td>40.4</td>
<td>32.0</td>
<td>80.0</td>
<td>93.3</td>
</tr>
<tr>
<td>Hammer-milled straw (treated) (0.5%)</td>
<td>55.8</td>
<td>35.8</td>
<td>76.2</td>
<td>60.0</td>
</tr>
<tr>
<td>Hammer-milled straw (treated) (1.0%)</td>
<td>11.2</td>
<td>25.4</td>
<td>51.4</td>
<td>85.8</td>
</tr>
<tr>
<td>Hammer-milled newsprint (1.0%)</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>8.6</td>
</tr>
<tr>
<td>Hammer-milled newsprint (treated) (0.5%)</td>
<td>6.4</td>
<td>19.2</td>
<td>10.5</td>
<td>22.5</td>
</tr>
<tr>
<td>Hammer-milled newsprint (treated) (1.0%)</td>
<td>2.8</td>
<td>35.7</td>
<td>10.7</td>
<td>31.7</td>
</tr>
</tbody>
</table>

1 Values are averages of duplicate assays
Table 4.2  Maximum extracellular enzyme production in dialysed culture fluids of *Orpinomyces* sp. WBR1 grown either statically or shaken for 7 days in media containing various cellulosic substrates

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Enzyme production (mIU/mL)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-glucosidase</td>
</tr>
<tr>
<td></td>
<td>shaken</td>
</tr>
<tr>
<td>Filter paper (0.5%)</td>
<td>8.8</td>
</tr>
<tr>
<td>Hammer milled straw (1.0 %)</td>
<td>7.4</td>
</tr>
<tr>
<td>Hammer-milled straw (treated) (0.5%)</td>
<td>6.2</td>
</tr>
<tr>
<td>Hammer-milled straw (treated) (1.0%)</td>
<td>6.8</td>
</tr>
<tr>
<td>Hammer-milled newsprint (1.0%)</td>
<td>0</td>
</tr>
<tr>
<td>Hammer-milled newsprint (treated) (0.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Hammer-milled newsprint (treated) (1.0%)</td>
<td>1.7</td>
</tr>
</tbody>
</table>

$^1$ Values are averages of duplicate assays
Straw-grown, static cultures of the *Neocallimastix* sp. DLS3, contained the highest activities of all 4 of the enzymes assayed. On the basis of these results, further investigations into substrate concentration were performed using static cultures of DLS3 grown on treated and untreated hammer-milled straw. The effects of varying concentrations of either treated or untreated hammer-milled straw on CM-cellulase, xylanase, β-glucosidase, and β-xylosidase activity in culture liquors of DLS3 are shown in Table 4.3.

Table 4.3  
*Maximum extracellular enzyme production in dialysed culture fluids of Neocallimastix sp. DLS3 grown statically for 7 days in basal medium M10 containing various concentrations of straw*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme production (mIU/mL)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>straw (untreated)</td>
<td>β-glucosidase</td>
</tr>
<tr>
<td>0.25%</td>
<td>27.8</td>
</tr>
<tr>
<td>0.5%</td>
<td>41.7</td>
</tr>
<tr>
<td>1.0%</td>
<td>25.1</td>
</tr>
<tr>
<td>2.5%</td>
<td>11.4</td>
</tr>
<tr>
<td>straw (treated)</td>
<td>β-glucosidase</td>
</tr>
<tr>
<td>0.25%</td>
<td>35.3</td>
</tr>
<tr>
<td>0.5%</td>
<td>33.3</td>
</tr>
<tr>
<td>1.0%</td>
<td>20.9</td>
</tr>
<tr>
<td>2.5%</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Generally, highest cellulolytic and xylanolytic activities were obtained using concentrations of 0.5% and 0.25% of untreated and treated straw, respectively, with the exception of β-xylosidase which produced comparable activities for the two treatments. Of the two substrates, untreated straw-grown cultures gave the highest enzyme activities. In view of these results the following parameters were

$^1$ Values are averages of duplicate assays
chosen for the investigation of the hydrolytic activity of the twelve strains of fungi:
- 0.5% untreated hammer-milled oat straw grown statically.

### 4.3.2 Extracellular production of some enzymes involved in degrading oat straw by 12 strains of anaerobic fungi

The performance of twelve strains of fungi grown in straw as measured by CM-cellulase, xylanase, β-glucosidase, β-xylosidase and cellobiase activity in culture liquors are shown in Table 4.4.

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Enzyme production (mIU/mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CM-cellulase</td>
<td>xylanase</td>
</tr>
<tr>
<td><strong>Neocallimastix sp.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLS3</td>
<td>1049.5 ± 107.1</td>
<td>10950.5 ± 246.8</td>
</tr>
<tr>
<td>ELX1</td>
<td>754.5 ± 34.6</td>
<td>8520.0 ± 231.9</td>
</tr>
<tr>
<td>LS11</td>
<td>736.0 ± 108.5</td>
<td>8250.5 ± 236.9</td>
</tr>
<tr>
<td>DLX2</td>
<td>689.5 ± 13.8</td>
<td>8800.5 ± 1668.7</td>
</tr>
<tr>
<td><strong>Piromyces sp.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSX1</td>
<td>792.0 ± 26.9</td>
<td>5840.0 ± 226.5</td>
</tr>
<tr>
<td>KSF2</td>
<td>782.0 ± 43.5</td>
<td>3540.0 ± 710.6</td>
</tr>
<tr>
<td>RSX1</td>
<td>651.5 ± 10.6</td>
<td>2870.0 ± 134.4</td>
</tr>
<tr>
<td>SS2</td>
<td>559.0 ± 102.5</td>
<td>4630.5 ± 901.6</td>
</tr>
<tr>
<td>LS4</td>
<td>437.0 ± 26.9</td>
<td>4630.0 ± 0.0</td>
</tr>
<tr>
<td>SN2</td>
<td>506.0 ± 21.2</td>
<td>2960.5 ± 6.4</td>
</tr>
<tr>
<td><strong>Orpinomyces sp.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBR1</td>
<td>255.0 ± 19.1</td>
<td>2590.0 ± 0.0</td>
</tr>
<tr>
<td>BCR1</td>
<td>244.5 ± 3.2</td>
<td>2270.5 ± 28.3</td>
</tr>
</tbody>
</table>

*Maximum extracellular enzyme production in dialysed culture fluids of 12 selected anaerobic fungi grown in 0.5% hammer-milled oat straw for 7 days. Each value represents the mean ± SD for duplicates to indicate the extent of data spread.*
The purpose Table 4.4 is to rank the isolates, which show large differences in enzyme activity, rather than to show statistical difference between different isolates. *Neocali\textit{m}ast\textit{i}x* strains produced more xylanase and β-xylosidase while equivalent amounts of the enzymes CM-cellulase, β-glucosidase and cellobiose were produced by both *Neocali\textit{m}ast\textit{i}x* and *Pi\textit{r}omy\textit{c}es* strains. Substantially lower amounts of all four enzymes were produced by the two *Or\textit{p}i\textit{n}om\textit{y}\textit{c}es* strains when compared to *Neocali\textit{m}ast\textit{i}x* and *Pi\textit{r}omy\textit{c}es* strains. The maximum levels of CM-cellulase, xylanase and β-xylosidase was considerably higher for *Neocali\textit{m}ast\textit{i}x* sp. DLS3 while *Pi\textit{r}omy\textit{c}es* sp. KSX1 produced the highest β-glucosidase and cellobiose activities.

Time courses for extracellular enzyme production of β-glucosidase, β-xylosidase, CM-cellulase and xylanase in culture liquors of *Neocali\textit{m}ast\textit{i}x* sp. DLS3 and *Or\textit{p}i\textit{n}om\textit{y}\textit{c}es* sp. WBR1 grown on hammer-milled oat straw (0.5%) are shown in Figure 4.1 (a) and (b). These figures illustrate the substantial differences in enzyme activities that exist between the monocentric strain, DLS3 and the polycentric strain, WBR1.

The relative performance of the twelve fungal isolates in three different screening methods is given in Table 4.5. This table ranks the strains in descending order according to the enzymatic performance as calculated from the maximum extracellular enzyme activities found in Table 4.4. There was no distinction between *Neocali\textit{m}ast\textit{i}x* and *Pi\textit{r}omy\textit{c}es* species in relation to enzymatic performance. The hydrolytic potential of the two genera are similar and enzyme activities produced by the monocentric isolates are substantially greater than those recorded by the polycentric isolates belonging to the genus *Or\textit{p}i\textit{n}om\textit{y}\textit{c}es*.

Close correlation exists between the two quantitative screens for the monocentric strains, that is, hydrolytic performance and 14C-cellulose solubilisation. However, a slight deviation does appear between the three *Pi\textit{r}omy\textit{c}es* strains KSX1, KSF2 and SN2. Apart from two *Neocali\textit{m}ast\textit{i}x* strains, LS11 and LS4, the remaining
monocentric strains bear quite close similarity between the semi-quantitative and quantitative screens.

Table 4.5 shows how the ranks deviate from each other and from this a rank correlation can be calculated. The coefficient \( r_{s} \) is Spearman's coefficient of rank correlation (Bishop, 1983). It ranges in value from +1, when there is complete agreement between two sets of ranks, to –1, when there is complete disagreement, that is when the ranking of one group is in exactly the opposite order. With reference to Table 4.5 the sum of the squared deviations is equal to 72 which gives an \( r_{s} \) value of 0.75. From this \( r_{s} \) value it can be stated that the agreement or correlation between enzyme activity rank and \(^{14}\)C-solubilisation rank is statistically significant.
Figure 4.1  Extracellular enzyme production in culture liquors of Neocallimastix sp. DLS3 (●, ○), and Orpinomyces sp. WBR1 (■, □) grown in 0.5% hammer-milled oat straw. (a) β-glucosidase and β-xylosidase, (b) CM-cellulase and xylanase
Table 4.5  
Relative ranking of twelve fungal isolates: Enzyme activity ranks vs $^{14}$C solubilisation ranks (Spearman’s rank correlation $r_s$)$^3$

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fungal Isolate</th>
<th>Genus</th>
<th>Source</th>
<th>Enzyme activity (x)</th>
<th>$^{14}$C solubilisation (y)</th>
<th>C-A$^1$</th>
<th>Deviation ($d$)</th>
<th>Deviation squared ($d^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS3</td>
<td>Neocallimastix</td>
<td>deer</td>
<td></td>
<td>1</td>
<td>1</td>
<td>2 (5)$^2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KSX1</td>
<td>Piromyces</td>
<td>kangaroo</td>
<td></td>
<td>2</td>
<td>6</td>
<td>2 (7)</td>
<td>-4</td>
<td>16</td>
</tr>
<tr>
<td>ELX1</td>
<td>Neocallimastix</td>
<td>eland</td>
<td></td>
<td>3</td>
<td>2</td>
<td>3 (10)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>KSF2</td>
<td>Piromyces</td>
<td>kangaroo</td>
<td></td>
<td>4</td>
<td>8</td>
<td>2 (6)</td>
<td>-4</td>
<td>16</td>
</tr>
<tr>
<td>LS11</td>
<td>Neocallimastix</td>
<td>sheep</td>
<td></td>
<td>5</td>
<td>3</td>
<td>1 (5)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>DLX2</td>
<td>Neocallimastix</td>
<td>deer</td>
<td></td>
<td>6</td>
<td>4</td>
<td>2 (5)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>RSX1</td>
<td>Piromyces</td>
<td>rhinoceros</td>
<td></td>
<td>7</td>
<td>7</td>
<td>1 (4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SS2</td>
<td>Piromyces</td>
<td>cattle</td>
<td></td>
<td>8</td>
<td>10</td>
<td>1 (3)</td>
<td>-2</td>
<td>4</td>
</tr>
<tr>
<td>LS4</td>
<td>Neocallimastix</td>
<td>sheep</td>
<td></td>
<td>9</td>
<td>9</td>
<td>2 (6)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SN2</td>
<td>Piromyces</td>
<td>cattle</td>
<td></td>
<td>10</td>
<td>5</td>
<td>1 (3)</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>WBR1</td>
<td>Orpinomyces</td>
<td>water buffalo</td>
<td></td>
<td>11</td>
<td>12</td>
<td>3 (11)</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>BCR1</td>
<td>Orpinomyces</td>
<td>banteng cattle</td>
<td></td>
<td>12</td>
<td>11</td>
<td>1 (5)</td>
<td>-1</td>
<td>1</td>
</tr>
</tbody>
</table>

$\sum d^2 = 72$

---

$^1$ Rank calculated as maximum rate of colour release

$^2$ Number in brackets represent actual score at day 8 of incubation

$^3$ $r_s$ calculated as $r_s = 1 - \frac{6 \sum d^2}{n(n^2 - 1)} = 1 - \frac{6 \times 72}{12 \times 143} = 0.75$
4.4 Discussion

This chapter documents the quantification of several enzymes produced by anaerobic fungi isolated from ruminant and non-ruminant herbivores and was performed as part of the screening programme to select for anaerobic fungi that have the ability to produce high levels of hydrolytic enzymes. Monocentric fungi were found to produce substantially larger amounts of enzyme than polycentric fungi, however, the number of polycentric strains under investigation was not large. Highest enzyme yields were recorded by cultures that were incubated statically which were grown in oat straw at a concentration of 0.5% in the medium.

All twelve strains isolated from ruminant and non-ruminant herbivores produced a number of enzymes that allowed them to hydrolyse straw (Table 4.4). The extracellular activities of β-glucosidase, CM-cellulase, cellobiase, β-xyllosidase and xylanase reached maximum levels in 5- to 7-day-old cultures in all twelve fungal strains. This is similar to the results obtained with Neocallimastix sp. LM1 and Piromyces sp. SM1 (Gordon and Phillips, 1989), Neocallimastix sp. R1 cellulase and xylanase (Lowe et al., 1987b) and N. frontalis cellulases (Mountfort and Asher, 1985; Pearce and Bauchop, 1985). CM-cellulase and xylanase activities are higher in straw-grown Neocallimastix strains in this study compared to Neocallimastix sp. LM1 (Gordon and Phillips, 1989) and Neocallimastix sp. R1 (Lowe et al., 1987b). Both β-glucosidase and β-xyllosidase activities are similar for Neocallimastix strains in this study and LM1, while cellobiase activities are similar for straw-grown Neocallimastix sp. R1. CM-cellulase, xylanase and β-glucosidase activities of Piromyces strains in this study are similar for the straw-grown Piromyces sp. SM1 (Gordon and Phillips, 1989), however, β-xyllosidase activities are higher in SM1 cultures. Neocallimastix strains N1 and N2 and Piromyces strain R1 grown in straw (Teunissen et al., 1993) gave comparable xylanase activities to those exhibited in this study while CM-cellulase produced by N1, N2 and R1 were lower. Piromyces strains in this study produced higher
β-glucosidase levels compared to R1 while N1 and N2 produced higher levels of β-glucosidase than *Neocallimastix* isolates in this study.

Borneman *et al.* (1989) investigated plant cell wall degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi grown on Coastal Bermuda Grass leaf blades. While the cellulase and xylanase yields of the monocentric isolates were comparable to those in this present study, the component extracellular enzyme levels of the polycentric strains were more varied. CM-cellulase activities of strains WBR1 and BCR1 were comparable to those in that particular study while xylanase activities were higher and both β-glucosidase and β-xylosidase activities were lower. In general, comparisons with published studies have been difficult to make because of the different experimental conditions employed. These comparisons also highlight the differences that can occur in enzyme complexes when using different substrates.

Polycentric and monocentric fungi found in the rumen are closely related and possess many common characteristics for carbohydrate utilisation and fermentation end-productions (Phillips and Gordon, 1995b), however, this present study has shown there are substantial differences in their capacity to produce fibre-degrading enzymes. The ability of the polycentric fungus *Orpinomyces* sp. WBR1 to produce active cellulolytic and xylanolytic enzymes on a number of different substrates was shown to be much lower in comparison to the activities recorded for the monocentric fungus, *Neocallimastix* sp. DLS3. The inability of the polycentric strain to produce enzyme activity comparable to that of the monocentric strain KSX1 may lie in the growth characteristics of these two morphologically different strains. Results show that some polycentric fungi can very rapidly degrade fibre (Gordon, 1990). The penetration of the rhizomycelium of polycentric fungi into lignified cell walls may provide an advantage in breaking down recalcitrant lignocelluloses (Akin and Rigsby, 1987). However, monocentric isolates have a broader pH range of enzymatic activities and a higher rate of extracellular enzyme production.
The inconsistent production of zoospores by polycentric fungi (Phillips, 1989; Borneman et al., 1989; Ho and Bauchop, 1991) may also explain the low levels of enzyme activity when compared to those obtained by the monocentric isolates. Borneman et al. (1989) suggested that if the fungi were to colonise plant material by extension of a rhizomycelium alone, then slower colonisation of fibre might occur than would be the case if zoospores were released, resulting in increased sites of attachment and therefore increased enzyme activities.

The semi-quantitative screen using cellulose-azure does not show any real correlation with the two quantitative screens when viewing the results of the two polycentric strains WBR1 and BCR1. However, a consideration must be made in that the $^{14}$C solubilisation rank is a reflection of the performance out of a total of 46 isolates. In this sense, WBR1 and BCR1 can degrade and solubilise cellulose equally well, but are unable to produce extracellular enzymes to the same capacity. It is important to note that this screening method selects those fungi which have the ability to produce extracellular enzymes. It is possible that polycentrics may not produce a great proportion of extracellular enzyme activity and much of the activity may be cell-associated. The variability in growth characteristics of monocentric and polycentric fungi may explain in part the discrepancies that occur between the qualitative and quantitative screens for the polycentric isolates. The ability of polycentric fungi to degrade cellulose-azure and solubilise radio-labelled cellulose is superior to its ability to produce extracellular enzymes. The different scores which occur between hydrolytic activity and the other two screens amongst the two polycentric strains is a consequence of their growth characteristics and suggests that growth characteristics are an important factor in biomass conversion by anaerobic fungi.

The effects of different growth substrates on enzyme activity were examined using cellulosic materials as it has been shown that soluble sugars are less effective inducers of cellulase than cellulose (Mountfort and Asher, 1985; Williams and Orpin, 1987a; 1987b). Oat straw was the best inducer for cellulosytic and xylanolytic enzymes for both DLS3 and WBR1. Gordon and Phillips (1989) also
obtained high enzyme activities of the monocentric isolates *Neocallimastix* sp. LM1, *Piromyces* sp. SM1 and *Caeomycyes* sp. NM1 grown in straw. Filter paper proved a good inducer of cellulytic activity, although this substrate had a slightly smaller effect on xylanolytic activity. To demonstrate the effectiveness of a complex substrate as an inducer of fibrolytic enzymes, newsprint was included as a growth substrate. Enzyme activity produced by DLS3 and WBR1 was significantly reduced when grown in this substrate suggesting that newsprint was not a good substrate for the production of high levels of cellulolytic and xylanolytic enzymes.

The effect of chemical treatment of straw and newsprint on enzyme activity gave similar results for both DLS3 and WBR1. Untreated oat straw-grown cultures produced higher enzyme activities than treated oat straw. This contrasts with the results obtained using newsprint as the growth substrate. In this case, treated newsprint significantly increased the production of enzymes by DLS3 while WBR1 only produced enzymes whilst being cultured on this substrate. It would appear that straw, in its native state, is a good inducer of fibre-degrading enzymes whilst newsprint requires a degree of pre-treatment in order to make the substrate accessible to the organism.

There was no substantial increase in the activity of cellulolytic and xylanolytic enzymes in shaken cultures of DLS3 and WBR1. In most cases the enzyme activity was lower compared to the corresponding static culture. It is possible that shaking the culture limited the ability of the organism to attach to the cellulosic particles which may affect the production of enzymes by the shaken culture. Mountfort and Asher (1985) studied the effects of shaking on CM-cellulase activity using two *Neocallimastix frontalis* fungi grown in various concentrations of filter paper cellulose. The yield of CM-cellulase in this study was improved by shaking in the case of only one strain.

Correlation between the two sets of data provided by enzyme activity and $^{14}$C solubilisation ranks was shown to be statistically significant (refer to Table 4.5).
The secondary screening methods of this chapter and the previous have demonstrated the differences in cellulolytic capability that can occur between species indicating that some isolates of the same genus hold more potential for commercial application than others. The following chapters report on the subsequent immobilisation of a select number of these anaerobic fungi with a view toward enhancing productivity through the re-use of cells during extended repeat-batch fermentation.
CHAPTER FIVE

Preparation of suitable propagules and the development of a procedure for the immobilisation of anaerobic fungi in calcium alginate beads

5.1 Introduction

Two activities have been identified as important in developing a microbial product. The first activity is the selection of a suitable microorganism followed by the development of a production process to utilise the selected microorganism(s). The screening process in this study was performed in an effort to obtain a pool of cultures with known fibre-degrading capabilities. The previous chapters have reported on a combination of qualitative and quantitative screening methods and have resulted in the selection of twelve strains of anaerobic fungi which demonstrate the ability to degrade cellulose-azure, solubilise radioactively labelled bacterial cellulose and to possess a number of active cellulolytic and xylanolytic enzymes. From this group, isolates can then be selected for further study on the basis of their suitability toward immobilisation. This chapter and those that follow describe the development of a procedure for the immobilisation of anaerobic fungi in calcium alginate and report on the application of immobilised anaerobic fungi in the production of cellulase.

A major issue in the development of an immobilisation procedure is the production of a suitable inoculum. Unlike yeast and bacteria it is very difficult to homogeneously immobilise living fungal mycelia although it has been attempted (Kopp and Rehm, 1983). Homogenisation can result in mechanical damage to cells and microbial contamination can increase during homogenisation. In view of this, entrapment of fungal spores and subsequent cultivation of the entrapped
spores into mycelia have been attempted (Tamada et al., 1986; Miranda and D'Souza, 1988).

Monocentric fungi readily produce zoospores in culture. Zoospores would therefore be the propagule of choice when attempting the immobilisation of monocentric fungi. On the other hand, polycentrics have indeterminate life cycles and are not dependent upon the formation of zoospores for their continued survival. Zoospores are infrequently produced by polycentrics or zoosporogenesis can be absent (Phillips, 1989), although a procedure for the induction of zoosporogenesis and release of zoospores in cultures has been developed (Ho and Bauchop, 1991).

The objectives of this present study were two fold. Firstly, the suitability of different propagules for the immobilisation of both monocentric and polycentric fungi was examined. This included investigations into the induction of zoosporogenesis in a number of polycentric fungi in an effort to release zoospores for use as propagules in immobilisation. The second objective was to optimise the immobilisation conditions in order to maximise fungal biomass within the alginate matrix while at the same time confining fungal growth to the bead. The potential of the cells to recover from stress arising from the immobilisation procedure is likely to be increased if the cells are suspended in nutrient or are in contact with complex nutrients. For example, algal cells have been entrapped in alginate which was dissolved in growth medium (Shi et al., 1987), while Leo (1990) found improved biomass distribution of P. chrysogenum when complex nutrients were incorporated into the bead. Therefore, one of the first modifications in the immobilisation procedure was the use of growth medium in the preparation of alginate. Other culture parameters that were altered during the immobilisation procedure in this present study included the use of basal medium M10 during the washing procedure and performing the beading procedure at 39°C. These modifications were performed with the aim of making the immobilisation conditions mild thereby reducing the severity of the entrapment process and increasing culture viability.
5.2 Materials and methods

5.2.1 Organisms

Two morphologically different anaerobic fungi were chosen to assess the amenability of the organisms toward immobilisation, namely *Piromyces* sp. KSX1 (a monocentric), isolated from a red kangaroo and a polycentric cattle isolate, *Orpinomyces* sp. 478P1. Strain 478P1 was isolated from digesta obtained from a fistulated cow (breed unknown) housed at C.S.I.R.O. Division of Tropical Crops and Pastures, Samford (Queensland, Australia).

Since the number of zoospores used as inoculum influences the success of growth in an immobilised matrix it was essential to choose a strain of monocentric fungus which produced zoospores in large numbers. Preliminary zoospore count studies revealed that out of the two top ranking monocentric isolates *Piromyces* sp. KSX1 produced zoospores in larger numbers than *Neocallichasmix* sp. DLS3. Generally, strain KSX1 produced zoospores in numbers that were two orders of magnitude higher than strain DLS3. Therefore, KSX1 was chosen in all subsequent immobilisation experiments.

Of the polycentric fungi *Orpinomyces* sp. WBR1 was initially selected for immobilisation studies. A procedure for the induction of zoosporogenesis and release of zoospores in this culture was attempted without success. Consequently, partially homogenised rhizomycelia of WBR1 was used as the propagules for immobilisation. Poor colonisation of the alginate beads resulted which was possibly due to the loss of viability of this culture during the homogenisation and gelation procedures. A programme investigating the possible release of zoospores of three existing polycentric fungi *Orpinomyces* sp. 141T1, 622T7 and 478P1 from the laboratory collection led to the selection of strain 478P1. Although failing to produce zoospores from strain 478P1 it was possible to transfer a viable inoculum using partially homogenised rhizomycelia to produce alginate beads
containing the growing fungus. Therefore, 478P1 was chosen in all subsequent immobilisation experiments involving polycentric fungi.

5.2.2 Preparation of propagules of anaerobic fungi for immobilisation

5.2.2.1 Preparation of zoospores of *Piromyces* sp. KSX1 for immobilisation

Inocula for immobilisation studies using *Piromyces* sp. KSX1 were grown in serum bottles containing basal medium M10 with the addition of 0.25% cellobiose. The cellobiose medium (25 mL) was inoculated using 0.25 mL from an actively growing fungus cultured on M10X medium and was grown for 24 hours at 39°C. At the time of harvest the zoospore count was approximately 10^5 zoospores per mL. Direct zoospore counts were obtained using a Hawksley Counting Chamber (Hawksley Ltd., Lansing, England) under phase contrast microscopy.

5.2.2.2 Induction of zoosporogenensis and release of zoospores in *Orpinomyces* sp. WBR1, 141T1, 622T7 and 478P1

A procedure for the release of zoospores in these fungi was performed based on the method of Ho and Bauchop (1991). All polycentric fungi were maintained on Cellulose Sloppy Medium (CSM) which contained 0.2% ball-milled cellulose and 0.1% agar (w/v) in basal medium M10. Induction media consisted of either Glucose Sloppy Medium (GSM) which contained 0.3% glucose and 0.1% agar in M10 or Cellobiose Broth (CELB) which contained 0.3% cellobiose in M10. Both GSM and CELB were prepared with 10% clarified rumen fluid. All media were prepared anaerobically in Hungate tubes (7.0 mL/tube) fitted with black butyl rubber septa and screw caps and autoclaved at 110°C for 25 minutes. For the induction of zoosporogenensis a 5% volume of a 4 to 5 day old culture was inoculated into GSM or CELB media and incubated at 39°C statically. The culture was shaken vigorously by hand for 20 to 30 seconds daily and monitored every 24 hours for sporangial development using a dissecting microscope. After 3
to 4 days growth, pieces of rhizomycelial mats producing sporangia were selected using a sterile syringe and transferred to fresh medium (either GSM or CELB). The culture was re-incubated for 30 to 40 minutes after which time the release of zoospores was examined using a light microscope. The polycentric fungi failed to release zoospores after this time.

Of the four polycentric under investigation, strain 478P1 appeared the most promising in regard to culture viability. Sporangia were induced in this strain, however, they failed to differentiate to form zoospores. A method for the preparation of a rhizomycelial suspension of this fungus was therefore devised.

5.2.2.3 Preparation of rhizomycelium of *Orpinomyces* sp. 478P1 for immobilisation

*Orpinomyces* sp. 478P1 was maintained on basal medium M10 broth (7 mL) in a Hungate tube containing ten to twenty 1 cm pieces of sisal (*Agave* sp.) fibre and leaf blade sections of couch-grass (*Cynodon dactylon*) as modified from Wubah *et al.* (1991a). A piece of sisal fibre containing the active growing fungus was transferred to fresh medium every 4-5 days. This method of inoculation was necessary since zoospore production was not a reliable characteristic with polycentric isolates.

Inocula for immobilisation studies using strain 478P1 were grown in 100 mL serum bottles containing basal medium M10 with the addition of 0.25% cellobiose. The cellobiose medium (25 mL) was inoculated using a piece of sisal fibre containing the active growing fungus and was grown for 5 days at 39°C. Throughout the incubation period the serum bottle, which contained 30 to 40 sterile glass beads (3 mm in diameter), was shaken by hand vigorously for approximately 1 minute every day. This was performed to prevent the formation of a rhizomycelial mat during incubation and resulted in a partially homogenised rhizomycelial suspension which was used as inoculum for all subsequent immobilisation experiments involving strain 478P1.
5.2.3 Materials for immobilisation

Sodium alginate (Manugel GMB; gift from Kelco A.I.L., Melbourne, Australia) was prepared as a 3% solution dissolved in basal medium M10 containing 0.25% glucose. The mixture was thoroughly homogenised with a hand blender (Braun) and dispensed into screw-cap Hungate tubes (4 mL) in an anaerobic chamber using a 5 mL syringe. The tubes containing the alginate/basal medium mixture was left to reduce in an anaerobic chamber before being sealed with black butyl rubber septa and screw caps and then autoclaved at 115°C for 30 minutes. An anaerobic solution of 0.1 M CaCl$_2$ (30 mL) was prepared under N$_2$ gas in 100 mL serum bottles and autoclaved at 121°C for 15 minutes. In immobilisation media containing glucose or cellobiose, the sugars were separately sterilised at 110°C for 20 minutes in concentrated solutions under a N$_2$ atmosphere and were added aseptically to basal medium M10 by syringe.

5.2.4 Immobilisation procedure

A number of modifications were performed on the immobilisation procedure and included:

(i) The addition of growth medium in Na-alginate;
(ii) The use of basal medium M10 during the washing of beads;
(iii) The incubation of serum bottles containing beads at 39°C;
(iv) Shaking the immobilised culture (polycentric strain only).

The success of each variation was assessed by photography after 24 hours incubation for the immobilised monocentric strain. The combination of four modifications gave rise to beads which were visually more colonised in which case they were incorporated into the general immobilisation procedure. The protocol for the immobilisation of monocentric and polycentric anaerobic fungi is given in Figure 5.1.
Rhizomycelial suspension
strain 478P1 (1mL) or

Zoospore suspension
(10^5 zoospores/mL)
strain KSX1 (1mL)

Vortex inoculum/Na-alginate suspension

Inoculum (1 mL)
Na-alginate (4 mL)

21 gauge needle

Immobilised cells
(~ 150 beads)

30 mL 0.1M CaCl₂

Wash immobilised cells *in situ* with 25 mL basal medium (twice)

Add 25 mL growth medium and incubate at 39°C statically for 24 hours then with shaking for a further 5 days for polycentrics

**Figure 5.1** The experimental procedure for the immobilisation of monocentric and polycentric anaerobic fungi in calcium alginate
5.2.4.1 Immobilisation of monocentric fungi

A 24 hour old 1 mL suspension of strain KSX1 containing approximately $10^5$ zoospores was aseptically transferred to 4 mL of alginate/medium mixture via a sterile 1 mL syringe. The inoculum and alginate/medium mixture was thoroughly mixed using a vortex and then withdrawn aseptically using a sterile 5 mL syringe fitted with an 18 gauge needle. This suspension was then added, dropwise, to a sterile anaerobic CaCl$_2$ solution (0.1 M) through the same syringe fitted with a 21 gauge needle. Each drop formed a spherical bead (diameter ~ 2.5-3.0 mm) after contacting the electrolyte solution and each 5 mL of alginate-zoospore suspension yielded approximately 150 beads. The ions necessary for ionotropic gelation can be toxic under prolonged conditions. Therefore, to minimise any possible deleterious effects, the immobilised cells were cured in calcium chloride for the minimum time allowable. Smidsrød and Skjåk-Bræk (1990) suggest curing times of 5 minutes to 30 minutes depending on the bead diameter. For the purpose of this present study, ten minutes resulted in beads that were sufficiently hardened. After this time, the CaCl$_2$ solution was removed using a sterile 50 mL syringe and the beads were washed twice with the addition of 25 mL of basal medium M10. During the washing procedure the serum bottles containing the beads were held in a 39°C water bath. Twenty five millilitres of growth medium (basal medium M10 with the addition of 0.25% glucose) was transferred and the immobilised fungus was incubated statically at 39°C for 24 hours. Addition and removal of medium was performed using sterile 50 mL syringes.

5.2.4.2 Immobilisation of polycentric fungi

Immobilisation of the polycentric fungus was similar to that previously described for the monocentric isolate. A 5 day old 1 mL suspension of partially homogenised rhizomycelium of strain 478P1 was used to inoculate the alginate/medium mixture. Beading and washing procedures were performed and growth medium consisting of basal medium M10 and 0.25% cellobiose was added. The immobilised fungus was initially incubated statically at 39°C for 24 hours before
being transferred to a reciprocating water bath (Paton Industries, South Australia) and shaken at 80 oscillations per minute. Shaking incubation was performed at 39°C for 5 days.
5.3 Results

The growth of anaerobic fungi in calcium alginate beads was affected by a number of factors:

(i) the condition of the inoculum. In the case of the monocentric isolate, the number of zoospores at the time of encapsulation was an important factor;
(ii) the addition of growth medium in the Na-alginate;
(iii) the use of basal medium to wash the beads rather than water;
(iv) temperature at which the immobilisation procedure was performed;
(v) shaking incubation in the case of the polycentric strain

These modifications were performed to reduce the severity of the immobilisation procedure and therefore increase the success of colonisation of the alginate bead by anaerobic fungi. Shaking the polycentric strain was carried out in order to confine the rhizomycelial growth to the bead.

5.3.1 Influence of immobilisation techniques on the growth of P. piromyces sp. KSX1

Preliminary immobilisation of strain KSX1 was performed in a 3% aqueous solution of Na-alginate. The beading procedure was performed at room temperature and water was used to wash the beads before growth medium (basal medium M10 containing 0.25% glucose) was added. It was obvious that the number of zoospores germinating into rhizomycelia was low since colonisation of the bead by the fungus was localised in a small number of mycelial aggregations (Figure 5.2a). The addition of growth medium in the inoculum/alginate mixture resulted in beads which were more evenly colonised by biomass (Figure 5.2b). The use of basal medium M10 instead of water for bead washing and performing the immobilisation procedure at 39°C resulted in further improvement in the degree of colonisation of the fungus (Figure 5.2c).
Figure 5.2  Influence of immobilisation techniques on the growth of Piromyces sp. KSX1

(a) 3% aqueous Na-alginate preparation
(b) 3% Na-alginate dissolved in basal medium M10 containing 0.25% glucose
(c) 3% Na-alginate dissolved in basal medium M10 containing 0.25% glucose; washes were performed in basal medium and cultures were held at 39°C during the washing procedure.

The beads were photographed 24 hours after incubation. Bars = 3 mm
5.3.2 Induction of zoosporogenesis and release of zoospores in *Oripinomyces* sp. 478P1

Although the use of GSM and CELB did induce the production of sporangia in strain 478P1, they failed to differentiate to form zoospores. Ho and Bauchop (1991) observed a similar result, rarely observing zoospores. For this reason, partially homogenised rhizomycelium was used as propagules for immobilisation in Ca-alginate.

5.3.3 Influence of immobilisation techniques on the growth of *Oripinomyces* sp. 478P1

In static incubations of strain 478P1 rhizomycelial growth was first visible after 48 hours with the rhizomycelial filaments extending throughout the solid matrix. Static incubation resulted in mycelial outgrowth forming a conglomerate mass containing a number of beads joined by rhizomycelium (Figure 5.3a). Shaking incubation resulted in beads that did not aggregate (Figure 5.3b and c). However, rhizomycelial growth was unable to be totally confined to within the alginate bead as can be viewed by the difference in bead size (Figure 5.3c). Unlike the monocentric strain which colonised all of the beads, approximately 30% of beads were colonised by strain 478P1. An increase of inoculum size from 20% to 40% did not increase the percentage of beads colonised by the fungus.
Figure 5.3  
*Influence of immobilisation techniques on the growth of Orpinomyces sp. 478P1. Effect of shaking the cultures*

(a) Immobilised Orpinomyces sp. 478P1 from a static culture forming aggregates of beads joined by rhizomycelia. Bar = 2 mm
(b) and (c) Immobilised Orpinomyces sp. 478P1 from shaken cultures. The beads were photographed 5 days after incubation. Bars = 3 mm
5.4 Discussion

This chapter describes the development of a procedure for the immobilisation of anaerobic fungi and has shown that strains belonging to the genera *Piromyces* and *Orpinomyces* can be effectively cultured in Ca-alginate under optimal conditions.

Selection of anaerobic fungi for immobilisation was based on their amenability toward the procedure. Consequently, the highest ranked monocentric strain obtained from the screening procedure was not chosen. Studies into the selection of suitable polycentric strains utilised four strains of *Orpinomyces* sp. that included three additional strains that were not part of the original isolation programme of this project. Polycentric fungi were isolated in low numbers in this study and by increasing the number of polycentric fungi under investigation the chance of successfully immobilising these type of fungi may be maximised.

Zoospores were suitable propagules for the immobilisation of *Piromyces* sp. KSX1. The viability of strain KSX1 zoospores entrapped in Ca-alginate was apparently not affected by the immobilisation procedure developed in this study with immobilisation of this monocentric strain recording 100% colonisation of the beads. Although polycentric strain 478P1 failed to produce zoospores it was possible to transfer a viable inoculum using partially homogenised rhizomycelia to produce alginate beads containing the growing fungus.

The monocentric isolate, KSX1, formed micro colonies inside the matrix and also at the periphery of the matrix. The life cycle of monocentric fungi consists of an alteration between a motile, zoosporic stage and a vegetative, zoosporangial stage and is a possible reason for the inability of strain KSX1 to invade the entire bead structure. The non-homogeneous distribution of biomass within the immobilisation matrix has been previously reported for fungi (Kopp and Rehm, 1984; Kautola *et al.*, 1985; El-Sayed and Rehm, 1986; Honecker *et al.*, 1989). From a process aspect there are several consequences of this non-homogeneity in biomass distribution. It leads to zonation of the microenvironment within the
bead which may modify intraparticle growth, metabolism, and product formation (Shi et al., 1987). Low concentrations of cells at the particle centre will generate significant unproductive regions and may influence particle density. The accumulation of cells at the surface may rupture the gel surface resulting in outgrowth (Tanaka and Irie, 1988).

In contrast to strain KSX1, the growing rhizomycelial filaments of the polycentric isolate, strain 478P1, were found to have the potential to colonise the entire matrix. The polycentric fungi in this sense are similar to aerobic filamentous fungi where growth occurs by the linear elongation of mycelial filaments. In static cultures immobilised 478P1 formed aggregates and it was found that outgrowth by this strain was arrested by shaking the culture. The manner in which shaking confines the fungus in the bead is probably twofold. Firstly, shaking the culture eliminates possible diffusion gradients, that is, the nutrients are readily available to the fungus which stops possible outgrowth. Secondly, the physical abrasion that occurs during shaking confines the fungus to the bead. The rhizomycelial filaments that are broken off are too small to be viable and thus fail to grow in the culture medium.

In early studies of inoculum production for immobilisation of strain 478P1 laboratory mixers with high shear screens were used to homogenise the rhizomycelium. However, the fungus did not survive this process as there was no visible fungal growth in the gel beads. A hand held blender was then used to disrupt the rhizomycelial mat and gave the same results as the laboratory mixer. There are two possible reasons for the inability to retain culture viability during homogenisation using these two methods. Firstly, the degree of homogenisation was too severe as the resulting rhizomycelial suspension was very finely cut. Secondly, it is possible that the inoculum may have become aerated during the homogenisation procedure due to the vigorous action of the mixing heads as was indicated by the medium changing colour. The method of manually shaking the serum bottle which contained the growing fungus and glass beads was a much milder form of disruption of the rhizomycelial mat. The mild disruption used in
this method resulted in rhizomycelial fragments that were a size such that they were able to survive and continue to grow throughout the alginate matrix. Furthermore, viability of the inoculum was retained since anaerobiosis was maintained throughout the procedure as homogenisation was performed within the serum bottle and transfer of the inoculum to the alginate was via syringe.

The modifications adopted in this study supported good growth in the immobilised matrix of this group of organisms. In developing an immobilisation procedure specific for the requirements of anaerobic fungi, the effect of differences in the growth habit of monocentric and polycentric fungi have been highlighted. While strain KSX1 had the ability to colonise 100% of the beads the biomass distribution of this strain was non-homogeneous and it was apparent that further additions of growth substrate were required for the biomass to fully occupy the gel matrix. This will be dealt with in the following chapter. The polycentric strain 478P1, on the other hand, while only colonising about 30% of the total beads in culture had an evenly distributed biomass that invaded the entire gel matrix. As long as some of the entrapped cells remain viable, the cells in the gel can grow to the desired density after entrapment.
CHAPTER SIX

Maximising the colonisation of alginate beads by *Piromyces* sp. KSX1 biomass using fed-batch techniques

6.1 Introduction

To maximise productivity, the ratio of mycelium to immobilising material in the ideal immobilised fungal aggregation should be high. In addition, the mycelium should be evenly distributed throughout the aggregate so that the total biomass can be maximised. The previous chapter described a procedure for the immobilisation of two strains of anaerobic fungi, namely *Piromyces* sp. KSX1 and *Orihominomyces* sp. 478P1. During the development of this procedure it was found that the two strains demonstrated differing capacities to grow within the alginate gel which was attributed chiefly to their differences in physical growth characteristics. In particular, the monocentric isolate strain KSX1, exhibited a non-homogenous distribution of biomass and was unable to fully occupy the gel matrix during a single batch incubation.

The objective of this study was to achieve increased colonisation of KSX1 biomass in alginate beads. This was attempted using a series of alternate repeat-batch and fed-batch culture techniques which resulted in the addition of growth substrate every 24 hours. A combination of information derived from light microscopy and scanning electron microscopy was employed to visualise the spatial distribution of immobilised cells. In addition, a quantitative measure of growth in the form of fungal cell wall chitin was used to determine whether maximum rhizomycelial growth in immobilised KSX1 cultures had been attained in the gel bead.
6.2 Materials and methods

6.2.1 Culture techniques for improving colonisation

To increase the colonisation of KSX1 biomass in alginate beads a culture establishment phase was devised (Figure 6.1). The cell immobilisation technique for this fungus was as described previously (Section 5.2.4.1). At 48 hours into the incubation, when the initial glucose in the medium was depleted, the spent culture liquor was removed. The beads were washed twice in situ with basal medium M10 and fresh growth medium containing 0.25% glucose was added. After 24 hours of re-incubation the glucose in the medium was consumed. At this point enough fresh glucose was added to reconstitute the initial concentration and the immobilised cell culture was re-incubated. After another 24 hour re-incubation the spent culture liquor was removed and fresh growth medium was added after the beads were washed. This combination of repeat-batch and fed-batch techniques resulted in the addition of growth substrate every 24 hours and was repeated until biomass in the gel bead reached a maximum as measured by cell wall chitin.

6.2.2 Sampling

During growth experiments, samples of growth medium (1.0 mL) were taken from the culture vessels at 24 hour intervals and assayed for substrate depletion. The samples were removed by sterile anaerobic procedures with 2.5 mL syringes and filtered using 0.2 μm syringe filters (Minisart, Sartorius Australia). For determination of cell wall chitin in the alginate bead the bottle contents were harvested at 48 hourly intervals.
1. Remove spent culture liquor from 2 day old immobilised KSX1 culture

2. Wash beads and add fresh growth medium

3. After 24 hours incubation add sterile concentrated glucose solution to reconstitute to initial concentration

4. Incubate for 24 hours

5. Remove spent culture liquor from 4 day old immobilised KSX1

6. Wash beads and add fresh growth medium. Repeat steps 1 to 6 until biomass is maximal in the gel bead

Figure 6.1 The experimental procedure for the culture establishment phase of immobilised KSX1
6.2.2.1 Glucose estimations

Glucose estimations were made in triplicate using a glucose oxidase-peroxidase diagnostic kit (Sigma chemicals). A 0.25 mL sample of appropriately diluted culture liquor was added to 2.5 mL aliquots of glucose-oxidase-peroxidase-chromogen solution. In the standard, 0.25 mL of a 0.0667 mg/mL glucose standard solution (prepared from a 1mg/mL glucose standard solution, Sigma Chemicals) was added instead of culture liquor. Following incubation at 37°C for 30 minutes the absorbance was determined at 450 nm in a Bausch and Lomb Spectronic 21 spectrophotometer.

6.2.2.2 Estimation of immobilised rhizomycelia

Fungal chitin was estimated as the total hexosamine content of cultures after acid hydrolysis as described by Gordon and Phillips (1989). In experiments involving biomass estimation, duplicate immobilised cultures of strain KSX1 were grown under identical conditions in 100 mL serum bottles containing 25 mL of growth medium. The entire contents of two bottles were harvested at each sample point. Each batch of beads (approximately 150 beads/bottle) were washed thoroughly in GDW and placed in 10% hexametaphosphate (BDH Chemicals Ltd., England) (100 mL) overnight to dissolve the alginate. The resulting free fungal mycelium was sedimented by centrifugation at 2000 x g (Beckman Instruments Inc., Palo Alto, CA) for 15 minutes at 0°C, and the pellets were washed twice with GDW. Duplicate samples from the fungal cultures were hydrolysed for 4 hours at 100°C with 6 mL of 6 N HCl contained in glass tubes sealed with Teflon-lined screw caps. The hydrolysate was cooled and allowed to settle before filtering through Whatman GF/C filter paper. Sub-samples of the hydrolysates (0.5-1.0 mL) were dried at 39°C under reduced pressure using a vacuum desiccator and NaOH trap (usually 18-24 hours). Hexosamine was measured colorimetrically with Erhlich reagent by using D-glucosamine hydrochloride as the standard (Chen and Johnson, 1983) in a Bausch and Lomb Spectronic 21 spectrophotometer. The chitin content
of the samples was calculated as the 1,4-anhydro-N-acetyl-2-deoxy-D-
glucopyranose equivalent.

6.2.3 Microscopic examination of immobilised rhizomycelium

6.2.3.1 Light microscopy

Colonisation of beads by rhizomycelium was assessed at various intervals throughout the growth phase using an Olympus BH2 phase contrast microscope. The beads were preserved in a solution of formal saline (10 g formalin in 90 g 10% saline).

6.2.3.2 Scanning electron microscopy

The surface of the immobilised fungal culture was also assessed by scanning electron microscopy (SEM) at various time intervals throughout the incubation. The beads were rinsed clean of growth medium with GDW and were pre-fixed in 2.5% (v/v) gluteraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 2 hours. The beads were then washed three times over 30 minutes in 0.1 M cacodylate buffer solution before being post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours. Dehydration of the sample was effected through an ethanol series from 30% through to 100%, then transferred to a critical point drier for 2 hours. The dry beads were mounted on an aluminium stub and gold coated in a Magnetron sputter coater. The beads were examined using a Joel (model 35C) scanning electron microscope.
6.3 Results

6.3.1 Spatial distribution of immobilised KSX1 cells as observed by light and scanning electron microscopy

To maximise the total biomass in the gel bead a number of substrate additions were performed during cultivation of immobilised KSX1. After 24 hours of incubation in growth medium, the zoospores of KSX1 in gel beads germinated and formed rhizomycelial mass which grew in localised areas in beads (see Figure 5.2 c). Figures 6.2 (a-d) show the appearance of the beads throughout various intervals of the incubation. The density of rhizomycelia in the interior and exterior of the beads is observed to increase as the beads were repeatedly transferred into fresh growth medium. Zoospores were observed under light microscopy in the growth medium at day 2 and fungal rhizomycelia in the medium was evident from day 3. Changes in the distribution of biomass of immobilised KSX1 on the surface of the gel beads were observed with increasing incubation time as illustrated by the scanning electron micrographs (Figures 6.3 a-d). By day 10 fungal rhizomycelia was produced to such an extent that the bead was overgrown and the surface could not be observed. Figures 6.4a and 6.4b are scanning electron micrographs which show an extensive network of rhizomycelium and the presence of a number of sporangia that were responsible for the release of zoospores into the culture medium.
Figure 6.2  Morphological development of immobilised KSX1 at different cultivation times. (a) beads before incubation; (b) beads after 2 days incubation; (c) beads after 8 days incubation; (d) beads after 10 days incubation. Bar = 3mm
Figure 6.3 Scanning electron micrographs of whole Ca-alginate beads containing growing Piromyces sp. KSX1

(a) bead after 2 days incubation; (b) bead after 4 days incubation; (c) bead after 8 days incubation; (d) bead after 10 days incubation. Bars = 100μm.

Note the presence of rhizomycelial growth near the gel surface (arrow) after 48 hours of incubation.
Figs 6.4a and b  Aspect of the surface of immobilised KSX1 with a high rhizomycelial density. The surface of the bead shows rhizomycelia (rh) and sporangia (sp) at day 10 of the incubation. Bars = 10 µm
6.3.2 Substrate depletion and fungal biomass during the culture establishment phase

Glucose was exhausted within 36 hours in the first batch and then every 24 hours after substrate addition (Figure 6.6). The pH of the culture medium dropped from 6.69 to 6.29 in the first batch. In subsequent batches the largest pH drop was from 6.54 to 5.71. The growth of immobilised KSX1 in glucose cultures was determined by measuring the amount of chitin produced in duplicate cultures. Maximum growth occurred after 8 days incubation, that is after 8 additions of growth substrate (Figure 6.7).

The fed-batch technique was shown to markedly improve bead colonisation when compared to beads which were kept in batch incubation with one substrate addition (Figure 6.5 a and b). Beads that remained incubated for 5 days with only one a single substrate addition did not further colonise the bead and appeared much the same as fed-batch beads at day 2 (compare Figures 6.2 b and 6.5 a). By inference, the amount of biomass as measured by chitin in a single batch bead culture is approximately equivalent to the amount of biomass in repeat-batch bead cultures at day 2. Therefore, with reference to Figure 6.7, the biomass of fed-batch beads at the end of the 10 day cultivation could possibly have increased as much as 3 times compared to beads in a single batch culture.

Figure 6.5 Comparison of biomass colonisation in (a) bead incubated with a single substrate addition and (b) fed-batch bead. Both beads were incubated for 5 days at 39°C statically.
Figure 6.6  Glucose and pH profiles of immobilised KSX1 grown in M10 containing 0.25% glucose

Figure 6.7  Cell wall chitin produced by immobilised KSX1 growing in sequential batch cultures of 0.25% glucose
6.4 Discussion

The method of alternate repeat-batch and fed-batch culture techniques adopted in this study was successful in improving the biomass colonisation of immobilised KSX1 cultures. However, the outer surface became completely covered by rhizomycelia by the end of the 10 day incubation period. Zoospores and rhizomycelia also appeared in the culture medium. Immobilised KSX1 cultures growing rapidly on the outer surface of the bead would release zoospores which then germinate to form free rhizomycelial growth in the culture medium. Coupled to this was the observation of sporangia associated with a network of rhizomycelia on the surface of these beads. These mature sporangia would conceivably release zoospores into the culture medium and germinate. This is further supported by Barbotin et al. (1990) who have shown that rapid growth near the gel surface induces a decrease in the rigidity and mechanical strength of the gel. Consequently, cavities near the surface rupture and release cells.

Although immobilised biomass of KSX1 increased as a consequence of further additions of substrate, the distribution of the biomass within the bead matrix was not homogeneous. It has been observed that the growth of cells is not uniform throughout the gel particle but often occurs in the surface region (Wada et al., 1980; Godia et al., 1987; Audet et al., 1988). Cells have been observed to grow within small cavities of the gel (Barbotin et al., 1990) while Jamuna and Ramakrishna (1990) showed that yeast cell growth mainly occurred in the periphery of the 4 mm diameter bead with some growth also evident within the core. Gosmann and Rehm (1986) observed the growth of Aspergillus niger near the surface of the bead and showed that after a second incubation of alginate beads, mycelia covered the outer bead surface as a fur-like coat. This non-ideal spatial distribution is exemplified by aggregation with mycelial growth on the surface and is a major problem that has to be overcome before immobilised fungi can find wide use in the fermentation industry (Kuek, 1986). Before attempting any investigation into the production of cellulolytic enzymes by immobilised anaerobic fungal cultures it is necessary to confine the growth of rhizomycelia to
the bead and minimise external biomass in the culture liquor. The ensuing investigation examines the effectiveness of various media manipulations in the control of free fungal rhizomyelia in the culture liquor of immobilised KSX1 cultures.
CHAPTER SEVEN

Reducing external biomass in immobilised *Piromyces* sp. KSX1 cultures through media manipulations

7.1 Introduction

Studies examining the growth and spatial distribution of immobilised KSX1 reported in the previous chapter showed that although increased colonisation of KSX1 in the bead was achieved, fungal growth was not entirely confined to within the alginate matrix. Growth of mycelia at the microbial aggregate-medium interface has proven to be a major problem to overcome for many fermentation operations involving immobilised growing cell systems and has been reported in a number of production systems (see Section 1.2.3.2). Kuek (1986) has reported a number of problems that would arise from mycelial growth at the aggregate-medium interface. First, hyphal fragments are easily broken off with possible subsequent growth into free mycelia. Second, free mycelia can cause clogging of outlets in continuous culture vessels. Further, the presence of mycelia at the interface increases culture viscosity. Finally, mycelial growth at the interface effectively decreases the density of the fungal aggregation, thus negating one of the advantages of immobilisation.

To avoid the overproduction of biomass, attempts have been made to manipulate immobilised cell metabolism into a state of 'metabolic uncoupling' by withholding selected nutrients or adding selective inhibitors with the aim of maintaining the rate of a desired reaction while reducing the growth rate. The most common method has been nutrient limitation, usually nitrogen or phosphate. An example of this strategy is the use of nitrogen-starved cells of *Clostridium acetobutylicum* (Reardon and Bailey, 1989). The limiting nutrients have
sometimes been supplied in small amounts on a semi-continuous basis (Förberg and Häggström, 1985). Lohmeyer et al. (1990) showed that an optimal phosphate supply allowed effective long-term cultivation and alkaloid production using immobilised *Claviceps purpurea*. On the other hand, Somerville et al. (1977) observed decreases in benzene production when using nutrient limited immobilised bacterial cells. Eikmeier et al. (1984) reported more uniform distribution of biomass and reduced outgrowth under nutrient limiting conditions. In Eikmeier's study growth of *A. niger* was peripheral using 0.1-0.2 g/L NH₄NO₃ but more uniform and sparse with 0.05 g/L. Similarly, for *A. niger* in calcium alginate phosphate levels of 0.075-0.01 g/L KH₂PO₄ caused a shift to peripheral growth which was more pronounced at 0.125-0.15 g/L (Honecker et al., 1989). In view of these findings, competing external biomass may be potentially minimised in immobilised KSX1 cultures by lowering the carbon substrate level and basal medium components.

Hence, the objective of this study was to investigate the effectiveness of these two media manipulations in the control of external fungal growth in the culture liquor of immobilised KSX1 cultures. The success of the manipulations was gauged by monitoring β-glucosidase production throughout repeat-batch fermentation. Ideally, production should be maintained during cultivation under nutrient limited conditions while the biomass is confined to the gel bead. In this experiment and those that follow, β-glucosidase was chosen for measurement over other enzymes in the cellulase complex on the basis of assay simplicity thereby allowing for a large number of assays to be performed over an extended period. β-glucosidase plays an important role in cellulose breakdown and is responsible for the hydrolysis of cellobiose or higher cellooligosaccharides to glucose. Through this action, β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is often the rate limiting step in cellulose hydrolysis (Kadam and Demain, 1989). Therefore adequate levels of β-glucosidase activity are essential when glucose is intended as the major end product of cellulose hydrolysis.
7.2 Materials and methods

7.2.1 Culture conditions

Two media manipulations were performed in an effort to minimise zoospore release from the gel beads and therefore reduce subsequent competing external biomass in the culture liquor. The first manipulation studied the effects of cellobiose at different concentrations in the medium. That is, 0.025%, 0.1%, 0.175% and 0.25%. The second approach investigated the effects of using basal medium M10 at one-tenth strength. In these experiments, mineral solution I, mineral solution II, volatile fatty acids, and haemin components were added at one-tenth the normal concentrations (refer to Table 2.1). Resazurin was added at normal strength while Na₂CO₃ was added at half the rate. The medium was prepared according to Section 2.2.1.2 and the pH of reduced, chilled medium was 6.4. Two millilitres of 10 M NaOH per 100 mL of medium was added and the resulting pH of the cooled, autoclaved medium was 6.7.

7.2.2 Repeat-batch fermentation

To study the effects of the two media manipulations repeat-batch fermentation of duplicate sets of immobilised KSX1 cultures were performed in 100 mL serum bottles containing 25 mL of production medium using approximately 150 beads per culture. Figure 7.1 provides a schematic representation of repeat-batch fermentation studies for immobilised KSX1 cultures. Initial culture establishment was achieved by growing the immobilised fungus in basal medium M10 containing 0.25% glucose for 10 days (Section 6.2.1). After culture establishment the spent medium was aseptically removed by sterile anaerobic procedures with 50 mL syringes and the beads were washed twice in 25 mL basal medium M10. Twenty five millilitres of production medium, either cellobiose at varying concentrations in normal strength or reduced strength M10 was added and the beads were re-incubated at 39°C statically. After 5 to 6 days incubation the spent medium was removed. Production medium was added in the usual
- Basal medium M10 plus 0.25% glucose

**Media manipulations during production phase:**
- Basal medium M10 plus cellobiose at varying concentrations
- Basal medium M10 (1/10\textsuperscript{th} concentration) plus cellobiose at varying concentrations

**Figure 7.1** *Schematic representation of repeat-batch fermentation studies in an attempt to reduce external fungal biomass in the culture liquor of immobilised Piromycetes sp. KSX1*
way after the beads were washed in basal medium.

7.2.3 Sampling

At the commencement of the production phase samples of culture liquor (1.0 mL) were taken from the culture vessels at 24 hour intervals for the measurement of β-glucosidase. The beads were harvested at the end of each batch, dissolved in 10% hexametaphosphate and chitin estimations were performed as an indicator of fungal biomass.

7.2.4 Analytical procedures

Fungal chitin estimations were as previously described (Section 6.2.2.2). β-D-glucosidase was assayed as described in Section 4.2.4.4 but without the use of microtitre plates. The reaction mixture consisted of 1.0 mL of a p-nitrophenol derivative of β-D-glucose solution (5 mM) in 10 mM MES-NaOH buffer (pH 6.0) and a known volume of suitably diluted enzyme solution (between 50-200 µL of culture liquor) in a total volume of 2.0 mL. Following incubation at 39°C for 30 minutes the reaction was terminated by the addition of 2.0 mL of 0.2 M Na₂CO₃. The amount of p-nitrophenol produced was determined from a standard curve by measurement of absorbance at 410 nm using a Bausch and Lomb Spectronic 21 spectrophotometer.
7.3 Results

7.3.1 Effect of cellubiose concentration on minimising external biomass

Highest β-glucosidase production was observed using cellubiose at 0.25% in the production medium (Figure 7.2). As the amount of added cellubiose in the medium was reduced a proportional drop in β-glucosidase was observed. At the end of the first batch β-glucosidase production in cultures containing 0.25% cellubiose was three times higher than those cultures containing 0.025% cellubiose. By the end of batch 6 β-glucosidase production in 0.25% cellubiose cultures was approximately ten times higher than that of cultures containing 0.025%. A similar trend exists for the amount of biomass in culture liquor as measured by cell wall chitin and the concentration of cellubiose in the production medium (Table 7.1). Approximately twice as much chitin was measured in cultures containing 0.25% cellubiose than those cultures containing 0.025% in batch 1. The amount of biomass decreased with each batch in cultures containing 0.025% cellubiose until only a small amount of chitin remained at the end of batch 6 (0.08 mg). This compares with 0.63 mg chitin which was measured in the liquor of cultures containing 0.25% cellubiose at the end of batch 6. The amount of free biomass in the medium of these cultures remained constant throughout the six batches.
Figure 7.2  
*Production of β-glucosidase by immobilised Piromyces sp. K5X1 grown in repeat-batch culture. The fungus was grown in medium M10 containing various concentrations of cellobiose. Numbers in parentheses represent batch number.*

Table 7.1  
*Fungal biomass as estimated by cell wall chitin (mg) in the culture liquor of immobilised Piromyces sp. K5X1 grown in repeat-batch cultures using various concentrations of cellobiose in the production medium.*

<table>
<thead>
<tr>
<th>Concentration of cellobiose in medium (%)</th>
<th>Fungal biomass in culture liquor (mg chitin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Batch 1</td>
</tr>
<tr>
<td>0.025</td>
<td>0.28</td>
</tr>
<tr>
<td>0.1</td>
<td>0.30</td>
</tr>
<tr>
<td>0.175</td>
<td>0.45</td>
</tr>
<tr>
<td>0.25</td>
<td>0.55</td>
</tr>
</tbody>
</table>
7.3.2 Effect of reducing basal medium M10 components to one-tenth normal concentration on minimising external biomass

In the first batch the use of one-tenth strength M10 resulted in a drop of approximately 75% drop in enzyme production as compared to a control culture growing in M10 at normal strength containing 0.25% cellobiose (Figure 7.3). β-glucosidase production continued to drop in cultures containing reduced strength M10 in the second and third batches until enzyme activity was very low. β-glucosidase was reduced in all these cultures regardless of cellobiose concentration. Minimal external biomass was observed in cultures containing reduced strength M10 during the first batch and by the third batch the culture liquor was visually free of any competing biomass. At the end of the third batch (day 27) cultures containing minimal basal medium were re-introduced into normal strength M10. The cultures were re-incubated and β-glucosidase production was monitored. Enzyme production was not restored indicating that the immobilised KSX1 cultures cultivated on reduced M10 were no longer viable.
Figure 7.3 Production of β-glucosidase by immobilised Piromyces sp. K SXI grown in repeat-batch culture. The fungus was grown in basal medium M10, at normal and one-tenth strength concentrations, and contained cellobiose at various concentrations.
7.4 Discussion

Repeat-batch cultivation of immobilised KSX1 using lowered cellobiose concentrations and basal medium components at one-tenth normal concentration substantially reduced the amount of external biomass in the medium. However, \( \beta \)-glucosidase production was not maintained in these treatments. The effects of altering substrate concentration and reducing basal medium components show that a complete medium consisting of a sugar source at a suitable concentration, nitrogen source and salts is essential for immobilised growing anaerobic fungal cells to maintain enzyme production.

The technique of immobilisation allows microbial cells to be cultured in a medium which encourages rapid biomass accumulation after which time they can be transferred to a medium which limits growth and one which is optimum for product formation. This technique is feasible only if the product is a secondary metabolite. The close relationship between biomass and enzyme production in this study suggests that the immobilised fungus must grow for continuous enzyme production to occur and as such the practice of nutrient limitation proved unsatisfactory.

The findings of this present study and those mentioned in the introduction of this chapter illustrate how differences in the physiology of organisms, that is, whether the product is a primary or secondary metabolite, dictates the cultural requirements for optimum product synthesis. With reference to the explicit objectives of this investigation, the practice of nutrient limitation failed to maintain enzyme production, although successfully confining rhizomycelial growth. \( \beta \)-glucosidase production was sustained in cultures which produced fungal growth. The question remains; what proportion of the biomass in the bead is responsible for enzyme activity? This is the subject under investigation in the following chapter.
CHAPTER EIGHT

Assessing enzyme production contributed by immobilised biomass of *Piromyces* sp. KSX1

8.1 Introduction

The previous chapter showed that β-glucosidase production can be continually sustained using growing immobilised KSX1 cultures in repeat-batch mode. However, although the amount of external biomass was reduced in those cultures which had lowered nutrient levels, enzyme production was not sustained. The external rhizomycelia in the culture liquor associated with these cellobiose-grown immobilised cultures of KSX1 prompted a study to ascertain whether enzyme activity was due to biomass in the gel bead or free biomass in the medium.

The objective of this study was to determine the basis of sustainability of enzyme production in immobilised KSX1 cultures where external rhizomycelial growth in the culture liquor was a problem. An experiment was designed to determine what portion of the biomass was responsible for enzyme production by incorporating uninoculated ('dummy') beads of Ca-alginate into the culture liquor which previously contained immobilised growing cells of *Piromyces* sp. KSX1. To assess enzyme activity contributed by immobilised biomass, β-glucosidase was monitored throughout repeat-batch incubations of normal and dummy bead cultures.
8.2 Materials and methods

8.2.1 Repeat-batch fermentations using dummy beads

Immobilised cell experiments using dummy beads were used to assess enzyme production contributed by immobilised biomass of KSX1 cultures. Two batches of duplicate immobilised KSX1 cultures were grown for 10 days according to the usual method (Section 6.2.1). After culture establishment in the gel beads the growth medium was removed from the two duplicate sets of immobilised KSX1 cultures and the beads were washed twice in 25mL basal medium M10. Twenty five millilitres of production medium containing 0.25% cellobiose was added and the cultures were re-incubated for 24 hours at 39°C without agitation. After this time the culture liquor from one batch of duplicate beads was then added to a duplicate set of dummy beads (approximately 150 beads of 3% uninoculated Ca-alginate gelled with basal medium M10). The second batch of beads continued to be incubated as normal bead cultures. Repeat-batch fermentation was employed as previously described (Section 7.2.2) using 25mL of cellobiose (0.25% in M10) as the production medium over six sequential batches.

Samples of culture liquor (1.0mL) were taken from both dummy and normal bead culture vessels at 24 hour intervals and filtered. β-glucosidase in the culture filtrates was measured according to Section 7.2.4 and the enzyme activities of the two treatments were compared.
8.3 Results and discussion

Enzyme production in normal and dummy beads in production medium containing 0.25% cellobiose is shown in Figure 8.1. β-glucosidase activity in normal cultures was typical in batch 1 (compare Figure 8.1 with Figure 7.2 at the same cellobiose concentration). Similarly, β-glucosidase starts to accumulate in the second duplicate set for the first 24 hours of incubation in the production medium until the culture liquor is transferred into serum bottles containing dummy beads. This is indicated by an arrow in Figure 8.1. Following this phase, β-glucosidase levels dropped off in these cultures to a maximum of 54 mIU/mL, a decrease of almost one half when compared to the maximum β-glucosidase produced in normal cultures. In the second batch enzyme production in normal cultures drop to approximately 100 mIU/mL and again in the third batch to 70 mIU/mL. β-glucosidase is sustained at approximately this level for three successive batches thereafter. In the third batch β-glucosidase begins to rise in dummy bead cultures and by the end of the sixth batch enzyme production is 55 mIU/mL. At this stage there is little difference between the β-glucosidase levels of normal and dummy beads.

Fungal biomass was seen to accumulate on the surface of dummy beads from the second batch onwards. In subsequent batches the rhizomycelial growth on the surface of these beads continued until the sixth batch when the dummy beads were visually similar to the beads in normal cultures (Figure 8.2). Sectioning the dummy beads with a scalpel blade revealed that the cell growth occurred in the periphery of the bead and no growth was evident within the core (Figure 8.3).
Figure 8.1  Comparison of $\beta$-glucosidase production between immobilised Piromyces sp. KSX1 and free rhizomycelia in culture liquor from a previous immobilised KSX1 culture. The fungus was grown at 39°C statically in medium M10 containing 0.25% cellobiose. The arrow indicates the time of addition of culture liquor from a 24 hour immobilised KSX1 culture to dummy beads.
**Figure 8.2**  
Comparison of outer rhizomycelal growth on gel surface on (a) dummy bead and (b) normal bead. Both beads were incubated in repeat-batch culture for 45 days. Bar = 3 mm

**Figure 8.3**  
 Dummy beads showing the extent of fungal growth after 45 days incubation. (a) whole bead illustrating surface growth; (b) cross-section of whole bead illustrating peripheral growth with no evident core growth. Bars = 5 mm
The use of dummy beads showed that the source of biomass responsible for enzyme activity varied depending on the stage of incubation. The results of this study demonstrate that colonised gel beads may be considered as a reservoir of cells carrying zoospores. It is most likely that there was continuous renewal of cells near the gel bead surface and in the medium and 'leaked' cells were issued from cells grown inside cavities for a number of batches. In the case of the dummy bead, the alginate matrix acted purely as an anchor to which viable zoospores attach and proliferate, subsequently generating new growth on the outer surface of the beads. $\beta$-glucosidase production was therefore a combination of both free rhizomycelia and that 'immobilised' rhizomycelia which became subsequently attached to the surface of the gel bead. It is likely that the biomass in the normal gel beads did contribute in part to enzyme production in earlier batches before losing viability as the fermentation proceeded.
CHAPTER NINE

The effect of cellobiose and ball-milled cellulose on the production of \( \beta \)-glucosidase by immobilised *Piromyces* sp. KSX1 in repeat-batch cultures

9.1 Introduction

In assessing \( \beta \)-glucosidase production contributed by immobilised biomass of strain KSX1 it was not possible to ascertain whether sustainability of enzyme production was due to rhizomycelia in the gel bead or in the culture liquor. While not entrapped within the alginate matrix as such, the free rhizomycelium did grow on the surface of the gel bead and continued to proliferate. In this sense, a portion of the fungus remained immobilised throughout the duration of the fermentation.

This chapter is concerned with the further study of the production of \( \beta \)-glucosidase using immobilised strain KSX1 in repeat-batch notwithstanding the inherent accumulation of external growth that occurred with this isolate. The specific objective of this study was to compare two substrates for their ability to produce extracellular \( \beta \)-glucosidase by immobilised KSX1 cultures. Enzyme production is lower in media containing mono- or disaccharides than in media containing plant cell-wall polymers (see Section 1.2.1.6). Therefore, in an effort to increase enzyme production, ball-milled cellulose was trialed in addition to cellobiose. In systems using immobilised aerobic microbial cells, the process performance is usually determined by the oxygen mass-transfer limitation (Furusaki and Seki, 1992). In the case of immobilised anaerobic cells, mass transfer of a substrate for the growth and/or the reaction is often rate-limiting (Sakaki *et al*., 1988). In particular, intraparticle diffusion effects on the reactivity of immobilised whole cell systems are important (Seki and Furusaki,
1985). Consequently, the effect of shaking immobilised KSX1 cells cultured on ball-milled cellulose was also examined.
9.2 Materials and methods

9.2.1 Repeat-batch fermentation

Repeat-batch fermentation using duplicate sets of immobilised KSX1 cultures were performed in 100 mL serum bottles containing 25 mL of production medium with approximately 150 beads per culture. The repeat-batch procedure was as previously described (Section 7.2.2). After culture establishment the immobilised cultures were grown in basal medium M10 containing either cellobiose (CB) at 0.25% or ball-milled cellulose (BMC) at 0.25% or 0.5%. Immobilised fungi cultured in cellobiose were incubated statically at 39°C. Due to the insoluble nature of BMC, immobilised cultures grown in this substrate were either incubated statically or with shaking using an orbital incubator at 80 oscillations per minute. For shaken cultures it was ensured that the medium remained reduced throughout the duration of the incubation.

9.2.2 Sampling

During repeat-batch fermentation, samples of culture liquor (1.0 mL) were aseptically taken at 24 hour intervals and the pH of the filtered culture liquor was measured immediately. β-glucosidase in the culture filtrate was also measured and when enzyme production reached a maximum the medium was aseptically removed by sterile anaerobic procedures with 50 mL syringes. Twenty five millilitres of fresh production medium was added after the beads were washed twice with 25 mL of basal medium M10. The beads were harvested at the end of each batch, dissolved in 10% hexametaphosphate and chitin estimations were performed as an indicator of fungal biomass.
9.2.3 Analytical procedures

Fungal chitin estimations and scanning electron microscopy procedures were as previously described (see Sections 6.2.2.2 and 6.2.3.2 respectively; all SEM was performed on a Philips model XL 30 in this study). Aryl-\( \beta \)-D-glucosidase was assayed as described in Section 7.2.4. Cellobiose in the culture liquor was determined after enzymatic hydrolysis with \( \beta \)-D-glucosidase glucohydrolase (EC 3.2.1.21) based on the method of Russell and Baldwin (1978). Cellobiose was assayed by adding 15 µL of sample to 40 µL of acetate buffer (0.1 M; pH 4.0) in an eppendorf tube. A 2-IU quantity of \( \beta \)-D-glucoside glucohydrolase (from almonds, Sigma G-0395) was then added, and the mixture was allowed to react for exactly 45 minutes at room temperature. The enzymatic reaction was stopped by raising the pH to approximately 10 with the addition of 3 µL of 1 N NaOH. The resulting glucose (from cellobiose) and the original glucose in the sample were assayed by the glucose oxidase-peroxidase procedure (see Section 6.2.2.1). Absorbance due to glucose present in the original sample before enzymatic hydrolysis of cellobiose was subtracted so that the resulting absorbance was indicative of the amount of cellobiose.
9.3 Results

9.3.1 Effect of ball-milled cellulose on β-glucosidase production

Highest β-glucosidase production was recorded in a static culture using BMC at a concentration of 0.25% in the medium (Figure 9.1). Maximum β-glucosidase using BMC at this concentration reached 84 mIU/mL after 8 days incubation in the first batch. However, this level was not sustained with maximum enzyme production declining to 37 mIU/mL in the second batch. Only two batches were performed using BMC due to the substantial drop in the production of β-glucosidase. Shaking had a deleterious effect on enzyme activity since these cultures recorded approximately half the β-glucosidase compared to corresponding static cultures during each batch. Slight quantities of free rhizomycelia were observed in the culture liquor of static cultures while the amount in shaken cultures was negligible.

![Graph showing enzyme activity over time with different concentrations and shaking conditions.]

Figure 9.1 Production of β-glucosidase by immobilised Piromyces sp. KSX1 grown in repeat-batch culture. The fungus was grown at 39°C statically or with shaking in medium M10 containing 0.25% or 0.5% ball-milled cellulose.
9.3.2 Effect of cellobiose on $\beta$-glucosidase production

Determination of suitable cellobiose concentration:
Studies using cellobiose at concentrations of 0.25% and 0.5% showed that $\beta$-glucosidase did not substantially increase with levels higher than 0.25% (Figure 9.2). Consequently, a concentration of 0.25% cellobiose in the production medium was used in all subsequent fermentations.

Figure 9.2  Production of $\beta$-glucosidase by immobilised Piromyces sp. KSXI. Determination of optimal cellobiose concentration
9.3.2.1 β-glucosidase production using 0.25% cellobiose

β-glucosidase production was sustained over 6 batches in repeat-batch fermentation studies using 0.25% cellobiose in the production medium (Figure 9.3). In the first batch, β-glucosidase reached a maximum of 107 mIU/mL of culture liquor but levelled off to between 60 and 80 mIU/mL for subsequent batches. The average β-glucosidase produced over this time was 69 mIU/mL. Cellobiose was consumed by day 2 in the first batch and day 3 in subsequent batches. Culture pH profiles dropped from a starting pH of around 6.7 to a minimum of 6.2 within three days.

Figure 9.3  Production of β-glucosidase by immobilised Piromyces sp. KSX1 grown in repeat-batch cultures. The fungus was grown at 39°C statically in medium M10 containing 0.25% cellobiose
Viable zoospores and rhizomycelium appeared in the culture liquor 24 hours into the incubation of each batch of immobilised KSX1 cultures. Even though zoospores were released from the gel beads, they maintained their shape during the 45-day-long operation. Figure 9.4 is a scanning electron micrograph of a bead harvested at the end of the last batch after enzyme production for 45 days. Closer examination of the bead surface using SEM revealed numerous sporangia associated with an extensive rhizomycelial network (Figure 9.5). It is likely that the zoospores were the source of propagules for the development of free rhizomycelia with each addition of fresh media.
Figure 9.4  Scanning electron micrograph of immobilised KSX1 after 6th batchwise incubation (45 days). Bar = 500 μm

Figure 9.5  The surface of immobilised KSX1 after 45 days incubation illustrating numerous sporangia and associated rhizomycelial network. Bar = 20 μm
Chitin data for immobilised and free rhizomycelia and β-glucosidase levels at the end of each batch are given in Table 9.1.

Table 9.1  *Fungal biomass as estimated by cell wall chitin (mg) in gel beads and in the culture liquor and β-glucosidase production of immobilised *Piromyces sp. KX1* grown in repeat-batch cultures using cellobiose at 0.25% in the production medium. Each value represents the mean ± SD for duplicates.

<table>
<thead>
<tr>
<th>Batch Number</th>
<th>β-glucosidase production (mIU/mL)</th>
<th>Fungal biomass in culture liquor (mg chitin)</th>
<th>Fungal biomass in gel bead (mg chitin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>106.5 ± 19.8</td>
<td>0.60</td>
<td>3.90</td>
</tr>
<tr>
<td>2</td>
<td>70.4 ± 11.3</td>
<td>0.67</td>
<td>4.58</td>
</tr>
<tr>
<td>3</td>
<td>75.0 ± 21.4</td>
<td>0.73</td>
<td>4.21</td>
</tr>
<tr>
<td>4</td>
<td>68.3 ± 10.1</td>
<td>0.68</td>
<td>4.52</td>
</tr>
<tr>
<td>5</td>
<td>72.6 ± 10.5</td>
<td>0.61</td>
<td>4.60</td>
</tr>
<tr>
<td>6</td>
<td>59.4 ± 14.8</td>
<td>0.69</td>
<td>4.75</td>
</tr>
</tbody>
</table>

Both free and immobilised biomass did not vary between batches. Chitin measurements showed that immobilised biomass was seven times greater than free biomass in the culture liquor. Figure 9.6 illustrates β-glucosidase production and associated levels of both free and immobilised rhizomycelia at the end of each batch. The plot of β-glucosidase produced at the end of each batch emphasises the increased enzyme production that occurred at the end of batch 1 even though no increase in either immobilised or free biomass was recorded at this time.
Figure 9.6 Fungal biomass (immobilised and free) and β-glucosidase produced by immobilised Pirromyces sp. KSX1 at the end of each batch of the repeat-batch fermentation. Each value for enzyme activity represents the mean ± SD for duplicates.
9.4 Discussion

In this study two substrates, namely ball-milled cellulose and cellobiose, were compared for their ability to produce extracellular β-glucosidase in immobilised KSX1 cultures. Ball-milled cellulose was found to be less effective in maintaining β-glucosidase production compared to the soluble sugar cellobiose. Cultures growing on 0.25% BMC produced higher levels of β-glucosidase than those cultivated on 0.5% BMC. Shaking the cultures containing BMC failed to increase enzyme levels with highest β-glucosidase being recorded in static cultures. Attachment of the cellulose substrate to the gel bead was detected in static cultures suggesting that the fungus requires adhesion to the substrate in order to proliferate. A role as initial colonisers in lignocellulosic digestion has been suggested (Bauhop, 1979), and the ability of anaerobic fungi to penetrate deeply into substrates inaccessible to other rumen microbes may be significant in this regard (Bauhop, 1981; Bauhop, 1983). It is probable that shaking, as performed in this study, did not permit the necessary attachment required in order for the fungus to completely utilise and degrade the insoluble substrate.

β-glucosidase production in immobilised Piromyces sp. KSX1 using 0.25% CB was maintained for 45 days over six consecutive repeat-batches, although free rhizomycelia was present in the culture liquor throughout the duration of the fermentation. Highest β-glucosidase activity was recorded at the end of batch 1 and continued to be produced throughout the entire fermentation. The decrease in production of β-glucosidase in batches following the first could be affected by estimation of the optimal time for changing the medium. This could affect cell viability. Since fungal biomass occurring in the culture liquor and gel bead did not vary considerably from batch to batch (refer to Table 9.1 and Figure 9.6) it could be inferred that the difference in β-glucosidase enzyme production between the first batch and the remaining five batches is due to a possible decrease in viable biomass in the bead that occurred in the succeeding batches. In examining the physiological aspects of immobilised cells Hahn-Hägerdal (1989) has suggested there may be an exponential growth phase with
concomitant high metabolic activity immediately after immobilisation with only a percentage of the cell population maintaining a high metabolic activity. This population of cells are either in close proximity to the surrounding medium and in a location where space is made available due to the release of cells. Similarly, it is conceivable that the peripheral regrowth of the fungus on the gel bead is responsible for enzyme production during the later batches.

The performance of KSX1 when compared to other reports utilising *Piromyces* strains in free cell systems gave varied results. *Piromyces* strain R1 (Teunissen *et al.*, 1993) produced 36 mIU/mL of β-glucosidase activity when grown on cellobiose. *Piromyces* strain KSX1 in this present study produced higher yields of β-glucosidase when compared to strain R1 producing a maximum β-glucosidase level of 107 mIU/mL in the first batch and a minimum of 59 mIU/mL in the last batch. Teunissen *et al.* (1991) reported extracellular β-glucosidase activities for two *Piromyces* strains, E2 and R1, grown on filter paper cellulose, which are comparable to β-glucosidase levels produced by immobilised KSX1 cultured on BMC. In another study, Borneman *et al.* (1989) found that *Piromyces* isolate MC-1 produced a maximum β-glucosidase activity of approximately 90 mIU/mL after 9 days incubation on Coastal Bermuda grass, a level which is equivalent to that produced by strain KSX1 in repeat-batch culture. Strain KSX1 also compares favourably with *Piromyces* strain E2 which produced a β-glucosidase activity of approximately 86 mIU/mL when cultivated in a 20 mL batch culture with filter paper as the carbon source (Dijkerman *et al.*, 1996a). A direct comparison demonstrating the cellulytic superiority of *Piromyces* sp. KSX1 with respect to enzyme yield can be made by comparing the data from Gordon and Phillips (1989). The cellobiose-grown *Piromyces* sp. SM-1 produced 11 mIU/mL of β-glucosidase activity after 7 days incubation. In comparison to SM-1, β-glucosidase activity is ten-fold higher at the end of batch 1 in immobilised KSX1 cultures.

The described system using *Piromyces* sp. KSX1 cells immobilised in alginate gel proved to be an innovative method in so far as maintaining repeat-batch
production of β-glucosidase over an extended period of incubation. However, the full advantage of immobilisation was not gained because of the accumulation of rhizomycelia in the culture liquor. Therefore, there was a requirement to use a different fungus which might be less likely to produce free rhizomycelia in the culture liquor when immobilised. Immobilisation studies using the polycentric fungus Orpinomycies sp. 478P1 produced promising results in regard to its ability to be immobilised without producing external rhizomycelium (Chapter 5). The amenability toward beading displayed by this strain warrants further investigation into the production of cellulolytic enzymes in immobilised form and is covered in the following experiment.
CHAPTER TEN

Repeat-batch production of cellulolytic enzymes by immobilised cultures of *Orpinomyces* sp. 478P1 using cellobiose in the production medium

10.1 Introduction

The previous chapter described a process for the repeat-batch production of β-glucosidase by immobilised *Piromyces* sp. KSX1. Although the production of this enzyme was maintained for a period of 45 days rhizomycelial growth was present in the culture liquor throughout the fermentation. In contrast to monocentric anaerobic fungi, polycentric fungi are not dependent upon the formation of zoospores for their continued survival. Their growth pattern resembles that of higher fungi, that is, they grow by hyphal extension. Immobilisation investigations using the polycentric fungus *Orpinomyces* sp. 478P1 showed that the growing rhizomycelial filaments of this strain had the potential to colonise the entire alginate matrix. It follows that polycentric fungi may be better adapted to immobilisation than monocentric fungi. Whether enzyme activity can be sustained in this form over an extended period of incubation remains to be determined.

Studies into the production of cellulolytic enzymes of anaerobic fungi have focused on monocentric fungi and there are relatively few reports which investigate the nature of polycentric fungi, particularly in regard to their ability to produce cellulase (see Section 1.2.1.7). The objective of this present study was to investigate the possibility of reusing the same active immobilised fungal aggregations of *Orpinomyces* sp. 478P1 in consecutive batch cultivations for the production of cellulolytic enzymes. Preceding investigations using immobilised strain KSX1 in repeat-batch fermentation monitored the production of
β-glucosidase only for assay simplicity (see Section 7.1). Additional characterisation of the resulting enzyme mix in the culture liquor of immobilised 478P1 was performed in this study by measuring the production of CM-cellulase and Avicelase throughout the fermentation. This was done to further examine the cellulytic potential of immobilised 478P1, that is, to determine the ability of the culture to produce endoglucanases and exoglucanases whilst using cellobiose in the production medium.
10.2 Materials and methods

10.2.1 Repeat-batch fermentation

Repeat-batch fermentation using duplicate sets of immobilised 478P1 cultures were conducted in 100 mL serum bottles containing 25 mL of production medium with approximately 150 beads per culture. The fermentation was performed in a similar manner as for immobilised K5X1 cultures (see Section 7.2.2.) but without a culture establishment phase. Immobilised cultures of strain 478P1 were grown in basal medium M10 containing 0.25% cellobiose (see Section 5.2.4.2) and subsequently cultured repeatedly in batch mode for enzyme production using the same substrate at the same concentration. All cultures were incubated in a reciprocating water bath at 39°C and shaken at 80 oscillations per minute.

10.2.2 Sampling

During fermentation studies, samples of culture liquor (1.0 mL) were taken at regular intervals and the pH of the filtered culture liquor was measured immediately and also assayed for substrate depletion. β-glucosidase in the culture filtrate was assayed on the day of sampling and when enzyme levels reached a maximum the medium was aseptically removed by sterile anaerobic procedures with 50 mL syringes. Twenty five millilitres of fresh production medium was added after the beads were washed twice with 25 mL of basal medium. The beads were harvested at the end of each batch, dissolved in 10% hexametaphosphate and chitin estimations were performed as an indicator of fungal biomass. The culture filtrates were stored at -20°C until required for Avicelase and carboxymethyl cellulase analysis. Freezing the supernatants was required due to the logistical demands of the assays.
10.2.3 Analytical procedures

Fungal chitin and scanning electron microscopy procedures were as previously described (see Sections 6.2.2.2 and 6.2.3.2 respectively; all SEM was performed on a Philips model XL 30 in this study). Cellulose in culture liquors was determined after enzymatic hydrolysis with β-D-glucosidase glucohydrolase (see Section 9.2.3).

10.2.4 Enzyme assay procedures

All enzyme reactions were linear over the period of assays. Blanks of enzyme without substrate and substrate without enzyme were included in all enzyme assays and sample values were corrected for any blank value.

CM-cellulase and aryl-β-D-glucosidase were assayed as previously described in Sections 4.2.4.2 and 7.2.4 respectively.

10.2.4.1 Avicelase

Avicelase assays were based on Wood and Bhat (1988) and Teunissen et al. (1991). The reaction mixture consisted of 0.75 mL of a 1 % (w/v) suspension of Avicel (microcrystalline cellulose, type PH 101, Serva, Heidelberg, FRG) in 0.2 M citrate-phosphate buffer (pH 6.0) and 0.25 mL of culture liquor. Following incubation at 39°C for 2 hours the reaction was stopped by placing the reaction tubes in boiling water for 5 minutes. The reaction mixture was transferred to 2.0 mL plastic eppendorf tubes and centrifuged at 8000 x g for 10 minutes (Micro Spin 245, Sorvall Instruments, Dupont, Wilmington, DE) to pellet residual Avicel and the liquor was analysed for reducing sugars using TZ reagent (see Section 4.2.4.1). Four millilitres of TZ reagent was added to 200 µL of reaction mixture and the tubes were capped and immediately heated in a boiling water bath for exactly 3 minutes. The tubes were cooled for 3 minutes in running water, dried and absorbance was determined at 660 nm in a LKB Utraspec II Biochrom
spectrophotometer. The concentration of reducing sugars was measured with D-glucose as the standard.
10.3 Results

10.3.1 β-glucosidase production using immobilised 478P1 in repeat-batch culture

Repeat-batch cultures of immobilised Orpinomyces sp. 478P1 were maintained for 30 days over four successive batches. Enzyme production profiles are shown in Figure 10.1. In the first batch β-glucosidase levels reached a maximum of 34 mIU/mL after 8 days incubation then declined to 22 mIU/mL at the end of the second batch. Enzyme levels were sustained at this level for two successive batches thereafter averaging 20 mIU/mL over the second, third and fourth batches. Celllobiose in the medium was consumed by day 3 in the first batch. In subsequent batches celllobiose depletion occurred by days 4 to 5. Culture pH profiles show a drop from a starting pH of around 6.7 to 5.9 in four days.

In the second batch, short rhizomycelial segments (ca. 0.5 mm in length) appeared in the culture medium and continued to be produced in subsequent batches. These rhizomycelial segments were tested for viability by culturing them on an agar plate containing M10X medium. No spreading colonies arose from the segments and it is possible that the rhizomycelial filaments that were broken off due to the shear abrasion as a result of shaking were too small to be viable and thus failed to grow in the culture medium. The viability of the immobilised fungus at the end of the 30 day long operation was checked by aseptically embedding a single bead onto an agar plate containing M10X medium. Figure 10.2 illustrates the outward growth of rhizomycelium of Orpinomyces sp. 478P1 which has been stained with 0.05% cotton blue in lactophenol and destained with GDW to remove background stain from the agar.
Figure 10.1 Production of β-glucosidase by immobilised Orpinomyces sp. 478P1 grown in repeat-batch cultures. The fungus was grown at 39 °C with shaking in medium M10 containing 0.25% cellulose

Figure 10.2 Viability of immobilised Orpinomyces sp. 478P1. Rhizomycelial outgrowth after 4 days incubation of a 30 day cultured bead. Bar = 2 cm
Scanning electron micrograph of a whole gel bead at the end of the fermentation period shows a fur-like coat of rhizomycelia on the surface (Figure 10.3) and peripheral rhizomycelial growth on the outside of the bead (Figure 10.4). It is possible that the action of shaking the cultures caused physical abrasion releasing nonviable filaments into the culture liquor while there was continual re-growth of the fungus at the microbial aggregate-medium interface.

Chitin data and corresponding β-glucosidase levels for immobilised 478P1 cultures at the end of each batch are presented in Table 10.1. Similarly, as for immobilised Pirinomycetes sp. KSX1 cultures, fungal biomass in the gel bead did not vary between batches.

Table 10.1  **Fungal biomass as estimated by cell wall chitin (mg) in gel beads and β-glucosidase production of immobilised Orpinomyces sp. 478P1 grown in repeat-batch cultures using cellulose at 0.25% in the production medium**

<table>
<thead>
<tr>
<th>Batch Number</th>
<th>β-glucosidase production (mIU/mL)</th>
<th>Fungal biomass in gel bead (mg chitin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34.1</td>
<td>1.32</td>
</tr>
<tr>
<td>2</td>
<td>22.0</td>
<td>1.30</td>
</tr>
<tr>
<td>3</td>
<td>20.2</td>
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Figure 10.3  Scanning electron micrograph of a whole gel bead of immobilised 478P1 at the end of the 30-day-long operation. Bar = 1 mm

Figure 10.4  Scanning electron micrograph of rhizomyelia found on the surface of a cultured gel bead after the 30 day batch. Note the absence of sporangia. Bar = 10 μm
10.3.2 CM-cellulase and Avicelase production using immobilised 478P1 in repeat-batch culture

Cellulase production from culture filtrates of immobilised 478P1 cultures was assayed over four consecutive repeat-batch cultures using Avicel PH-101 and carboxymethyl cellulose as substrates (Figure 10.5). Cellobiose-grown cultures of immobilised 478P1 cultures contained levels of both Avicelase and CM-cellulase. Enzyme production would be lower than expected bearing in mind that the culture liquors were frozen prior to being assayed for Avicelase and CM-cellulase. Although direct comparison of enzyme production cannot be made with other polycentric strains isolated in this project, that is, Orpinomyces sp. WBR1 and BCR1 and Anaeromyces sp. WRP1, immobilised 478P1 cultures produced similar CM-cellulase activity when compared to straw-grown cultures of the two Orpinomyces strains WBR1 and BCR1 (Table 4.4). No previous measures of Avicelase were performed therefore comparisons could not be made. The extracellular production of CM-cellulase and Avicelase reached maximum levels by day 5 of each repeat-batch. CM-cellulase appeared in the culture medium after 24 hours of incubation while Avicelase began accumulating slightly later, approximately 2 to 3 days into the incubation. Both maximum Avicelase and CM-cellulase were higher in the first batch compared to subsequent repeat-batches. In the first batch CM-cellulase reached a maximum of 228 mIU/mL after 5 days incubation then declined to 140 mIU/mL in the second batch. Avicelase in the first batch recorded 1.4 mIU/mL after 5 days incubation and similarly declined to a maximum activity of 0.9 mIU/mL in the second batch. No decrease in extracellular cellulolytic production in successive batches occurred thereafter. This trend is similar to that observed for β-glucosidase production in the same repeat-batch experiment (Figure 10.1).
Figure 10.5 Production of CM-cellulase and Avicelase by immobilised Orpinomyces sp. 478P1 grown in repeat-batch cultures. The fungus was grown at 39 °C with shaking in medium M10 containing 0.25% cellobiose.
10.4 Discussion

In repeat-batch cultivations, immobilised *Orpinomyces* sp. 478P1 could be successfully used for four consecutive batches. Cellulolytic enzyme activity was maintained during the 30-day-long incubation and no deterioration of the gel beads was observed. In contrast to the immobilised monocentric fungus strain KSX1, no viable free rhizomycelium arose in the culture liquor of immobilised 478P1 cultures. Although there was no apparent increase in biomass during the incubation period it is possible that continual re-growth of rhizomycelia at the gel bead surface maintained cell activity. As in immobilised KSX1 cultures, highest β-glucosidase activity was recorded at the end of batch 1 and continued to be produced throughout the entire fermentation.

Immobilised cells of *Orpinomyces* sp. 478P1 produced significant amounts of extracellular CM-cellulase and Avicelase over the 30 day incubation. β-glucosidase activities of cellobiose-grown cultures of immobilised strain 478P1 were approximately double those produced by straw-grown *Orpinomyces* sp. WBR1 and BCR1 while CM-cellulase activities were similar for all three isolates (see Section 4.3.2). Avicelase produced by immobilised 478P1 cultures was found to be substantially lower than CM-cellulase and β-glucosidase levels. Borneman et al. (1989) similarly recorded significant differences between Avicelase and CM-cellulase/β-glucosidase levels. In that particular study, the two *Orpinomyces* isolates PC-2 and PC-3 grown on Coastal Bermuda grass recorded similar CM-cellulase activities but higher β-glucosidase and Avicelase activities compared to immobilised 478P1 cellobiose cultures (refer to Table 1.5 in the Literature Review). CM-cellulase activities obtained in this present study are higher in comparison to the results obtained with glucose-grown *Orpinomyces joyonii* strain 19-2 (Yanke et al., 1996). Extracellular endoglucanase activity produced by *O. joyonii* strain 19-2 was 15.3 mIU/mL when grown on glucose while Avicel-grown cultures produced slightly higher endoglucanase activity (23.6 mIU/mL). This is an expected result as enzyme activities are likely to be higher when cultures are grown on cellulosic substrates than on soluble sugars.
Ultimately, comparisons are difficult to make because of the various assay conditions employed in different laboratories. Furthermore, the composition of the multicomponent enzyme complex varies according to the growth conditions.

The research in this chapter described the first attempt to utilise immobilised polycentric fungi in an extended fermentation for the production of cellulolytic enzymes. Although β-glucosidase levels were higher in the immobilised monocentric culture, strain KSX1 (69% more β-glucosidase was produced by KSX1 cultures during the first batch under the same culture conditions; compare Figure 9.3 with Figure 10.1), the immobilised polycentric strain 478P1 did not produce any viable growth in the culture medium and retained its ability to produce extracellular enzymes for four consecutive repeat-batches. Long term viability of the culture has been demonstrated in this experiment and is another one of the various advantages encountered when working with immobilised cells. It is possible that the fermentation could have proceeded longer if the incubation was continued considering that the immobilised fungus was still viable.
CHAPTER ELEVEN

β-glucosidase production in free cell batch cultures of Piromyces sp. KSX1 and Orpinomyces sp. 478P1: comparison with corresponding cultures using immobilised rhizomycelia

11.1 Introduction

The technique of immobilisation offers improvement in the production of cellulase for those organisms exhibiting growth-dissociated enzyme synthesis. A close relationship between biomass and enzyme production was a phenomenon observed in all repeat-batch experiments in this project suggesting that the immobilised fungus must grow for continuous enzyme production to occur. In this study β-glucosidase production was measured in free cell batch cultures of Piromyces sp. KSX1 and Orpinomyces sp. 478P1 and the specific growth rates and enzyme productivities were calculated. The objective was to elucidate the pattern of enzyme synthesis in these organisms and therefore determine whether strains KSX1 and 478P1 produce β-glucosidase in a growth-associated or -dissociated manner. An assessment of immobilisation as a process for the production of cellulytic enzymes by anaerobic fungi can be gained from this information.

Further evaluation of the immobilisation process was performed by comparing specific β-glucosidase production in immobilised and free cultures of strains KSX1 and 478P1 operating under the same conditions. In addition, enzyme production in immobilised and free cell cultures of these two strains were compared to assess the superiority of monocentric and polycentric isolates in regard to enzyme yield.
11.2 Materials and Methods

11.2.1 Batch fermentation of free cells of *Piromyces* sp. KSX1 and *Orpinomyces* sp. 478P1

Batch cultures of strains KSX1 and 478P1 were performed in 100 mL serum bottles containing 25 mL basal medium M10 with the separate addition of cellobiose to a concentration of 0.25%. Inocula for KSX1 and 478P1 have been previously described (see Sections 5.2.2.1 and 5.2.2.3 respectively). A 1 mL suspension of either a 24 hour culture for strain KSX1 or a 5 day-old culture for strain 478P1 was used to inoculate duplicate cultures which were grown at 39°C without agitation.

11.2.2 Sampling

Samples of culture liquor (1.0 mL) were taken aseptically at regular intervals for the determination of substrate losses and β-glucosidase production and the pH of the filtered culture liquor was measured immediately. Chitin estimations were performed on one set of duplicate cultures at each time interval as an indicator of fungal biomass.

11.2.3 Analytical procedures

Fungal chitin and residual cellobiose in culture liquors were determined as previously described (see Sections 6.2.2.2 and 9.2.3 respectively). Aryl-β-D-glucosidase was assayed as described in Section 7.2.4. Specific β-glucosidase production was expressed as IU per milligram of chitin where one IU of β-glucosidase produced was the amount of the enzyme which liberated 1 micromole of *p*-nitrophenol per minute.
11.3 Results and discussion

11.3.1 β-glucosidase production using free cells of *Piromyces* sp. KSX1 and *Orpinomyces* sp. 478P1 in single batch cultures

Growth and β-glucosidase production by free cells of *Piromyces* sp. KSX1 and *Orpinomyces* sp. 478P1 was followed during a single batch in medium M10 containing 0.25% cellobiose. Figures 11.1 a and b illustrate the pattern of cell growth, cellobiose depletion, β-glucosidase accumulation and culture pH of strains KSX1 and 478P1 respectively, during cultivation on the cellobiose medium.

The freely suspended cells of strain KSX1 started to grow after 24 hours of incubation and the most active cell growth was observed at approximately 2 days (Figure 11.1a). Cellobiose in the medium was consumed by day 2 of the incubation. Culture pH profiles showed a drop from a starting pH of around 6.7 to 6.2, which was sustained until the end of the batch. The maximum total cell growth of 1.5 mg of chitin was achieved after 4 days and levelled off, decreasing slightly until the end of incubation. β-glucosidase activity accumulated from the start of incubation and increased to the end of the incubation period. The increase in enzyme activity that is observed from day 4 to 6, although smaller in comparison, was accompanied by a non-growth period of the fungus at the same stage.

Strain 478P1 started to grow after 24 hours incubation and the most active cell growth was observed at around 3 days (Figure 11.1b). Cellobiose in the medium was consumed by day 3 of the incubation while culture pH profiles showed a drop from a starting pH of around 6.7 to 5.9 in four days. Days 3 to 4 represent a 24 hour period in which maximum growth and maximum rate of enzyme production occurred concomitantly. Following this, growth levelled off to a maximum of 1.30 mg of chitin by the end of the 9 day incubation period. However,
Figure 11.1  Time course of fungal growth, cellubiose depletion, β-glucosidase production and culture pH of free cells of (a) Piromyces sp. KSX1 and (b) Orpinomyces sp. 478P1 in medium M10 containing 0.25% cellubiose. Each value represents the mean ± SD of duplicate cultures.
β-glucosidase activity continued to accumulate in the culture liquor forming a plateau by day 8.

The results in Figures 11.1 a and b show some difference between Orpinomyces sp. 478P1 and Piromyces sp. KSX1. As expected, it is observed that as strain 478P1 stopped growing the pH of the medium ceased to fall. However, strain KSX1 grew rapidly for 4 days yet the pH of the medium ceased to drop after only 2 days. Although there appears to be some basic differences in the fermentation capabilities of the two organisms they are regarded as uncertain being based on a small number of data points.

11.3.2 Relationship between rhizomycelial growth and synthesis of β-glucosidase in Piromyces sp. KSX1 and Orpinomyces sp. 478P1

The relationship between rhizomycelial growth and β-glucosidase production in free cell batch cultures of strains KSX1 and 478P1 is presented in Figures 11.2 a and b respectively. Specific growth rates and specific enzyme productivities were derived from biomass and β-glucosidase data obtained from free cell batch cultures (Figures 11.1 a and b).

Figures 11.2 a and b show that the specific productivity of β-glucosidase synthesis is proportional to the specific rate of growth in KSX1 and 478P1 cultures. Maximum specific rate of enzyme formation and maximum rate of growth occurs during day two of the incubation for strain KSX1 and around day three for strain 478P1. The plots of cell concentration, enzyme production, specific growth rate, and specific rate of enzyme formation presented in Figures 11.2 a and b closely resemble the ‘Type I’ schematic process patterns of the production of hydrolases (Enatsu and Shinmyo, 1978). Thus classifying the production of β-glucosidase by these two strains of anaerobic fungi as a growth-associated pattern. The previous observation made in free KSX1 cultures where enzyme activity increased whilst growth was in decline (Figure 11.1 a) was only true for one data point (day six of the incubation). On interpretation of the data sets, however, Figures 11.2 a and b
Figure 11.2  Relationship between rhizomycelial growth and synthesis of β-glucosidase in (a) Piromyces sp. KSX1 and (b) Orpinomyces sp. 478P1
clearly show a pattern of growth-associated enzyme synthesis where specific productivity of β-glucosidase synthesis is proportional to the specific rate of growth of the organism.

The physiology of β-glucosidase production in strains KSX1 and 478P1 is therefore not biphasic; a phenomenon reported in cellulase production studies involving *Trichoderma* sp. (Mandels *et al.*, 1975; Ryu *et al.*, 1979). In these studies the conditions for maximum cellulase production was not the same as conditions for optimum growth. Immobilisation is a technique specific for the exploitation of the synthesis of secondary metabolites. Therefore, any attempts to increase product formation by altering the production medium would be ineffective due to the growth-associated synthesis of β-glucosidase by immobilised strains KSX1 and 478P1.

**11.3.2 Evaluating the potential application of immobilised *Piromyces* sp. KSX1 and *Orpinomyces* sp. 478P1**

Histograms comparing the specific β-glucosidase production of immobilised and free cell cultures of *Piromyces* sp. KSX1 and *Orpinomyces* sp. 478P1 are presented in Figures 11.3a and b.

**11.3.2.1 Specific β-glucosidase production of immobilised KSX1 in repeat-batch culture versus free cell KSX1 in a single batch**

Calculations of specific enzyme production are based on Figure 11.1a for free cell cultures and Table 9.1 (Section 9.3.2.1) for immobilised cultures. At the end of 6 days incubation specific β-glucosidase production in free cell cultures measured 2.0 IU per mg chitin (Figure 11.3a). Specific β-glucosidase production in immobilised repeat-batch cultures was calculated on the basis of either free rhizomycelia present in the culture liquor, immobilised rhizomycelia or total biomass; that is, both free and immobilised rhizomycelia. Specific enzyme production calculated using total biomass or immobilised biomass was substantially lower than that obtained in the single batch free cell culture. Specific enzyme production calculated using free rhizomycelium in the culture liquor was
approximately equivalent to that obtained in all batches except the first. It is most likely that specific β-glucosidase of free biomass in this first batch is an over estimation since the true assessment of viable biomass could not be gauged in this initial batch.

11.3.2.2 Specific β-glucosidase production of immobilised 478P1 in repeat-batch culture versus free cell 478P1 in a single batch

Calculations of specific enzyme production are based on Figure 11.1b for free cell cultures and Table 10.1 (Section 10.3.1) for immobilised cultures. The histogram (Figure 11.3b) illustrates specific β-glucosidase production of immobilised and free cell cultures Orpinomyces sp. 478P1. Specific β-glucosidase production of immobilised 478P1 cultures was calculated using biomass in the gel bead since the rhizomycelial filaments remained nonviable in the liquor of these cultures throughout the duration of the incubation. However, it is possible that non-viable filaments can still produce β-glucosidase activity. In this case, slightly lower specific β-glucosidase levels would be expected if the biomass of the free non-viable filaments were taken into account. At the end of 9 days incubation specific β-glucosidase activity measured 0.30 IU/mg chitin in free cell cultures. In immobilised cultures, similar measures of specific β-glucosidase production were obtained at the end of each batch since the biomass in the gel bead was similar to that measured in the free cell culture at the end of the incubation. The exception to this was the increased enzyme activity obtained in the first batch. This is represented as a 42% increase in specific β-glucosidase production using immobilised 478P1 cultures compared to a single batch of free cells. Despite a drop in specific enzyme production in subsequent batches, existing immobilised biomass continued to produce β-glucosidase at levels comparative to free cell cultures. It is possible that cell viability was higher in batch 1 compared to subsequent batches thus accounting for the higher enzyme production that was produced during this initial batch.
Figure 11.3  Specific $\beta$-glucosidase production of free and immobilised cultures of (a) Piromyces sp. KSXI and (b) Orpinomyces sp. 478P1
The specific $\beta$-glucosidase activities were approximately seven fold higher in immobilised and free cultures of KSX1 compared to 478P1 cultures (compare Figures 11.3 a and b). However, the merits of repeat-batch culture in immobilised KSX1 cultures were equivocal because rhizomycelial growth in the culture liquor was a confounding problem throughout the fermentation. The amount of cell leakage from immobilised KSX1 cultures was constant throughout the six batches (Table 9.1) whereas no measurable chitin was found in the culture liquor of the polycentric culture, strain 478P1. As such, this strain proved to be operationally superior to the monocentric culture, KSX1.
CHAPTER TWELVE

General discussion

In general, attempts to improve cellulase production have been based on the selection of a suitable organism and the development of an efficient production process to utilise the chosen isolate. In this project, a production process, novel to anaerobic fungi, was developed in an effort to improve cellulase production. The present research has focused on two key objectives. Firstly, an isolation and screening programme was established to obtain cultures which have both the ability to produce high levels of hydrolytic enzymes and be suitable for immobilisation. Secondly, a production process incorporating immobilised fungal rhizomycelia was developed for the production of cellulolytic enzymes. Together, these investigations provided useful data for the evaluation of the potential application of immobilised anaerobic fungi to produce cellulolytic enzymes.

12.1 Selection of anaerobic fungi exhibiting high cellulolytic capability

In this project anaerobic fungi were nominated as good sources of cellulase and an isolation and screening program was initiated in an attempt to find isolates of anaerobic fungi that have the potential to produce high levels of this enzyme. The success of this first stage was assessed by comparing the extracellular enzyme yields of the selected isolates with published studies reporting on enzyme production of other strains of anaerobic fungi. These comparisons show that although many of the enzyme levels produced by the isolates in this project were comparatively higher than those reported, some were lower, making it difficult to gauge the success of the isolation and screening procedure. They have highlighted the differences that can occur in the enzyme complex when using different substrates. Variability in enzyme yields can also occur depending on the culture conditions and assay conditions. In general, the strategies used in this project
allowed for the selection of anaerobic fungi which produced enzyme levels that were similar to those commonly achieved to date. It is important to note, however, that enzyme activities are not specifically related to biomass and therefore accurate comparisons with published data are difficult to make.

**Identification of cellulolytic isolates at the isolation and primary screening stage.** The isolation procedure resulted in the selection of a large number of organisms from a variety of ruminant and non-ruminant herbivores. Generally, the fungal strains isolated from the various herbivores were of the same genera as those found in other isolation studies. A screening test using cellulose-azure was performed on 46 isolates as a primary means of identifying cellulolytic strains of fungi. All isolates were capable of degrading C-A, however, not all fungi degraded the dyed substrate to the same end point. By comparing the degree of dye release produced by individual isolates relative ranks of cellulolytic activity were obtained. This semi-quantitative measure enabled broad differences in the cellulolytic capability of the isolates to be identified.

**Identification of isolates with the best potential for producing high cellulase levels using secondary screening.** Further screening tests on the 46 isolates were undertaken to quantify the capacity of the different fungal strains to degrade cellulose and therefore select those organisms that were the most cellulolytic. The combined results of two test methods used in the secondary screen allowed to differentiate between isolates of the same genus on the basis of their cellulolytic activity.

The first of these tests used radioactively labelled cellulose to determine rates of cellulolysis. The $^{14}$C-cellulose screen resulted in the selection of 12 strains out of the 46 that were isolated and produced a general ranking where isolates belonging to the genus *Neocallimastix* performed better than *Piromyces* species while the two *Orpinomyces* isolates were ranked in the last two places (Table 3.2). However, it was observed that strains belonging to both *Neocallimastix* and *Piromyces* genera were also included in the 34 isolates that were eliminated in this
screening test (Table 3.3). These results have shown that relative ranks should be considered as specific to the individual strain rather than being representative of other isolates that are members of the same genus.

This was further exemplified in the second screening test that examined the hydrolytic potential of the 12 isolates. These investigations showed that the ranks were not consistent among isolates of the same genus (Table 4.5). That is, neither members of the genera *Neocallimastix* nor *Piromyces* performed consistently better than the other. In a comparison of the influence of carbon substrates on the production of fibre-degrading enzymes of anaerobic fungi, Yanke *et al.* (1996) found that while the enzyme activities were functionally similar, there were likely to be significant variations in the enzyme systems of these fungi. Their results strongly suggest enzyme systems of each fungal species are unique. Likewise, Teunissen *et al.* (1991) have demonstrated differences in cellulolytic yields between species belonging to the genera *Piromyces* and *Neocallimastix*. Valuable information can therefore be gained by direct comparisons of the cellulolytic systems of anaerobic fungi, particularly if the isolates are being sought for their commercial potential.

A measure of the effectiveness of the screening systems can be gained by comparing the extracellular enzyme activities of the 12 selected fungi (Table 4.4) with studies reporting on enzyme production of other strains of fungi (refer to Tables 1.3, 1.4 and 1.5 in the Literature review). Extracellular CM-cellulase and xylanase levels produced by all ten monocentric cultures in this project were higher when compared to those enzyme titres produced by monocentric isolates in batch culture reported by Lowe *et al.* (1987b); Borneman *et al.* (1989); Teunissen *et al.* (1991); Yanke, *et al.* (1996); Zhu *et al.* (1996; 1997). CM-cellulase and β-glucosidase yields produced by monocentric isolates in a study conducted by Dijkerman *et al.* (1996a) were higher compared to the ten monocentric isolates in this present study while xylanase yields were lower. Monocentric strains MC-1 and MC-2 (Borneman *et al.*, 1989) produced lower extracellular β-glucosidase and β-xylosidase yields when compared to monocentric strains in this study.
β-glucosidase levels of the 10 monocentric strains of this study were comparable to those produced by *Neocallimastix* sp. R1 (Lowe *et al.*, 1987c) while they were higher when compared to those levels produced by *N. hurleyensis* (Zhu *et al.*, 1996; 1997).

The two polycentric strains examined in this study, WBR1 and BCR1, produced similar CM-cellulase levels when compared to the polycentric strains PC-1, PC-2, and PC-3 (Borneman *et al.*, 1989). While xylanase yields were higher in strains WBR1 and BCR1 when compared to Borneman’s polycentric strains, β-glucosidase and β-xylosidase yields were lower in the polycentric cultures of this present study. CM-cellulase, β-glucosidase, xylanase and β-xylosidase levels in *O. joyonii* (Yanke *et al.*, 1996) and an *Orpinomyces* sp. (Zhu *et al.*, 1997) were all substantially lower when compared to the component extracellular enzyme levels of WBR1 and BCR1.

### 12.2 Immobilisation of anaerobic fungi and repeat-batch culture as an approach to cellulase production

The second key area of study was the development of a process which utilised immobilised fungal rhizomycelia for the production of cellulytic enzymes. Immobilisation was chosen over traditional free cell fermentations for two reasons. Firstly, immobilisation allows for the exploitation of organisms which exhibit growth-dissociated synthesis of enzymes. Secondly, it has the operational advantage of using the microbial cells repeatedly and continuously.

The screening procedures produced a pool of cultures with known capabilities to degrade fibre. From this pool, fungal strains were assessed for their ability to be immobilised. This project reports on the immobilisation of anaerobic fungi for the first time. Therefore, the primary objective of this part of the study was to devise a model system for the immobilisation of these organisms. This system should include both the ability to produce immobilised rhizomycelia that did not overgrow bead surfaces and also the ability to repeat-batch for the production of cellulase.
Choice of propagules in the production of immobilised rhizomycelia. The production of immobilised fungal aggregations was the first step in the development of an immobilised production process and the limitations encountered during the development of this procedure have provided further insight into the life cycle and growth patterns particular to monocentric and polycentric fungi.

Amenability toward immobilisation rather than fibre-degrading superiority formed the basis for the selection of strains for immobilisation. Zoospores were identified as suitable propagules for immobilisation of the monocentric isolate, strain KSX1. This strain was selected for immobilisation studies on the basis of its ability to produce zoospores in large numbers when compared to Neocallimastix sp. DLS3 which was the highest ranked monocentric isolate. To examine how the different morphological types of fungi responded to immobilisation polycentric fungi were also investigated. Polycentric fungi were isolated in low numbers in this study with only two strains retained for immobilisation studies. Thus, in order to maximise the chance of successfully immobilising these fungi a number of polycentrics belonging to the laboratory collection were also assessed for their ability to be immobilised. These studies led to the selection of Orpinomyces sp. 478P1 which was used in subsequent immobilisation studies.

There have been no previous reports on the immobilisation of anaerobic fungi. Therefore, the first attempts at immobilisation using zoospores of KSX1 were based on methods used for aerobic fungi with modifications to accommodate the anaerobic requirements of this strain. In these initial experiments fungal colonisation in the bead was poor, due primarily to the severity of the entrapment process. A number of modifications were introduced with the aim of making the immobilisation conditions mild and resulted in improved fungal colonisation of the bead. The modifications developed in the beading procedure give indirect evidence that zoospores are fragile.
The inability of strain 478P1 to produce zoospores meant that partially homogenised rhizomycelia was alternatively used as propagules. This polycentric fungus developed as a mycelial network inside the beads whereas strain KSX1 tended to grow in microcolonies and was unable to invade the entire matrix. In the latter case it is possible that only sporangia are surviving during the immobilisation procedure. Consequently, immobilisation may be more suited to the vegetative growth cycle of polycentric fungi.

While strain KSX1 colonised all of the beads, strain 478P1 colonised approximately 30%. In order to achieve the maximum number of beads colonised by the fungus it is important to transfer a viable inoculum. In an assessment of the different types of propagules that could be used in the immobilisation of anaerobic fungi it was nominated that zoospores would be better propagules than homogenised rhizomycelia. The inability to use zoospores as propagules for the immobilisation of strain 478P1 placed certain restrictions on the ability to immobilise this fungus and the use of homogenised rhizomycelia is a possible reason for the low rate of colonisation displayed by this strain.

Rhizomycelial fragments that resulted from the homogenisation process were quite large (3-4 mm) which is a possible reason for this low rate of colonisation. Fragments of ca. 0.5 mm in length were observed in the culture medium of immobilised 478P1 cultures as a result of shaking (Section 10.3.1). However, these fragments were found too small to be viable since they failed to grow in the culture medium. Therefore, the means to increasing the percentage of beads that are colonised by the polycentric fungus may lie in producing rhizomycelial fragments smaller than 3 mm but large enough to remain viable. Studies into alternate methods of homogenisation that investigate optimum rhizomycelial fragment length, possibly leading to better rates of bead colonisation, are thus open to examination. Given that homogenisation of rhizomycelia reduces inoculum viability the ability to achieve 100% colonisation rates in polycentric fungi may lie in the use of zoospores as propagules for immobilisation. Although the induction of zoospores in polycentric fungi was unsuccessful in this project...
further studies into the generation of zoospores in these fungi should not be ruled out.

*Attempts to confine rhizomycelia to the gel bead.* Rhizomycelia of strain 478P1 was confined to the surface and subsurface of the gel bead due to the vegetative growth cycle this fungus. In contrast, immobilised KSX1 cultures growing rapidly on the surface of the bead released zoospores that germinated to form free rhizomycelial growth in the culture liquor. Although easy separation of the cells from the product is considered to be one of the important merits in immobilised cells systems, such an advantage was not fully exploited in immobilised KSX1 cultures due to easy leakage of growing cells from the gel bead into the medium. Free mycelial growth in the culture liquor defeats the purpose of immobilisation. The accumulation of free mycelia in the production of citric acid by immobilised *Aspergillus niger* was a contributory factor to the process being assessed as no better than traditional batch fermentation (Borglum and Marshall, 1984).

Attempts at avoiding the overgrowth of rhizomycelia in immobilised KSX1 cultures by lowering nutrients were performed with limited success. In these instances a reduction in external rhizomycelial biomass was accompanied by a reduction in enzyme activity. The findings of Chapter 7 together with the conclusions made from Chapter 11 provides a possible explanation as to why attempts to confine rhizomycelia to gel beads were unsuccessful. That is, β-glucosidase was only produced when the immobilised fungus was growing. The exception to this was the free culture of *Pirimyces* sp. KSX1 (Fig 11.1 a) which showed some β-glucosidase activity in the absence of growth in the latter stages of its incubation. Apart from this, the production of β-glucosidase was closely linked with the active growth of this culture (see Section 11.3.2). An alternate method to nutrient limitation may be to eliminate or minimise zoospore release which would reduce subsequent cell growth in the culture liquor. Coating of gel beads is one possible method to minimise zoospore release and has been performed to avoid cell release and also to increase mechanical and chemical stability. A method for immobilisation of viable cells in alginate fibres with
double layers is described by Tanaka et al. (1989). In this method, the Saccharomyces sp. was restricted to the inner layer while the outer layer helped to prevent cell leakage. It is important to note, however, that coating the gel beads may compromise substrate diffusion.

*Sustained enzyme production using immobilised cells in repeat-batch culture.* The ability to achieve reasonable colonisation of the alginate bead allowed for the evaluation of the potential use of immobilised fungi to produce cellulolytic enzymes. Although the screening procedures allowed for the quantification of several cellulolytic and xylanolytic enzymes, studies involving immobilised cells quantified β-glucosidase only. These experiments investigated the ability of beads to be repeatedly used for enzyme production therefore it was not essential to quantify enzymes of the whole cellulase spectrum. β-glucosidase was chosen on the basis of assay simplicity and the rationale for choosing the measurement of this enzyme over other enzymes in the cellulase complex are outlined in Chapter 7.

Production of β-glucosidase was maintained for 45 days over six consecutive repeat-batch cultures using immobilised KSX1 cultures in the celllobiose medium. However, the full advantage of immobilisation was not taken because of the growth of free rhizomycelia in the culture liquor. Assessment of the relative contribution to enzyme production by immobilised and free biomass in KSX1 cultures (Chapter 8) showed that the alginate gel bead possibly acted simply as an anchor to which viable zoosporos could attach and proliferate, subsequently generating new growth on the outer surface of the beads. Strain KSX1 produced equivalent levels of β-glucosidase activity throughout the batch cultures when compared to a small batch culture of Piromyces sp. strain E2 (Dijkerman et al., 1996a; see Table 1.3). However, β-glucosidase levels doubled when strain E2 was cultured in larger 10 L batch cultures.

Immobilised cells of Orpinomyces sp. 478P1 produced β-glucosidase during four consecutive repeat-batch cultivations totalling 30 days of incubation.
β-glucosidase levels produced by strain 478P1 in repeat-batch were double compared to batch and continuous-flow cultures of an *Orpinomyces* sp. (Zhu et al., 1997; see Table 1.5). However, *Orpinomyces* sp. PC-2 and PC-3 (Borneman et al., 1989) grown on Coastal Bermuda Grass (CBG) produced β-glucosidase levels that were approximately four times higher than those obtained in repeat-batch culture of strain 478P1. It is important to note here, the yield differences that can occur in enzyme complexes when using different substrates.

Throughout the repeat-batch procedure the alginate beads of both strains KSX1 and 478P1 maintained their shape. However, it was observed that gel beads of KSX1 had lost some strength. This weakening of the gel network may have been caused by various factors such as mechanical tension produced by the growing fungus and removal of Ca²⁺ by phosphates in the medium. Immobilised cultures of 478P1 were comparatively stronger owing to their ability to invade the whole alginate matrix. The instability of the calcium alginate beads is a factor limiting the wide application of this technique. In a study investigating immobilised *Penicillium chrysogenum* strains in penicillin production, El-Sayed and Rehm (1986) found that maximum instability of the alginate beads occurred when a decrease of mycelia in the central layers took place by autolysis and the outer layers reached maximal mycelial capacity. This led to a destruction of the beads and the release of the mycelia into the fermentation medium to form a thick suspension.

Although the production of β-glucosidase was sustained throughout repeat-batch cultures of KSX1 and 478P1, an increase in enzyme production in the first batch was a phenomenon observed in all repeat-batch experiments and was possibly due to the higher cell viability in the bead that occurred at this time. In immobilised KSX1 cultures it is probable that the biomass in the bead contributed to enzyme production in this first batch, thus accounting for the increase in β-glucosidase activity. However, β-glucosidase production was lower in subsequent batches owing to the possible decrease in immobilised cell viability. It
is likely that peripheral regrowth of the fungus on the gel bed is responsible for sustaining enzyme production in batches following the first.

12.3 Evaluation of the potential application of immobilised anaerobic fungi in the production of cellulolytic enzymes

The conclusion made from the assessment of the literature that the physiology of cellulase synthesis in *Trichoderma* sp. can be exploited by immobilisation of the fungus provided no indication that immobilisation would be a successful process for the production of cellulase in anaerobic fungi. Published studies reporting on the physiology of cellulase production by anaerobic fungi have not examined the relationship between rhizomycelial growth and the synthesis of cellulase. Therefore, an investigation examining the success of immobilisation as applied to these organisms was therefore warranted.

*The relationship between cell growth and enzyme production.* This study has demonstrated that strains KXS1 and 478P1 can be both immobilised and used for the production of cellulolytic enzymes. However, the synthesis of β-glucosidase in these fungi was found to be growth-associated. The relationship between rhizomycelial growth and β-glucosidase production in free cell cultures of KXS1 and 478P1 (Figures 11.2 a and 11.2 b, respectively) showed that the maximum specific rate of β-glucosidase synthesis was proportional to the maximum specific rate of growth. The inability of strains KXS1 and 478P1 to produce β-glucosidase in a growth-dissociated manner provides an explanation as to why the full potential of immobilisation could not be employed. The unsuccessful practice of nutrient limitation (Chapter 7) used in immobilised KXS1 cultures attests to the close relationship between biomass and enzyme production as it was found that a complete medium was essential for immobilised growing anaerobic fungal cells to maintain enzyme production.

*Operational advantage of immobilised cell systems* Immobilisation not only allows for the exploitation of organisms that exhibit growth-dissociated synthesis of enzymes, it also has the operational advantage of using microbial cells
repeatedly and continuously. Although the physiology of cellulase synthesis in anaerobic fungi was found to be growth-associated immobilisation of the fungus offered the advantage of the repeat-batch use of cells with the accumulation of extracellular enzymes after each batch. Thus, operational gains were the key issues in assessing the potential use of immobilised anaerobic fungi in the production of cellulyolytic enzymes. The repeat-batch system was operationally more efficient than the free cell batch cultures because immobilisation removed the need of reculturing the cells for every single batch. Culturing of new cells requires time and preparation. Therefore, by reusing existing biomass in repeat-batch mode the amount of labour can be reduced, although it is important to note that this process uses the same amount of medium as a single batch.

*Free versus immobilised rhizomycelia.* A comparison of specific β-glucosidase production in immobilised and free cultures of strains KSX1 and 478P1 operating under the same conditions was made in Chapter 11. The comparison made here is based on 478P1 cultures since specific enzyme production in immobilised KSX1 cultures was difficult to gauge because β-glucosidase production probably involved both immobilised and free biomass in strain KSX1 (Figure 11.3a). Specific β-glucosidase production in batch 1 of immobilised 478P1 cultures was almost double of that produced by free cells in a single batch culture (Figure 11.3b). Specific β-glucosidase production in batches 2, 3 and 4 was lower, though specific enzyme production in these batches remained equivalent or slightly greater compared to the free cell culture.

### 12.4 Optimisation studies

A review of previous investigations reporting on the production of cellulase from anaerobic fungi (Section 1.2.1.7) has shown that there is scope for the improvement of enzyme production by modifying the culture medium and growth conditions. Optimisation studies of these factors may lead to improvements in enzyme production using immobilised cells.
Substrate. The choice of carbon source is an important parameter in optimising cellulase production. The investigations reviewed in the literature (Tables 1.3, 1.4 and 1.5) reported on the production of a number of fibre-degrading enzymes using various monomeric and polymeric substrates. Generally, cellulose has been considered to be the best inducer for producing the whole spectrum of cellulase components. The nature of cellulose can greatly affect the inductive formation of the cellulase complex. Easily metabolised substrates, such as cellobiose, support rapid cell growth, give high productivity, but yield less cellulase activity, while poorly metabolised substrates are not degraded sufficiently to support adequate growth and give low productivity but may yield more cellulase activity.

It has been reported that cellulase production is lower in media containing mono- or disaccharides than in media containing plant cell-wall polymers (see Section 1.2.1.6). Therefore, ball-milled cellulose was used in an effort to increase enzyme production. β-glucosidase levels in the first batch of immobilised KSX1 cultures using ball-milled cellulose as the substrate were found to be lower than those produced by cultures when cellobiose was used as the carbon source (compare Figures 9.1 and 9.3). Immobilising the fungus may have prevented it from adequately utilising the insoluble substrate.

Cellobiose was used in this study because it was soluble and produced high levels of β-glucosidase (compare β-glucosidase activity of straw-grown cultures of KSX1, Table 4.4, with that obtained in cellobiose-grown immobilised and free cultures of strain KSX1). In addition to β-glucosidase, levels of Avicelase and CM-cellulase were also produced by immobilised 478P1 cultures when grown on cellobiose indicating that the fungus was able to produce the whole spectrum of cellulases while growing on the disaccharide. Growth of KSX1 external to the bead established that immobilisation was an inconclusive culture strategy for this organism. Consequently, further enzyme characterisation apart from β-glucosidase was not carried out. It was therefore not possible to determine how the production of β-glucosidase activity correlated to Avicelase and CM-cellulase activity in immobilised KSX1 cultures. This presents certain limitations when
basing and comparing enzyme activities solely on β-glucosidase activity. Furthermore, differences that can occur in enzyme complexes when using different substrates cannot be gauged.

Cellulase production in immobilised cultures could be further improved by using a substrate that not only supports good growth but also produces high enzyme activity. The use of soluble celldextrins with degree of polymerization (DP) between 3 and 8 as a carbon source may significantly improve enzyme production while at the same time be appropriate substrates for use in an immobilised cell system. Chen and Wayman (1991) investigated inducers derived from waste paper for their use in cellulase production by T. reesei. Partially enzymatically saccharified newspaper containing celldextrins of various degrees of polymerization was found to be the most effective inducer of cellulase production, probably resulting from the wide spectrum of its breakdown products.

*Scale-up.* The use of fermentors is another important parameter in optimising the production of cellulase and allows for the scale-up of the process. Periodic replacement of medium was useful in the repeat-batch production of cellulolytic enzymes using immobilised anaerobic fungi in the serum bottle system used in this project. This method was technically simple to perform anaerobically, however, the use of serum bottles did not allow for the large-scale production of cellulase. Investigations into the use of repeat-batch fermentors using immobilised anaerobic fungi is one possible area open to examination. In order to achieve a high concentration of enzymes, a high concentration of cells in a fermentor must be achieved for long periods of time. An alternative process which maintains a high concentration of cells is immobilisation on an inert support. Production medium can be passed over these immobilised cells for the production of enzymes. Packed-bed reactors would be ideal in this situation and are designed for the continuous passage of production medium through the culture vessel and over the immobilised cells. Since immobilisation allows for the easy recovery of cells the spent medium can be removed and new medium can be replaced upon utilisation of the substrate. However, cell overgrowth has proven to
be a major problem to overcome for long-term operation in packed-bed reactors (Vega et al., 1988). In which case, it is unlikely to be useful in the large-scale production of cellulase enzymes using those strains of anaerobic fungi where cell release is a problem.

Other than the few reports that have investigated semi-continuous and continuous-flow culture (Hillaire and Jouany, 1990; Teunissen et al., 1992a; Zhu et al., 1996; 1997) little attention has been given to the growth of anaerobic fungi under these culture conditions. Most anaerobic fungal research in the laboratory has been conducted with cultures growing in closed batch cultures due primarily to the difficult task of setting up continuous-flow cultures with filamentous fungi. Immobilisation, therefore, has the potential to reduce the difficulties associated with filamentous fungi such as clogging of filter units.

*Scale-up potential using immobilised anaerobic fungi.* Immobilised polycentric fungi may hold particular promise in scale-up operations due to their operational superiority over the monocentric fungi since this type of fungus did not produce any viable growth in the culture medium. Although β-glucosidase production was low in immobilised 478P1 cultures compared to immobilised KSX1 cultures there is potential in obtaining polycentric strains that have higher enzyme activity than that displayed by strain 478P1. Borneman et al. (1989) showed that the β-glucosidase activities of two monocentric isolates, MC-1 and MC-2, were similar to those of three polycentric isolates, PC-1, PC-2 and PC-3 (Table 1.5). The β-glucosidase levels produced by these cultures are similar to those produced by immobilised KSX1 cultures. The enzyme yields produced by isolates PC-1, PC-2 and PC-3 provide evidence that it may be possible to select polycentric strains with higher cellulolytic capabilities than those few that were isolated in this present study by isolating a larger number and targeting those that have high cellulolytic capabilities.
In this project, anaerobic fungi, selected on the basis of their high cellulolytic capacity, were used in the form of immobilised growing cells to construct a process to produce cellulolytic enzymes. The ability of immobilised cells to produce significant amounts of extracellular enzyme over an extended fermentation period provided the basis of improvement over free mycelia systems. An assessment of the relationship between rhizomycelial growth and β-glucosidase production has revealed that the physiology of cellulase synthesis by anaerobic fungi is different to their aerobic counterparts and in attempting the immobilisation of these unique organisms a further understanding of the patterns of enzyme synthesis was obtained.
REFERENCES


APPENDICES
Time course of the release of radioactivity from U-[^14]C]-labelled bacterial cellulose by selected anaerobic fungi

A-1 SOLUBILISATION OF ^14C CELLULOSE BY ELX1

A-2 SOLUBILISATION OF ^14C CELLULOSE BY LS11
A-11 SOLUBILISATION OF $^{14}$C CELLULOSE BY WBR1

**REPLICATE:**
- No. 1
- No. 2
- No. 3

**Graph:**
- Y-axis: Solubilisation (DPM/mL x 10^3)
- X-axis: Time (hours)
**APPENDIX 2**

Table A.2  \textit{Maximum solubilisation rates of eliminated strains}

\begin{tabular}{|l|l|l|l|l|}
\hline
Strain name & Source & Genus & Ave. DPM/mL x 10^2 per 24h & Cellulose-azure rank \textsuperscript{1} \\
\hline
SX1 & cattle & \textit{Piromyces} & 60.47 & 1 \\
KSS1 & kangaroo & \textit{Piromyces} & 57.98 & 1 \\
LS13 & sheep & \textit{Piromyces} & 51.11 & 2 \\
DLF1 & deer & \textit{Neocalimastix} & 50.81 & 2 \\
LS12 & sheep & \textit{Piromyces} & 48.35 & 1 \\
KSF1 & kangaroo & \textit{Piromyces} & 47.74 & 1 \\
DLN2 & deer & \textit{Neocalimastix} & 43.58 & 2 \\
DLN1 & deer & \textit{Neocalimastix} & 40.52 & 2 \\
LN10 & sheep & \textit{Neocalimastix} & 39.75 & 2 \\
BLN1 & banteng & \textit{Neocalimastix} & 36.63 & 1 \\
BLN2 & banteng & \textit{Neocalimastix} & 36.11 & 1 \\
SX2 & camel & \textit{Piromyces} & 18.04 & 1 \\
\hline
\end{tabular}

RUN I: (DLS3 rate = 68.51 DPM/mL x 10^3/d)

\begin{tabular}{|l|l|l|l|}
\hline
KSF3 & kangaroo & \textit{Piromyces} & 39.49 & 1 \\
LN8 & sheep & \textit{Neocalimastix} & 36.44 & 1 \\
BLX1 & banteng & \textit{Neocalimastix} & 35.49 & 2 \\
SS1 & sheep & \textit{Piromyces} & 34.11 & 1 \\
LN6 & sheep & \textit{Neocalimastix} & 32.94 & 1 \\
LS10 & sheep & \textit{Neocalimastix} & 32.88 & 2 \\
LN1 & sheep & \textit{Neocalimastix} & 30.03 & 1 \\
LN9 & sheep & \textit{Neocalimastix} & 29.87 & 1 \\
LS8 & sheep & \textit{Neocalimastix} & 24.93 & 1 \\
LS9 & sheep & \textit{Neocalimastix} & 23.60 & 2 \\
SN1 & cattle & \textit{Piromyces} & 11.14 & 1 \\
LN3 & sheep & \textit{Neocalimastix} & n.d.\textsuperscript{2} & 2 \\
\hline
\end{tabular}

RUN II: (DLS3 rate = 46.48 DPM/mL x 10^3/d)

\begin{tabular}{|l|l|l|l|}
\hline
WLX1 & buffalo & \textit{Neocalimastix} & 48.06 & 1 \\
WRP1 & rhinoceros & \textit{Anaeromycetes} & 45.15 & 1 \\
ELX2 & eland & \textit{Neocalimastix} & 42.97 & 2 \\
DLX1 & deer & \textit{Neocalimastix} & 41.56 & 3 \\
DLX3 & deer & \textit{Neocalimastix} & 36.58 & 1 \\
BSX1 & banteng & \textit{Piromyces} & 36.33 & 1 \\
CAR1 & camel & \textit{Orpinomyces} & 29.54 & 3 \\
RNX1 & rhinoceros & \textit{Caecomyces} & n.d. & 1 \\
RSX2 & rhinoceros & \textit{Piromyces} & n.d. & 2 \\
KSS2 & kangaroo & \textit{Piromyces} & n.d. & 1 \\
\hline
\end{tabular}

RUN III: (DLS3 rate = 50.91 DPM/mL x 10^3/d)

\begin{tabular}{|l|l|l|l|}
\hline
\textsuperscript{1} Rank calculated as maximum rate of colour release \\
\textsuperscript{2} Not determined. Growth of these cultures on ^14C-cellulose was very poor

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Time course of the release of radioactivity from U-$^{14}$C-labelled bacterial cellulose by eliminated anaerobic fungi:
Run I

A-12 SOLUBILISATION OF $^{14}$C CELLULOSE BY SX1

A-13 SOLUBILISATION OF $^{14}$C CELLULOSE BY KSS1
A-16 SOLUBILISATION OF $^{14}$C CELLULOSE BY LS12

Replicate:
- No. 1
- No. 2
- No. 3

Solubilisation (DPM/mL x 10^3)

Time (hours)

A-17 SOLUBILISATION OF $^{14}$C CELLULOSE BY KSF1

Replicate:
- No. 1
- No. 2
- No. 3

Solubilisation (DPM/mL x 10^3)

Time (hours)
A-22 SOLUBILISATION OF $^{14}$C CELLULOSE BY SX2

REPLICATE:
- No. 1
- No. 2
- No. 3

A-23 SOLUBILISATION OF $^{14}$C CELLULOSE BY DLS1

REPLICATE:
- No. 1
- No. 2
- No. 3
Time course of the release of radioactivity from U-[14C]-labelled bacterial cellulose by eliminated anaerobic fungi: Run II

A-24 SOLUBILISATION OF 14C CELLULOSE BY KSF3

A-25 SOLUBILISATION OF 14C CELLULOSE BY LNS

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A-26 SOLUBILISATION OF $^{14}$C CELLULOSE BY BLX1

REPlicate:
- No. 1
- No. 2
- No. 3

Solubilisation (DPM/mL x 10^3)

Time (hours)

A-27 SOLUBILISATION OF $^{14}$C CELLULOSE BY SS1

REPlicate:
- No. 1
- No. 2
- No. 3

Solubilisation (DPM/mL x 10^3)

Time (hours)
A-28 SOLUBILISATION OF $^{14}$C CELLULOSE BY LN6

REPLICATE:
- No. 1
- No. 2
- No. 3

Solubilisation (DPM/mL x 10^4)

Time (hours)

A-29 SOLUBILISATION OF $^{14}$C CELLULOSE BY LS10

REPLICATE:
- No. 1
- No. 2
- No. 3

Solubilisation (DPM/mL x 10^4)

Time (hours)
Time course of the release of radioactivity from U-[\textsuperscript{14}C]-labelled bacterial cellulose by eliminated anaerobic fungi: Run III

A-36 SOLUBILISATION OF \textsuperscript{14}C CELLULOSE BY WLX1

![Graph showing solubilisation of \textsuperscript{14}C cellulose by WLX1 over time]

A-37 SOLUBILISATION OF \textsuperscript{14}C CELLULOSE BY WRP1

![Graph showing solubilisation of \textsuperscript{14}C cellulose by WRP1 over time]
A-38 SOLUBILISATION OF $^{14}$C CELLULOSE BY ELX2

![Graph showing solubilisation of $^{14}$C cellulose by ELX2 with replicates No. 1, No. 2, and No. 3.](image)

A-39 SOLUBILISATION OF $^{14}$C CELLULOSE BY DLX1

![Graph showing solubilisation of $^{14}$C cellulose by DLX1 with replicates No. 1, No. 2, and No. 3.](image)
A-40 SOLUBILISATION OF $^{14}$C CELLULOSE BY DLX3

REPLICATE:
- No. 1
- No. 2
- No. 3

Solubilisation (DPM/mL x 10^3)

Time (hours)

0 20 40 60 80 100 120

A-41 SOLUBILISATION OF $^{14}$C CELLULOSE BY BSX1

REPLICATE:
- No. 1
- No. 2
- No. 3

Solubilisation (DPM/mL x 10^3)

Time (hours)

0 20 40 60 80 100 120