STUDIES ON ANAEROBIC FUNGI FROM COW AND BUFFALO RUMEN WITH RESPECTTO CELLULOLYTIC ACTIVITY AND FIBRE DEGRADIBILITY

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ANIMAL NUTRITION

BY
ASHIS KUMAR SAMANTA

DIVISION OF DAIRY CATTLE NUTRITION
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)
KARNAL – 132001 (HARYANA), INDIA

1997

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DEDICATED

TO

KRISHNA

ABSTRACT

The microbiology of different segments of digestive tract, especially the rumen is extremely complex due to the presence of large number of organisms, the shifting populations and interactions among the various microbial groups. During mid seventies, the discovery of rumen flagellates as of fungal origin, dismissed the earlier hypothesis of non-existence of anaerobic fungus in the nature. The present investigation confirmed the presence of anaerobic fungi in rumen and caecum of riverine buffalo and cattle. These fungi were able to attack, weaken and partially or fully degrade plant fibre. In riverine buffalo, the population ranged from 1.10 x 10^3 to 5.41 x 10^3/ml while in the case of cattle, it ranged from 1.29 x 10^3 to 5.14 x 10^3/ml. In fecal samples of riverine buffalo, the population ranged from 6.5 x 10^3 to 6.8 x 10^3/g fecal DM. The dietary combination of oats fodder and wheat straw in equal proportion gave the maximum fungal population. Both monocentric and polycentric fungus were present in the rumen of buffalo and cattle. Out of 17 selected isolates, B3 isolate (*Piromyces* sp.) possessed maximum endoglucanase activity (17.5929 mIU/ml) on cellobiose media. Dried fungal cells were also good source of certain critical amino acids, viz. lysine (7.17 g/100 g total amino acids) and methionine (3.81 g/100 g total amino acids). The pH optima for endoglucanase, cellobiase and xylanase were 6.5, 6.0 to 6.5 and 6.5, respectively.

Crude enzyme from extracellular culture supernatant was fractionated with ammonium sulfate (60% saturation). The precipitated enzyme was attempted for purification on hydroxylapatite, gel filtration (superose-12) and anion exchange (mono Q) columns. With 60% ammonium sulfate, about 58% yield was obtained with only two-fold purification. This step helped in concentrating enzyme for subsequent purification on chromatographic columns. Subsequent purification on either hydroxylapatite column or superose-12 (gel filtration) column resulted in considerable loss in enzyme activity with little improvement in purification. However, purification on mono Q column led to separation of four isoenzymes of endoglucanase. The relative abundance of these isoenzymes were 18.2% (peak C1), 17.1% (peak C2), 21.8% (peak C3) and 43.3% (peak C4). Purification of xyalanse was attempted by fractionation with ammonium sulfate and separation on mono Q anion exchanger. Xyalanase activity was resolved into two peaks on mono Q column. Xyalanase in peaks X1 and X2 were purified to 129 and 17 folds and their relative abundance were 36.3% and 63.7%, respectively. Endoglucanase and xylanase existed in several isomeric forms. On SDS-PAGE endoglucanase gave four activity bands, having MW of 100 kDa, 66 kDa, 39.8 kDa and 33 kDa. Xylanase gave four activity bands with the MW of 177 kDa, 100 kDa, 63 kDa and 50 kDa.

Under *in vitro* system, with wheat straw as substrate, the total gas production ranged from 57 to 99 ml by different types of inoculum used during 48 hours of incubation. The values for IVDMD and IVOMD differed significantly among various treatments, viz. mixed rumen inoculum (T1), fungi only (T2) and bacteria only (T3). The addition of fungal isolate (B3) as probiotic did not give any significant effect regarding degradability or TVFA production over mixed rumen inoculum. Anaerobic fungi have been evolved over millions of years to occupy a unique niche in the digestive tract of herbivorous animals. So, we should judiciously select the dietary combinations for ensuring maximum fungal population in order to get an optimum benefit out of these organisms.

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CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON ANAEROBIC FUNGI FROM COW AND BUFFALO RUMEN WITH RESPECT TO CELLULOLYTIC ACTIVITY AND FIBRE DEGRADABILITY"** submitted by **Mr. ASHIS KUMAR SAMANTA** in partial fulfilment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL NUTRITION** of the National Dairy Research Institute (Deemed University), Karnal, India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

25/6/97

Dated 25th June**, 1997**

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MAJOR ADVISOR & CHAIRMAN
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STUDIES ON ANAEROBIC FUNGI FROM COW AND BUFFALO RUMEN WITH RESPECT TO CELLULOLYTIC ACTIVITY AND FIBRE DEGRADABILITY

By
ASHIS KUMAR SAMANTA

A thesis submitted to
the National Dairy Research Institute (Deemed University), Karnal
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Chapter 1

Introduction

1. INTRODUCTION

Ruminants as a class of mammals stand apart and unique in the sense that by virtue of the vast hoards of microbial population inhabiting their large dilated segment of the digestive tract, i.e., reticulorumen, they are able to degrade fibrous feed like cereal straws and other cellulosic materials into energy yielding substrates, for their utilization by the host animal after absorption. The discovery of rumen protozoa in 1893 was the first identification of microorganism in the ruminant stomach. The role of bacteria in the fermentation of plant material became well known as a result of the genius of Pasteur. It was inferred before the turn of the century that rumen microorganisms fermented fibre anaerobically and the end products of fermentation are acids and gases. Today we know that these acids are mostly the volatile fatty acids and the gases are mostly carbon dioxide, methane, hydrogen, etc.

Rumen bacteria and protozoa have been extensively studied with respect to their classification, characterization and the enormous metabolic role they play in the degradation of feed material. Hungate (1966) has done a pioneering work in this direction, evolving also the classified methodology for the cultivation of the rumen organisms. Another class of rumen microorganisms i.e., rumen flagellates were noticed long back but received attention and prominence only recently by the researchers. Rumen flagellates, possessing a spherical to ovoid cell and a single flagellum were described by Liebetanz (1910) and by Braune (1913). Since then, very few references to this type of organism have been made in the literature and no mention was made by Hungate (1966) or by Jensen and Hammond (1964). These flagellates have been reported to be fairly abundant in young calves before the ciliates develop (Eadie, 1962) and in adult sheep their number ranged from 2 x 10^3 to 3 x 10^4 per ml (Warner, 1966). It appears that these organisms have been overlooked, perhaps due to the lack of knowledge of their identity. But it was only in 1975, when Orpin first referred that flagellates, free living in rumen ecosystem were in fact zoospore of phycomycete fungus, thus, setting at rest the earlier notion that these are of protozoal origin. The possession of rhizoid by the reproductive body (sporangium) confirmed that the organism might be fungal rather than protozoal; for, protozoal reproductive bodies do not normally possess rhizoids. Fungi possessing life histories (alternating between two phases, i.e., the motile flagellated phase and the non-motile vegetative reproductive phase) were identical with that of aquatic phycomycetes. However, Orpin's flagellates possessed more flagella (up to 14) than the maximum of 2 previously recorded for aquatic phycomycete zoospores (Sparrow, 1960) and 2 and 9 recorded for *Callimastix cyclopis*, a member of the Blastocladiales (Varva and Joyon, 1966). The latter, too, were previously regarded as a flagellate protozoa.

It is now well established that fungi are also permanent member of rumen anaerobic ecosystem along with bacteria, protozoa, yeast and phage particles. In the rumen, anaerobic fungi are closely associated with the fibre particles, which they rapidly colonize after they have been ingested by the animal (Bauchop, 1981). The fungi attack preferentially the lignified tissues such as sclerenchyma and the fibres of the vascular bundles (Bauchop, 1979, 1981; Grenet and Barry, 1988). It is now well known that fungi possess strong hydrolytic activities against several polymers of the plant cell walls except lignin (Orpin and Joblin, 1988; Fonty and Joblin, 1990). The important members of rumen fungi are *Neocallimastix frontalis* (Orpin, 1975), *Sphaeromonas communis* (Orpin, 1976), *Piromonas communis* (Orpin, 1977b), *N. patriciarum* (Orpin and Munn, 1986), *N. joyonii* (Breton *et al.*, 1990), *Orpinomyces bovis* (Barr *et al.*, 1989), *Ruminomyces elegans* (Ho *et al.*, 1990), *Piromyces spiralis* (Ho *et al.*, 1993b). However, their efficiency in degrading the plant nutrients varies according to the species and strain. The cellulolytic activity of *Sphaeromonas* is generally lower than that of *Neocallimastix* and *Piromonas* (Gordon and Philips, 1989). During the degradation and fermentation of cellulose by the anaerobic fungus, the end products are formate, acetate, lactate, ethanol, carbon dioxide and hydrogen (Orpin and Joblin, 1988; Fonty and Joblin, 1990). Because of their ability to produce hydrogen, rumen fungi interact with methanogenic bacteria (Bauchop and Mountfort, 1981; Mountfort *et al.*, 1982; Fonty *et al.*, 1988; Joblin *et al.*, 1990; Marvin-Sikkema *et al.*, 1990). In the event of interaction with methanogens, fungal cellulolytic activity increases and the fungal metabolism is shifted towards the production of acetate at the expense of ethanol and lactate. Because of their high cellulolytic and hemicellulolytic activities, rumen fungi may occupy the same ecological niches as the cellulolytic bacteria and either compete or act synergistically with them to efficiently degrade the feed ingested by the animal.

At present the research work on the microbiological and biochemical aspects of anaerobic fungi in the rumen of cattle is going on in a few laboratories to see its precise role in fibre degradation, especially its cellulase system and its availability as a potential organism for enhancing cellulase activity in rumen. This is being done with the twin objectives of, either to go for their large scale cultivation and then use as a probiotic feed additive or alternatively use the fungi as a source of cellulase gene for the genetic manipulation of rumen bacteria or the fungi itself.

The precise role and overall contribution of anaerobic fungi to the degradation of fibrous plant substrates in the rumen remains unclear (Stewart *et al.*, 1990). If we consider the buffalo, there are only a few reports available which are again on swamp buffalo mostly. The riverine buffaloes of the Indian subcontinent have been shown to perform better on fibrous diets than cattle, by virtue of their slightly different bacterial and protozoal profile in rumen (Pradhan *et al.*, 1991). It may be, therefore, worthwhile to undertake the comparative studies on the two species with respect to microbiological and biochemical aspects of rumen fungi, about which the information is totally lacking today. Thus, the present investigation was carried out with the following objectives;

1. To enumerate total zoospore count in the rumen of cow and buffalo kept on different dietary roughage:concentrate ratios.

2. To examine and select fungal isolates from cow and buffalo rumen for potential cellulolytic activity.

3. To attempt partial purification of the cellulase enzyme from the most promising cellulolytic fungal isolates and to study its fibre degrading ability *in vitro*.

Chapter 2

Review of Literature

2. REVIEW OF LITERATURE

Ruminants are able to use plants with a high fibre content as feedstuffs, because of the breakdown of these materials by a complex microbial population in the reticulo-rumen, which is practically analogous to a large fermentation vat. For many years, only specific groups of anaerobic bacteria and, perhaps to a lesser extent, protozoa were thought to be responsible for this degradation. In the mid 1970s, however, the existence of anaerobic fungi as one of the rumen inhabitants was reported (Orpin, 1975) and latter on their active role on fibre degradation was established (Orpin, 1977a, 1977b). Before the discovery of Orpin's flagellate, even just the idea of the existence of anaerobic fungi, lacking mitochondria, possessing hydrogenosome surviving through mixed acid fermentation, was instantly dismissed. Because, one of the major metabolic differences between molds and bacteria was thought to be that there were no anaerobic molds, either obligatory or facultative in nature (Foster, 1949). This belief was proved wrong after a series of experiments led by Orpin. He reported on several aspects of the fungi, including their obligately anaerobic nature, the presence of chitin in the cell walls and their colonization on plant fibre (Orpin, 1975, 1976, 1977a, 1977b). Rumen fungi were able to grow on rumen simulating media, indicating the optimum pH about 6.5 to 6.7 and temperature around 39°C and that their nutrient requirements were met by the media. The semisynthetic basal media (Caldwell and Bryant, 1966), in which yeast extract, tryptone and a mixture of volatile fatty acids replaced rumen fluid, was also able to support the growth of anaerobic fungi. However, absolute requirements for growth have not been determined for most of the fungi. Orpin (1989) observed that *N. patriciarum* requires heme, D-biotin and thiamin or its precursors, sulfur is required in reduced form and the nitrogen requirement is met through ammonium ions or amino acids.

Rumen fungi extensively colonized the lignin containing tissues of the forages and appeared to be active in fiber degradation. Fungi were more prevalent in ruminants fed high fibre diets than in those fed less fibrous ones (Bauchop, 1979, 1981). In 1983, Bauchop remarked: "Our failure to recognize the anaerobic fungi for what they were, was attributable not only to mycological dogma, but also to the practice among rumen microbiologists of working with strained rumen fluid and discarding the solid digesta with which the vegetative stage of the fungus are associated". However, with regard to rumen flagellates in buffalo, there is still paucity of information on their morphological, taxonomical, biochemical and metabolic aspects. In the following text, an attempt has been made to compile whatever information could be collected and present a review on the different aspects of anaerobic fungi:

2.1 TAXONOMY OF ANAEROBIC FUNGI

Because of the unusual nature of the zoospores of some of the first isolates and the obligatory anaerobic nature of all isolates, the taxonomic position of these organisms has required some modifications by fungal taxonomists. A new family is created (Neocallimasticaceae) to accommodate these organisms (Heath *et al.*, 1983). Uptil now, five different genera are recognised based on monocentric or polycentric thallus, filamentous or bulbous rhizoids and mono or polyflagellated zoospores (Theodorou *et al.*, 1996), whereas species are differentiated according to zoospore ultrastructure (Munn *et al.*, 1988; Munn, 1994). However, there is little consensus on the characteristic features for defining the genera and species of anaerobic fungi, and the current criteria are questionable (Munn, 1994; Trinci *et al.*, 1994). According to Barr (1988) and Barr *et al.* (1989), the current systematics of rumen anaerobic fungi is as follows:

Division	Eumycots
Subdivision	Mastigomycotina
Class	Chytridiomycetes
Order	Spizellomycetales
Family	Neocallimasticaceae
Genera	*Neocallimastix, Piromyces, Caecomyces, Anaeromyces and Orpinomyces.*

Neocallimastix, Caecomyces and *Piromyces* are monocentric whereas *Anaeromyces and Orpinomyces* are polyentric (Trinci *et al.*, 1994). However, *Caecomyces* are sometimes polycentric also. Other characteristic features of different genera are as follows; *Neocallimastix*: polyflagellated zoospores and having extensive filamentous rhizomycelium; *Piromyces*: uniflagellated zoospores, filamentous rhizomycelium; *Caecomyces*: uniflagellated zoospores, spherical holdfasts; *Anaeromyces*: uniflagellated zoospores, filamentous rhizomycelium; *Orpinomyces*: Polyflagellated zoospores, filamentous rhizomycelium.

Analysis of sequences of 18s ribosomal RNA from 18 strains was used to clarify the phylogenetic relationships among anaerobic fungi, aerobic chytrids and other eukaryotes (Theodorou *et al.*, 1996). There was a similarity to the extent of 97 to 99% in rRNA sequence between a limited range of species of anaerobic fungi (Dore and Stahl, 1991). Bowman *et al.* (1992) placed the anaerobic fungi within the chytridiomycetes. Based on the analysis of the highly conserved internal transcribed spacer 1 (ITS1) region and its adjacent sequences from 18s rRNA gene clusters, Li and Heath (1992) and Li *et al.* (1993) also classified the anaerobic fungi under Chitridiomycetes. However, the possession of flagella by zoospores is not a trivial difference and suggest that a family, separate from the Neocallimasticaceae (which will continue to contain polyflagellated anaerorbic fungi), should be erected to accommodate the monoflagellated genera (Munn, 1994). But the systematics of the anaerobic fungi requires further refinement as information will be collected on these unusual organisms.

2.2 ESTABLISHMENT OF ANAEROBIC FUNGI IN THE RUMEN

A newly born ruminant lacks the microbial flora found in the adult and acquires it only after contact with the older animals. Large protozoa do not form the cysts and die rapidly in air (Hobson, 1971) and are thought to be transferred by direct contact with saliva. Rumen bacteria are transferred between animals through saliva, aerosols and also the fecal material (Hungate, 1966). Fonty *et al.* (1987) found that anaerobic fungi became established in normal young lambs within 8 to 10 days after birth in the presence of adult animals, although their survival was not guaranteed, as young animals did not possess a fully functional rumen. Lowe *et al.* (1987d) demonstrated that anaerobic fungi could be isolated from the esophageal preparations of saliva and from feces in sheep. This indicated that mode of the transmission of the fungi could be by direct oral contact, by aerosol and through faecal contamination.

2.3 LIFE CYCLE AND REPRODUCTION'

Anaerobic rumen fungi showed asexual type of reproduction during which sporocyst and zoospores were formed within 26 to 32 hours and it was similar to that of the chytids (Orpin, 1975; Lowe *et al.*, 1987a). The life cycle of anaerobic fungi consists of an alteration of generations between the cellulolytic, vegetative reproductive stage, which occurs principally on the digesta particles (Orpin, 1975; Orpin and Letcher, 1979) and the motile flagellated zoospore stage, which occurs principally in the rumen liquor. The cycle starts with the differentiation of zoospores in the sporocyst and their release into the rumen shortly after offering feed to the animal. The reproductive bodies (sporocyst) are stimulated to produce and liberate zoospores in response to soluble components of the host's diet. Flagellates remain motile in rumen fluid for several hours before their settlement and encystment over plant fragments (Lowe *et al.*, 1987a; France *et al.*, 1990). Generally, within 30 to 60 minutes after release from the sporangium, the zoospores get attached to plant fragments, mainly on stomata, damaged tissues and then loose their flagella and become encysted (Orpin, 1975; Heath *et al.*, 1986). The germination of spore is characterized by an increase in sporal cell size and by the formation of highly branched or bulbous rhizoids. The rhizoids penetrate the plant material, produce hydrolytic enzymes and take the nutrients from the medium. After 14 to 20 hours, the zoospores cease to grow and a septum is formed at the base, delimiting the zoosporangium from the rhizoidal system (Lowe *et al.*, 1987b). Cessation of zoosporangial growth marked the onset of zoosporogenesis. In the animals fed once a day, liberation of zoospores from the vegetative stages occurs soon after the animal is fed. With *Neocallimastix*, a maximum population density of zoospore was recorded within 15 to 30 minutes after feeding but with *Sphaeromonas* and *Piromonas*, the peak occurred about 60 minutes after feeding (Orpin, 1975, 1976). Haem appears to play a major role in zoosporogenesis of rumen fungi but under *in vitro* conditions, zoosporogenesis is not stimulated. This indicates that haem may act synergistically with other dietary components for stimulating zoosporogenesis (Orpin, 1977b).

In the monocentric type, the fungi develop either endogenously or exogenously. In the endogenous development, encysted zoospore retains the nucleus and gets transformed into zoosporangium, whereas during exogenous development, the nucleus is migrated out of the zoospore and the zoosporangium is formed in the germ tube or sporangiophore (Karling, 1978; Barr *et al.*, 1989; Ho *et al.*, 1993c). In both the cases, only one sporangium is formed per thallus. Nuclei are preseht and multiply within the zoosporangium developed endogenously. This causes the rhizoidal system to be devoid of nucleus. Thus, in monocentric fungi, after zoosporogenesis, the remaining thallus is autolyzed without further development (Lowe *et al.*, 1987a, 1987b).

In polycentric fungi, following encystment, the zoospore gets transformed into germinating rhizoid where nucleus is also migrated (Barr *et al.*, 1989; Gaillard *et al.*, 1989). The zoospore then became redundant (Breton *et al.*, 1990). A highly branched, nucleated rhizomycelium is developed. Zoosporangium is transformed into sporangiophores either singly or in groups upto six. The development of sporangia may be either intercalary or terminal on the rhizoids (Ho *et al.*, 1990). Unlike monocentric fungi, polycentric fungi possessed indeterminate life cycles and were less dependent on the formation of zoospores for their continued survival.

2.4 DISTRIBUTION OF ANAEROBIC FUNGI

Anaerobic fungi have been isolated worldwide from different countries from numerous foregut and hindgut fermenting mammalian herbivores. It was first isolated in the United Kingdom from the rumen of sheep. Later on their presence in digestive tract were reported from New Zealand, Australia, Canada, Czechoslovakia, China, Denmark, Ethiopia, France, Holland, Indonesia, Japan, Malaysia, Norway, Russia, Tanzania, Spain, U.S.A. etc. Anaerobic fungi are present in ruminants (cattle, water buffalo, sheep, goat), ruminants like animals (llama, vicuana, impala etc.) and herbivorous animals possessing a hindgut fermentation (horse) (Orpin and Joblin, 1988; Bauchop, 1989; Milne *et al.*, 1989; Teunissen *et al.*, 1991). These were also reported in the hindgut of largest herbivore, i.e., elephant (Milne *et al.*, 1989) and the smallest herbivore, i.e. blue duiker (Dehority and Varga, 1991). Discovery of anaerobic fungi from feces of sheep (Lowe *et al.*, 1987d) led to the investigation of these organisms from other herbivores. Later on, these anaerobic organisms were also found to be present in the digestive tract of rodent, mara (Teunissen *et al.*, 1991). Efforts to isolate these tiny creature from anaerobic muds and landfill sites proved unsuccessful (Orpin and Joblin, 1988; Theodorou and King-Spooner, 1989). Thus, these group of fungi were present in typical micro-anaerobic environment of digestive tract of different herbivores only.

Feces contained substantial population of anaerobic fungi, which declined very slowly after drying in such a way that organisms identical in appearance to ruminal chytridiomycetes could still be cultured up to 10 months later (Theodorou *et al.*, 1990a). Related studies showed that zoospores and vegetative thalli, are highly sensitive to periods of exposure to low temperatures and air (Lowe *et al.*, 1987b; Trinci *et al.*, 1988; Milne *et al.*, 1989). Motile zoospores were not detected in fecal contents, although structures like fungal zoosporangium was observed. Thus, it was proposed that the life cycle of anaerobic chytridiomycetes, like that of many of their aerobic counterparts, consisted of three stages: the motile zoospore, the vegetative thallus and the aerotolerant survival stage, i.e., resistant zoosporangium (Theodorou *et al.*, 1996). Two other reports suggested that anaerobic fungi produced survival structures (cysts or spores). Although survival was not demonstrated, Orpin (1981) reported on the appearance of thick walled zoosporangia in the caecal contents of the horse. Perhaps more significant was the report of Joblin (1981), which showed that anaerobic fungi could be maintained in sisal broth up to 7 months. A survival stage in ruminal fungi would explain their transfer through the entire digestive tract. These survival structures might be disseminated via feces to herbage in the field and thus enabling the transfer of anaerobic fungi between herbivores. With respect to host nutrition, loss of fungal biomass from the digestive tract via feces represents yet another drain on the conversion efficiency of plant biomass in the ruminal ecosystem. Anaerobic fungi isolated from hindgut fermenters were assigned to genera *Caecomyces* (Gold *et al.*, 1988) and *Piromyces* (Teunissen *et al.*, 1991). However, the isolation of *Neocallimastix* strain from the tapir (*Tapirus terrestris*), a hindgut fermenter, suggested that the genus *Neocallimastix* was not restricted to foregut fermenting herbivores. These organisms were also found in omasum, abomasum, small intestine, large intestine, caecum and rectum (Davies *et al.*, 1993).

2.5 ISOLATION OF ANAEROBIC FUNGI

In order to isolate a pure culture of anaerobic fungi from its natural environments, it is essential to use repeated subculture, antibiotics and some form of physical separation such as the growth of isolated colonies on agar medium (Theodorou *et al.*, 1996). Hungate's roll tube technique (Hungate, 1966) for culturing bacterial anaerobes was ideally suited to study these fungi. Orpin (1975) isolated *N. frontalis* from sheep rumen by overlaying the sloppy agar medium (containing antibacterial antibiotics) with 0.2 ml of the large particulate (LP) fraction of rumen fluid, followed by gassing with oxygen free carbon dioxide and incubating at 39°C. Within 2 days of incubation, *Neocallimastix* flagellates and fungus like growth were observed within the medium. The top 5 cm of medium was then removed aseptically by aspiration and drops of the underlying sloppy agar containing Neocallimastix flagellates were overlaid on fresh culture medium. The new culture was stoppered and shaken gently to distribute the inoculum in the top of medium and was incubated at 39°C. Successive subcultures eliminated bacteria leaving *Neocallimastix* as the sole flagellates.

The isolation procedure of Joblin (1981) and Akin *et al.* (1983) involved straining rumen fluid through muslin cloth, mixing the filtrate with molten agar medium plus antibiotics and preparing roll tubes. Bauchop and Mountfort (1981) used strained rumen fluid to make enrichment cultures in sloppy agar medium containing antibiotics. After three subcultures in this medium, the culture was transferred to liquid medium and single colony was picked up with a syringe and washed in buffer. Methanogenic bacteria, which were not eliminated by the first antibiotic treatment, were then removed using chloramphenicol. Lowe *et al.* (1985) isolated anaerobic fungi from rumen digesta of sheep and cattle and purified using a plate culture technique. As per this method, fungal enrichment was achieved by incubating 1 ml rumen digesta with 0.1 g milled barley straw and 10 ml liquid medium B containing antibiotics and lysozyme. After 5 days incubation, culture were transferred to the anaerobic chamber and colonized particles were used to inoculate plates of cellulose agar containing antibiotics and lysozyme. Fungal colonies having diameter upto 2 cm appeared following the incubation of the plates at 39°C. This isolation procedure depended upon the ability of rhizoids to grow radially outwards from the inoculum and from contaminating bacteria, Sijtsma and Tan (1993) isolated *Neocallimastix* and *Piromyces* from the rumen of sheep by using medium M1 containing cellobiose (0.5%) in the presence of penicillin G (100 μg per ml), chloramphenicol (50 μg per ml) and streptomycin sulphate (200 μg per ml) to suppress bacterial growth. After 8 days of incubation at 39°C, thallus, several zoosporangium and zoospores were observed. After a serial dilution, a pure fungal culture was obtained.

2.6 ENUMERATION

During studies on the diurnal changes in the concentrations of microorganisms in the rumen of sheep fed limited diets once daily, there was a significant increase in the population density of the flagellate, *Callimastix frontalis*, after one hour the sheep were fed. The population density then dropped to a minimum at 8 to 10 hours after feeding, after which little change occurred until the sheep was fed again (Warner, 1966). The number of *Callimastix* in any sample could be estimated by counting microscopically all the callimastix in 0.01 ml of the sample (Orpin, 1974). *Callimastix frontalis* was morphologically distinct (Braune, 1913) from the other flagellate protozoa, with characteristic movements and it was easily counted. Through this direct microscopic count, it was estimated that the population density of flagellates ranged from 3.9×10^5 to 2.2×10^7 per ml in case of defaunated sheep; while in faunated sheep it ranged from 4.2×10^3 to 6.4×10^6 per ml (Orpin, 1976). Animals consumed appreciable amounts of fibre, large number of fungal sporangia were found attached to stem fragments selected at random from rumen contents of sheep before morning feeding (Bauchop, 1979). Highest numbers were found with chaffed lucerne diet containing stalky material than pelleted lucerne diet. Phycomycetous fungal sporangia or zoospores were not detected microscopically in rumen contents of sheep on diets low in fibre.

The number of fungi in strained rumen fluid was determined by diluting samples from the stirred solution through roll tubes containing antibiotics. At 6 hours after inoculation, discrete fungal thalli were visible under a dissecting microscope. A count of the thalli present after 20 hours indicated that the rumen fluid sample contained 2×10^4 viable zoospores per ml (Joblin, 1981). But in glucose containing tube the number of zoospores detected was approximately twice of that found in tubes containing either cellobiose or cellulose.

Ruminal fungi associated closely with fiber during forage degradation in ruminants (Bauchop, 1981). Sporangia of various morphologies were produced by fungi which colonized plant cell walls. These sporangia were enumerated by light microscopy (Akin, 1987). The total number of sporangia on both cut edges of a leaf blade was divided by leaf blade width to give the sporangial number per millimeter of leaf cut edge. For evaluation of the relative numbers of sporangia per site on the leaf surfaces, the numbers of sporangia within five randomly selected field (x 20 objective lens; area, 0.55 mm^2) on the mid-rib surface of the blade were averaged. The number of sporangia on leaf blades at cut edge (per millimeter) ranged from 17.2 ± 8.5 to 25.4 ± 13.2 and the value at surface (per site) ranged from 8.5 ± 1.3 to 9.6 ± 7.9 following 6 to 48 hours of *in vitro* studies. However, monitoring sporangia did not take into account the biomass of rhizomycelia which were extensive at certain time of their life cycle. Thus, Akin (1987) suggested the use of chitinase for assessment of fungi associated with plant fiber.

Ushida *et al.* (1989) developed *in situ* method for enumeration of fungal population size where agar strips (size 3 x 3 x 15 mm) containing glucose, xylose, cellulose, xylan, cysteine hydrochloride were incubated in rumen through nylon bag (100 μm size) for 24 hours. Fungal zoosporangia developed on the strips were counted in calibrated fields. The number ranged from 5.7 x 10^3 to 12 x 10^3 per cm^2. Based on the technique of most probable numbers, an end point dilution procedure was developed to enumerate anaerobic chytridiomycetes as thallus forming units (Theodorou *et al.*, 1990a). This method did not distinguish between zoospores and thalli, but permitted enumeration of fungal populations with respect to their ability to digest plant cell walls. Through this technique, the estimated population of fibrolytric anaerobic fungi in reticulorumen ranged from 2.20 x 10^4 to 4.6 x 10^4 thallus forming unit (TFU) per gram of ruminal dry matter, whereas total population was within the range of 0.94 x 10^8 to 8.37 x 10^8 TFU per gram.

Population sizes of rumen anaerobic fungi were generally estimated by the roll tube method using appropriate antibacterial agents (Joblin, 1981). The estimates from this method were, therefore, based on the colony forming ability of fungal zoospores free of bacterial interference. However, viable zoospore counts might not necessarily correlate well with the fungal population size (Bauchop, 1981). Moreover, fungal muralytic activity increased when bacteria were eliminated (Windham and Akin, 1984; Ushida *et al.*, 1989), suggesting the risk of over-estimation of fungal population size by this method. Thus, direct counts of fungal colonies on plant fragments incubated in the rumen might be a more direct and suitable method for estimation of relative size of fungal populations.

2.7 ENUMERATION MODEL

Zoospores and thalli remained at steady state in the rumen (France *et al.*, 1990). With the combined knowledge of passage rate and life cycle, the number of digesta associated thalli could be determined from the concentration of zoospores in rumen fluid. Fungal life cycle could be compartmentalized as in Fig. 2.1. According to differential calculations developed by Theodorou *et al.* (1996), the number of digesta associated thalli ranged from 50 to 170 per cent of the number of zoospores.

Another model, i.e., particle size model was conceptualized by France and Theodorou (1994). This model assumed that the number of colonized particles in the rumen was always less than the number of fungal thalli attached to them. Thus, if it was possible to fragment colonized particles in such a way that each fragment ultimately contained only one fungal thallus, then enumeration of the thallus population by counting the fragmented colonized particles was comparatively easy.

2.8 PRESERVATION OF FUNGAL CULTURE

Rumen anaerobic phycomycetes remained viable only for 2 to 5 days in glucose broth (Orpin, 1976) and 7 to 10 days in sisal broth (Bauchop, 1979). Cultures in broth or an agar with and without glycerol as a cryoprotectant failed to retain viability after storage at low temperature (-60°C). However, apparently senescent cultures on sisal agar which was stored at 39°C could be re-established by the addition of glucose.

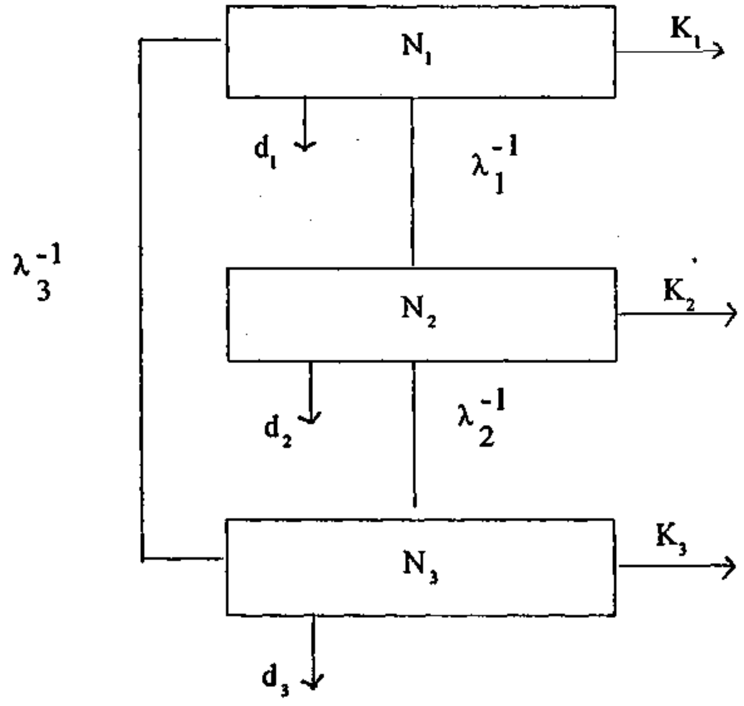

Fig. 2.1 Compartmental model for enumeration of anaerobic fungal population. N1, N2 and N3 represent population of zoospores, immature thalli and mature thalli, respectively. The arrowed lines indicate fluxes, the rate constant pertaining to each flux is shown against the corresponding arrowed line (France *et al.*, 1990).

Glucose broth (5 ml) was added and it was shaken vigorously to mix the agar and liquid phases. After 2 to 3 days of incubation, the culture was actively grown again. This provided a useful method of maintaining cultures of anaerobic fungi without frequent subculturing (Joblin, 1981). By this method, cultures remained viable in sisal broth at 39°C upto 7 months.

Cryogenic preservation of the anaerobic rumen chytridiomycete, *N. patriciarum* was possible by two-stage freezing procedure (Yarlett *et al.*, 1986a). It involved initial freezing and storage for 24 hours in solid carbon dioxide before transfer to either at -80°C freezer or liquid nitrogen. Immersion directly into liquid nitrogen resulted in no survival of cultures. Best long term preservation conditions were obtained by using 5 per cent dimethyl sulfoxide as cryoprotectant in liquid nitrogen. Storage under carbon dioxide or at -80°C gave good recoveries for a limited period (several months) which might be useful to laboratories require short term storage of the fungus and did not possess the necessary liquid nitrogen facility. Storage at -80°C for 1 year gave approximately 40 per cent survival compared to that in liquid nitrogen. Recovery of cultures after cryogenic storage of vegetative growth in solid carbon dioxide or at -80°C was enhanced by the addition of 5 per cent dimethyl sulfoxide to the medium.

2.9 TYPES OF ANAEROBIC FUNGI AND THEIR MORPHOLOGY

The first rumen fungus isolated was polyflagellated *Neocallimastix frontalis* (Orpin, 1975). The flagellates averaged about 20.6 x 8.7 μm. Though shape was variable, it was basically ovoid. The cells were frequently irregular in shape soon after release from the sporangium, but most of these became regular within 2 to 3 minutes. Locomotion was effected by the rapid beating of up to 14 flagella which formed a single locomotory organelle, curved in three dimensions up to 36.6 μm long and 2.5 μm thick. In live preparations, the individual flagella adhered closely and in fixed preparations the flagella spreaded out. The fresh cells were actively motile and the maximum linear speed recorded was 5 mm per minute, timed over a 200 μm grid of a haemocytometer chamber. Linear motility was rare, the usual movement found to be erratic and gyratory. Occasionally, the flagellates cease this erratic movement and the flagella become inert while the cell moves amoeboidly for 10 to 15 minutes. After this, cell becomes rounded and further starts the active movement by means of re-curved flagella.

The size of sporangia producing the flagellates *in vivo* varied from 21 x 9 μm to 74 to 52 μm. Some of the sporangia possess a single rhizoid of up to 140 x 8.5 μm, which is frequently branched. Under, *in vitro* conditions, the sporangia are capable of growing to larger dimensions, up to 155 x 83 μm, with rhizoids of up to 1380 μm in length. These rhizoids are repeatedly branched, but in no instance there is more than one sporangium on each rhizoid system. The number of flagellates produced per sporangium ranged from 2 to 38. Due to some anomalies, the isolate referred as *N. frontalis* by Orpin was subsequently renamed as *N. patriciarum* (Orpin and Munn, 1986). Later on, the isolates like PN1 (Bauchop and Mountfoot, 1981), L2 (Marvin-Sikkema *et al.*, 1992), CS36, CL16, XY6, CS4, XS26 (Sijtsma and Tan, 1993) were also classified under *Neocallimastix* because of their monocentric growth, branched rhizoid and multiflagellated zoospores.

In 1976, Orpin isolated another rumen fungus - *Sphaeromonas communis*. Assuming the similarity between *Sphaeromonas* isolates and Liebetanz's protozoans, Orpin retained the generic name used by Liebetanz (1910). However, due to differences between Liebetanz's description of his isolates and those described by Orpin, which was mainly to stress their fungal status, the genus *Sphaeromonas* was renamed as *Caecomyces* (Gold *et al.*, 1988). Type species are: *Caecomyces communis* (isolated from sheep rumen) and *C. equi* (isolated from horse caecum). The *Caecomyces* flagellates were usually irregular in shape immediately after release from the sporangia (Orpin, 1976). Most become spherical within 2 to 3 minutes with an average diameter of 7.95 μm, but a few remained elliptical and measured up to 10.5 μm long. Each zoospore possessed a single flagellum (24.9 μm long) and it was posteriorly oriented during cell motility. Within 30 to 120 minutes, the motility decreased and elliptical spore became spherical. Later on, movement eventually ceased, flagellum detached and the refractivity of the cell increased. The non-motile stage of *Caecomyces* flagellates was indistinguishable from that of *Neocallimastix flagellates*.

The vegetative stage, i.e., sporangia of *Caecomyces* occurred in rumen fluid with or without a limited thallus consisting of a single, sometimes branched rhizoid. It might be monocentric or polycentric in nature (Trinci *et al.*, 1994). The large sporangia were variable in shape, but were usually ovoid with maximum axial dimensions of 95 x 64 μm (Orpin, 1976). Initial germination of the cell appeared to occur by an outgrowth of the cell, producing a thick (up to 8 μm wide) rhizoid. On this, up to seven spherical bodies of unknown function developed before the development of usual single sporangium. Occasionally, there were 2 to 3 sporangia. The maximum number of flagellates were seen to be liberated from a sporangium was 114.

Piromonas communis, a rumen anaerobic fungus, was isolated and characterized by Orpin (1977b). The genus has been renamed as *Piromyces*. Type species are *Piromyces communis* (Gold *et al.*, 1988), *P. mae, P. deembonica, P. rhizinflata* (Li *et al.*, 1990), *P. minutus* (Ho *et al.*, 1993a), *P. spiralis* (Ho *et al.*, 1993b). The *Piromyces* flagellates were irregular in shape immediately after release from sporangia and became regular elongated in shape after 2 to 3 minutes with average axial dimensions of 7.1 x 14.6 μm (Orpin, 1977b). Zoospores were monoflagellated and the average length of flagella was 28.8 μm. The flagellum was posteriorly oriented whilst the cell was in motion. During period of less activity, the cell became more rounded, assuming the appearance of *Neocallimastix* flagellates. Occasionally, the flagellates ceased swimming, the flagellum became inert and the cell moved in an amoeboid fashion. After amoeboid movement, the cell again became elongated and flagellar motility reappeared. After 1 to 3 hours of rapid motility *in vitro*, or an unknown period *in vivo*, the flagellate became more refractile and subsequently lost their flagellum. Then germination started by the growth of single rhizoid from a terminal position on the cell. Occasionally, the flagellum remained attached to the germinating cell *in vitro* and subsequent growth of the vegetative stage was by elongation of the rhizoid and enlargement of the sporangium. The sporangium was provided with single, non-septate highly branched rhizoid, up to 365 μm cylindrical to ovoid. The number of flagellates produced per sporangia ranged from 2 to 78 depending on its age.

Ho and Bauchop (1991) demonstrated polycentric anaerobic fungi, viz. LL, LC2 and *Ruminomyces elegans* (C2) from the rumen of cattle. But the genus *Ruminomyces* was renamed as *Anaeromyces* (Trinci *et al.*, 1994). The zoospores were polyflagellated in case of LL and LC2, but monoflagellated in case of ***Anaeromyces elegans.*** Both LL and LC2 produced large rhizomycelia, comprising of extensively branched hyphae, which could be tubular and uniform in diameter. Sporangiophores usually developed from the wider hyphae. Many sporangiophores can be formed from one hyphae and these can be both intercalary and terminal. Development of sporangia usually occurred after 3 to 4 days growth in all media. The sporangia produced were usually spherical. *A. elegans* produced a much smaller but more compact rhizomycelia than LL and LC2. The hyphae were highly branched, giving a bead or sausage like appearance (frequently, small lobed or disc like structures). Other species was ***A. mucronatus,*** an isolate from sheep rumen (Breton *et al.*, 1990). The polycentric fungus -*Neocallimastix joyonii* is renamed as *Orpinomyces joyonii* (Trinci *et al.*, 1994). This genus was characterized by polyflagellated zoospores and filamentous rhizomycelium. In India, Malakar and Walli (1995) reported that the shape of sporangia from cattle and buffalo are of great variety including cylindrical, ovoid, globose etc.

2.10 ZOOSPORE ULTRASTRUCTURE

Zoospore was the first stage and also the motile stage in fungal life, and its shape was variable. In case of *N. patriciarum*, the cell surface was irregularly scalloped (Munn *et al.*, 1988). Flagella generally arise from one pole. There were about 9 to 15 flagella, alternating in two rows. These contained usually (9x2) + 2 arrangement of microtubules which were extended up to three quarters of the length of the flagella. The terminal part of each flagellum was narrow and contained only a few microtubules. The cells contain a nucleus and a variety of inclusions: large globular aggregates and helical arrays of ribosome like particles, amorphous electron dense globules, glycogen etc. From electron micrographs of a series of thin sections, Marvin-Sikkema *et.al.,* (1992) showed that zoospore of *Neocallimastix* sp. L2 possessed 9 flagella with typical configuration of 9 + 2 axonema. A circumflagellar ring surrounded the flagellum near the entrance region of the zoospore. In the kinetosomes, spurs, scoops and skirts were visible.

Neocallimastix sp. did not contain structures recognizable as mitochondria (Munn *et al.*, 1981; Heath *et al.*, 1983). Electron micrographs of the fraction enriched in hydrogenase activity contained finely granular microbody like organelles, about 0.5 µm in diameter (Yarlett *et al.*, 1986b). These organelles bore no similarity to mitochondria but were morphologically similar to hydrogen evolving organelles possessed by certain anaerobic protozoa and termed 'hydrogenosomes'. These were shown to be involved in energy generation and had a close association with structures involved in cell motility (Muller, 1980).

2.11 ENERGY METABOLISM OF ANAEROBIC FUNGUS

Anaerobic fungi were unable to produce energy either by aerobic or anaerobic respiration because of absence of mitochondria within the cell (O'Fallon *et al.*, 1991). The cells were not able to prevent the accumulation of reducing equivalents by the electron transport chain, where oxygen acted as an electron acceptor in aerobic respiration and nitrate, sulphate, fumarate, triethylamine oxide served the corresponding role in anaerobic respiration (Stanier *et al.*, 1987). Instead of this, anaerobic fungi metabolized energy by anaerobic fermentation of carbohydrate where carbohydrate acted as both electron donor and acceptor. Since part of the energy was retained in the substrate, anaerobic fermentation was less efficient energy yielding process than aerobic process. The yield coefficient (mg biomass produced per mg glucose utilized) for a fungus like *N. hurleyensis* (0.62) grown anaerobically was much lower than for a fungus like *Geortrichum candidum* (4.50) grown aerobically (Caldwell and Trinci, 1973; Lowe *et al.*, 1987b).

Some microbody like organelles with a finely granular matrix, slightly smaller in size were present in zoospore as well as in vegetative stage of anaerobic fungus. Previously, these were designated as amorphous globules (Munn *et al.*, 1981) but were actually hydrogenosomes (Yarlett *et al.*, 1986b). These organelles were capable of coupling the metabolism of hexose to cellular energy formation with the concomitant generation of hydrogen. Two pathways of hydrogen (H_2) formation were known; both involved pyruvate as intermediate, but they differed in the mechanism of end products formation. In one pathway, used by *Clostridia* (Gray and Gest, 1965) and protozoa

(Bauchop, 1971; Yarlett *et al.*, 1981), electrons for proton reduction were derived from the conversion of pyruvate into acetyl CoA by pyruvate:ferredoxin oxidoreductase. In the second pathway, which occurred in the Enterobacteriaeceae (Gray and Gest, 1965), electrons for proton reduction were derived from formate dehydrogenase. The absence of detectable formate dehydrogenase and formate hydrogen lyase in extracts of *N. patriciarum* indicated the absence of later pathway of hydrogen evolution. However, hydrogenase, malic enzyme and NADPH:ferredoxin oxidoreductase were detected under all growth conditions and differential fractionation of homogenates showed that these activities were associated with the particulate fraction. This was similar to the distribution of enzyme activities found in hydrogenosome from some anaerobic protozoa (Muller, 1980; Yarlett *et al.*, 1981).

2.12 METABOLIC TRANSFORMATION OF GLUCOSE

Primary pathway for glucose metabolism was established in the anaerobic rumen fungi *N. patriaciarum* (Yarlett *et al.*, 1986b) and *N. frontalis* EB188 (O'Fallon *et al.*, 1991). The presence of certain glycolytic enzymes but the absence of detectable glucose-6-phosphate dehydrogenase suggested that glycolysis was the sole mechanism of glucose metabolism in anaerobic fungi. Glycolysis in *N. frontalis* EB188 was coupled to malate dehydrogenase, malic enzyme and specified hydrogenosome reactions (O'Fallon *et al.*, 1991). Pyruvate, as in most of life forms, was a pivotal compound. The major fermentation end products of *N. frontalis* EB188 were acetate, ethanol and lactate with the concomitant generation of hydrogen (H_2). Despite a similarity in basic concept, O'Fallon's metabolic diagram differed in several important respects from that of Yarlett's concept. Namely, the phosphoenolpyruvate carboxykinase and pyruvate kinase reactions described by Yarlett *et al.* (1986b) were not likely to occur in the direction towards glycolysis. Pyruvate produced by glycolysis was converted via pyruvate carboxylase into oxaloacetate, which in turn was reduced by malate dehydrogenase to malate. This malate was transported into the hydrogenosomes, where hydrogenosomal enzymes generate energy by coupling malic enzyme oxidation of malate to electron transfer via NADH:ferredoxin oxidoreductase, ferredoxin and hydrogenase to proton reduction, with the concomitant generation of hydrogen.

2.13 ROLE IN FIBER DEGRADATION

Orpin (1977a) first observed that flagellates of *N. frontalis* and *P. communis* attached themselves to stomata, lateral spikes and regions of damaged plant tissues. The zoospores of anaerobic rumen fungi were observed to attach and encyst on the plant leaves and guinea grass fragments by 15 minutes after incubation in rumen (Bauchop, 1981; Ho *et al.*, 1988a). The zoospores shed their flagella very rapidly and attached themselves by means of material of unknown nature. It might be due to the fact that zoospores of *N. frontalis* attached on their flagellar pole with fibriller material, radiated out as fine fibrils from the point of attachment (Munn *et al.*, 1981). However, according to Ho *et al.* (1988b), the materials of attachment were not distinctly fibrillar but spreaded out irregularly at the point of attachment.

Shortly after attachment, the zoospores germinated with a single germ tube which then subjected to branching to form rhizoids. Attachment and germination of zoospores occurred mainly on damaged plant tissues, such as cut ends and damaged epidermal surfaces of grass fragments. If plant fragments were intact with covered cuticle, then zoospores attached and germinated predominantly at the stomata (Ho *et al.*, 1988a). Subsequently, single rhizoid grows parallel to the length of the cell for some distance before branching and penetrating other cells on either side. Following contact with undamaged cell walls, many of the rhizoids developed special appressorium like structures for penetration. Development of appressoria by the fungi for penetration of the cell wall probably accelerated the spread of the rhizoids from cell to cell, enabling maximum colonization of plant tissues. Then extensive root like systems of rhizoids with numerous appressoria penetrated and ramified within both thin walled (parenchyma and mesophyll) and thick walled cells (sclerenchyma and vascular tissues). As more rhizoids colonized and penetrated the plant cells, disruption and break down of the fiber took place and the areas of degradation appeared in the mesophyll, ground parenchyma and sclerenchyma.

2.13.1 DRY MATTER/CELL WALL DEGRADATION

Antibiotics provided a way to manipulate the various microbial groups to evaluate fungal contribution to fiber digestion in the rumen. Fiber loss due to the fungi was slightly higher at 24 and 48 hours but was significantly less at 96 hours. It was observed that ruminal fungi in selective culture could potentially degrade plant fiber at similar rates and to similar extent to that of bacterial population (Akin and Rigsby, 1987). Under *in vitro* system, in presence of streptomycin and penicillin, fungi caused a dry matter (bermuda grass and pangola grass) loss of 72.4 to 74.2 per cent (Akin *et al.*, 1989). Irrespective of the substrate, the maximum amount of dry matter degraded by *N. frontalis* was reached after 4 days of culture. In contrast, the degradation by *O. joyonii* was more progressive: greatest degradation was reached after 6 days for maize stem and 8 days for wheat straw (Roger *et al.*, 1993). These two fungi degraded maize stem with the same effectiveness (approximately 60% of DM was solubilized at the end of the culture). Sijtsma and Tan (1993) observed that after an incubation period of 8 days, *Neocallimastix* species degraded upto 89 per cent (w/w) of the isolated cell walls while *Piromyces* species strain CS1 degraded 65 per cent (w/w) of the cell walls.

2.13.2 DEGRADATION OF PHENOLICS AND LIGNIN

Anaerobic rumen fungi preferentially colonized and degraded lignified cell walls, which were the least biodegradable plant components (Akin *et al.*, 1983). The ability to degrade and utilize lignin anaerobically will be an important attribute for rumen microorganisms. Early studies with [^{14}C] lignin or [^{14}C] carbohydrate in lignocellulose of highly lignified and poorly digested cord grass indicated that the loss of radiolabelled lignin was not the result of its oxidation into carbon dioxide but it was due to its solubilization (Akin and Benner, 1988). Incubation of radiolabelled oat plants with different pure, monocentric fungal culture indicated that 30 to 38 per cent of the initial radioactivity was solubilized and negligible carbon dioxide was produced (Gordon and Phillips, 1989).

Hindrance of plant cell wall biodegradation appeared to be associated with *in situ* structural relationships between different types and quantities of cross-linking bonds between cell wall polymers rather to any one specific cell wall component (Ford and Elliot, 1987; Forsberg and Cheng, 1990). The extent of cross-linking by ferulic and para-coumaric acid dimers appeared to correlate best with low biodegradability (Hartley and Ford, 1989). Furthermore, the esterification of feruloyl and para-coumaroyl groups to arabinoxylans were shown to limit the release of cell wall pentoses (Hespell and O'Bryan, 1992). Recent studies indicated that rumen fungi were able to produce both para-coumaroyl and ferulolyl esterases (Borneman and Akin, 1990; Borneman *et al.*, 1990). The extracellular para-coumaroyl esterase secreted by the anaerobic fungus *Neocallimastix* strain MC-2 released para-coumaroyl groups from O-[5-O-{(E)-para-coumaroyl}-α-L-arabinofuranosyl]-(1→3)-O-ß-D-xylopyranosyl-(1→4)-D-xylopyranose (PAXX). The extracellular feruloyl esterases (FAE-I and FAE-II) cleaved ferulic acid from O[5-O-{(E)-feruloyl}-α-L-arabinofuranosyl]- (1→ 3) -O-ß-D-xylopyranosyl-(1→4)- D-xylopyranose (FAXX).

2.13.3 PHYSICAL DEGRADATION OF FIBER BY FUNGI

Abilities to degrade extensively certain lignified tissues, to partially degrade and weaken the more resistant tissues, and to penetrate the cuticle barrier in forages were characteristics, which indicated that rumen fungi were better equipped than rumen bacteria to degrade the structural barriers in plants. Therefore, fungi might be considered to have an ability to modify particle size or other fiber characteristics that relate to intake or passage of fiber through the intestinal tract (Welch, 1986). In the feeding study with sulfur fertilized warm season grass, the fungal population was stimulated which, in turn, increased the feed intake. It was thought to be influenced by the ability of fungi to attack and weaken anatomic barriers of fiber (Akin *et al.*, 1983). In a study to evaluate quantitatively the potential of mixed bacterial and mixed fungal population to weaken structural barriers *in vitro* (Akin *et al.*, 1989) indicated that stems incubated with fungi got significantly weaker than those incubated with bacteria. Particle size was not altered by any microbial group but fungal filaments degraded tissues most refractory to degradation in both grass and legume stems. *Caecomyces* isolates were able to fibrillate plant fragments due to the expansion of its bulbous rhizoid (Joblin, 1989). Therefore, rumen fungi appeared to have the ability to alter the characteristics of fibrous residues; thus potentially aided in mastication and fiber flow in the digestive tract and subsequently increased the feed consumption.

2.14 FACTORS AFFECTING SPORANGIAL COLONIZATION ON PLANT MATERIAL

The colonization of lignified tissues by rumen fungi is of interest in ruminant nutrition, because these tissues are the ones most limiting to digestibility (Akin, 1986). Electron microscopic investigations showed that colony development was initiated on the lignin-containing tissues preferentially (Akin *et al.*, 1983). Although chemotaxis was shown with soluble sugars in rumen fungi (Orpin and Bountiff, 1978), it was not known whether chemotaxis to cell wall components was important in colonization of specific plant cell walls. After colonization of the refractory lignocellulose, fungal rhizoids or hyphae partially or totally degraded plant walls. Lignin containing tissues those were partially degraded by rumen bacteria, such as leaf blade sclerenchyma but were extensively and often completely utilized by rumen fungi (Akin and Rigsby, 1987). Other types of lignin containing tissues which were totally resistant to bacterial degradation, such as xylem and sclerenchyma ring tissue in stems, were degraded partially by fungi. With some rumen fungi, appressorial like structures that produced a penetration peg, a system similar to that of plant pathogenic phycomycete fungi, appeared to be involved in degrading plant cell walls (Ho *et al.*, 1988b). In addition to the greater degradation of lignified tissues by rumen fungi compared to bacteria, another attribute of fungi was their ability to penetrate the cuticle of grass leaf blades. However, fungal colonization over plant biomass was not uniform. It depended upon several factors, viz., diet, time after feeding, system of keeping animal (grazing or stall-fed), soil status, fungal type, pH etc.

2.14.1 GEOGRAPHIC FACTOR

Whole rumen fluid from the Australian ruminant source degraded bermuda grass leaf blades at a faster rate and to a greater extent than that from the U.S.A. Inclusion of cyclohexamide or streptomycin and penicillin with the rumen fluid indicated that the potential bacterial activity was less in the Australian inoculum, while that for eucaryotic microorganisms (fungi) was greater (Akin *et al.*, 1989). Rumen fungi from the Australian inoculum were particularly more active on the leaf surface of both forages. The development of large number of oval sporangia on leaf surfaces in the Australian inoculum compared with the predominance of globose and fusiform morphotypes in the

U.S.A. inoculum suggested that different fungi were present in the inocula from the two geographic regions. Possibly, the greater degradation from the Australian inoculum was due to the ability of these fungi to extensively penetrate the leaf surface and attack underlying tissues by the rhizomycelium. A higher number of oval sporangia on the leaf surfaces of both bermuda grass and pangola grass for the Australian inoculum suggested that a predominant fungal type might be particularly important in establishing a niche for attacking and penetrating the leaf surface (Akin *et al.*, 1989).

2.14.2 SPECIES

Fungal colonization and development on rice straw and palm press fibre were shown to be similar in both cattle and buffalo (Ho *et al.*, 1991).

2.14.3 DIET

Not all feed ingredients allowed the proliferation of anaerobic fungi at the same rate in the rumen. In case of sheep consuming appreciable amounts of fiber (pelleted or chaffed lucerne), large numbers of sporangia were found attached to stem fragments selected at random from the rumen contents, before morning feeding (Bauchop, 1979). Highest numbers were found with chaffed lucerne diet because of mere stalky material as compared to pelleted lucerne diet.

Fungal populations were stimulated with the alfalfa diet to a much greater extent than that observed with coastal bermuda grass, though NDF per cent was more in case of coastal bermuda grass (69.7%) than alfalfa diet (32.8%). Therefore, the general statement that most fibrous diets support the greatest population of fungi may no longer be valid and indicated that factors other than fiber content affects fungal development (Windham and Akin, 1984). The exact factors in alfalfa responsible for stimulation of fungal development were not known. Akin (1987) observed large differences in the association of sporangia with different grasses from various field plots, at the time of incubation with rumen fluid and penicillin and streptomycin. The number of sporangia on cut edge (per mm) on different grasses were as follows: coastal bermuda grass 27.7 ± 14.3, coastcross 1 bermuda grass 4.2 ± 1.5, experimental low digestible bermuda grass 31.9 ± 21.9, experimental high digestible bermuda grass 14.0 ± 9.5, pangola grass 4.4 ± 4.2, tall fescue 3.3 ± 2.4 and orchard grass 1.0 ± 1.2.

Fungal zoospores generally attached and germinated mainly on damaged plant tissues, viz. cut ends and damaged epidermal surfaces of the grass (guinea) fragments (Ho *et al.*, 1988a). But in case of undamaged grass, fungal attachment took place at the stomatal region. Dimensions of the germinated zoospores were generally 7.0-14 x 8.5 x 17 μm and rhizoids up to 70 μm long are observed. The rhizoids were present mostly in thin walled tissues such as epidermis (stems and leaves), ground parenchyma (stems) and mesophyll (leaves). The rhizoids grew parallel to the length of the plant cell for some distance before branching and penetrating other cells on either side. The rhizoids were found to be especially long if it was in a sclerenchyma fibre or vascular element.

It was speculated that the presence of large amount of soluble carbohydrates in the rumen liquor not only inhibited the adhesion of cellulolytic bacteria to feed particles (Fonty *et al.*, 1987) but also hindered the germination of zoospores (Orpin and Bountiff, 1978). The zoospores preferred to get attached to those feed particles which stayed longer in the rumen and were able to provide the full duration of life cycle, i.e., about 24 hours (Grenet *et al.*, 1989).

Rye grass at the leafy stage was an unfavourable feed item for the fungi. This feed item did not cause any hindrance for the establishment of fungi in the rumen, but it reduced their development (Grenet *et al.*, 1989). The use of very young grass (rich in nitrogen, soluble carbohydrates and highly digestible) allowed extensive fermentations in the rumen and gave a higher level of volatile fatty acids. In addition to this, due to little lignification and lesser retention time in rumen, younger grass gave little scope to fungi for attachment and subsequent development. Differential colonization by rumen fungi could be explained in two ways (Grenet *et al.*, 1989) either by a selective colonization of the various composition of the substrate. Soyabean tegument (rich in cellulose and hemicellulose) seemed to be favourable to the development of fungi. However, again it was more difficult to explain why lucerne stems were more favourable than maize stalks.

2.14.4 TIME

Under *in vitro* system, incorporation of streptomycin and penicillin stimulated the development of sporangia on the leaf surface of bermuda grass from 24 to 96 hours. The number of sporangia per site (of bermuda grass) at different hours of incubation were as follows: 6 hours 0, 24 hours 1.2 ± 0.3, 48 hours 10.0 ± 1.6, 72 hours 8.2 ± 4.4 and 96 hours 4.8 ± 3.4 (Akin and Rigsby, 1987).

Fungal zoospores were found to get attached to rice straw fragments 15 minutes after rumen incubation and by 30 minutes many germinated (Ho *et al.*, 1991). Similar rapid attachment of zoospores to plant fragments in the rumen was reported by Bauchop (1981) and Ho *et al.* (1988b). The zoospores attached predominantly on stomata, cracks, fissures and cut surfaces of the rice straw. By 1 to 3 hours, same fungi developed an extensive hyphal system in the thin walled parenchyma cells of the rice straw. Some of the hyphae produced lobed or disc shaped 'appresoria' with penetration pegs penetrating cell walls. After 6 hours in the rumen, the thick walled lignified tissues, viz. sclerenchyma and vascular elements, were colonized by the fungi. Degradation of the cell wall took place in the thin walled tissues which were extensively colonized by fungi. Some degradation was also observed in the thick walled tissues with profused fungal colonization. Sporangia of the fungi were small, mostly spherical or ovoid. At 24 hours, fungal colonization of the straw fragments was very profused and dense networks of hyphae were observed in the sclerenchyma and vascular tissues. By 48 hours, much of the softer thin walled tissues of the rice straw fragments were degraded and the remaining sclerenchyma and vascular tissues were extensively colonized by fungi. In place of large spherical and ovoid sporangia, a large number of smaller ovoid structures having diameter of 8 to 15 μm (germinated zoospores or young sporangia) were present both outside and within the cells (Ho *et al.*, 1991). These young sporangia/germinated zoospores did not produce extensive network. Most of the hyphae produced were thick and short. Some of the larger sporangia appeared shrunken or collapsed with part of the sporangial walls disintegrated or with openings or pores in the walls. Similar type of collapsed sporangia were observed on guinea grass, incubated in the rumen of cattle and Swamp buffalo and they were probably the remains of sporangial wall after the release of zoospores (Ho *et al.*, 1988a). Although the degradation of tissues was quite extensive at 72 hours after rumen incubation, but extensive fungal growth was not observed. The number of sporangia was very much less than that at 24 and 48 hours (Ho *et al.*, 1991).

Roger *et al.* (1993) observed that after 15 hours of incubation with *Neocallimastix frontalis*, the tissues of straw and maize stem were almost completely covered by an abundant mycelium. After 24 hours, the inner part of the parenchyma was degraded and after 48 hours it was separated from the stem. At 72 hours of incubation, the degradable tissues (phloem and parenchyma) disappeared.

2.14.5 FREQUENCY OF FEEDING

Beet diet (comprised of 8.2 kg Capax fodder beet and 7.4 kg lucerne hay) offered in single meal did not cause fungal colonization on substrates suspended through nylon bag. The number of fungi was also low in rumen fluid. But during offering the same beef diet in six different meals, fungi attached to the substrates and number of fungi in rumen fluid was also more (Grenet *et al.*, 1989).

2.14.6 SPECIES OF FUNGUS

Members of the genus Caecomyces seemed to be the least capable of penetrating plant fiber among all the anaerobic fungi (Gordon and Phillips, 1989; Roger *et al.*, 1992). This was due to the presence of spherical holdfasts unlike mycelial rhizoids of *Neocallimastix, Orpinomyces, Anaeromyces* and *Piromyces* species. Thus, *Caecomyces* were unable to penetrate deeply into plant tissues (Joblin, 1989; Fonty *et al.*, 1990).

2.15 INTERACTION OF RUMEN FUNGI WITH OTHER MICROBES

Anaerobic chytridiomycetes fungi are part of the complex microbial consortium that colonize and degrade plant materials in the ruminal ecosystem (Orpin and Joblin, 1988). By virtue of their characteristic evolutionary nature, these fungi are able to thrive on strict and competitive anaerobic rumen ecosystem. In such complex rumen ecosystem, both synergistic and competitive microbial interactions take place. The

The presence of methanogenic bacteria in fungal culture increased cellulose fermentation by 5 to 10 per cent (Marvin-Sikkema *et al.*, 1990). There was a shift in the fermentation products to more acetate and less lactate, succinate, ethanol and formate. When *Selenomonas ruminantium* was used as a hydrogen consuming organism in co-culture with *Neocallimastix* sp. strain L2, both the rate and level of cellulolysis were increased. The beneficial effects of *S. ruminantium* might be resulting from a lowered electron sink products by the fungus, from consumption of electron sink products or hydrogen by bacteria or from competition for free sugars which in pure culture could exert an inhibiting effect on cellulolysis (Marvin-Sikkema *et al.*, 1990).

2.16 PLANT BIOMASS DEGRADING ENZYMES

Anaerobic fungi inhabiting the alimentary tracts of ruminants as well as non-ruminant herbivorous mammals plays a crucial role in the digestion of cellulose, hemicellulose, pectin, starch, protein etc. present in plant cell (Bauchop and Mountfort, 1981; Orpin, 1983/84, Lowe *et al.*, 1987b; Mountfort and Asher, 1988; Gordon and Phillips, 1992). The following enzymes are produced by anaerobic fungi: carboxymethyl cellulase or endoglucanase (Lowe *et al.*, 1987c; Barichievich and Calza, 1990; Wilson and Wood, 1992), exoglucanase or avicelase (Pearce and Bauchop, 1985), ß-1,4-glucosidase (Li and Calza, 1991a), hemicellulase (Lowe *et al.*, 1987c, Mountfort and Asher, 1989) including xylanase (Teunissen *et al.*, 1993a), and xylosidases (Hebraud and Fevre, 1988, 1990; Calza, 1991a; Garcia-Campayo and Wood, 1993; Teunissen *et al.*, 1993a; Chen *et al.*, 1994), pectin lyase (Gordon and Phillips, 1992), feruloyl and paracoumaroyl esterase (Borneman *et al.*, 1990, 1991, 1992), amylase and amyloglycosidase (Mountfort and Asher, 1988; Pearce and Bauchop, 1985), protease (Wallace and Joblin, 1985; Michel *et al.*, 1993). Most of the enzymes produced by anaerobic fungi were secreted in the culture supernatant and only a minor quantity of enzyme resided at cellular level (Li and Calza, 1991a).

Since the discovery of bacteria, search is still continuing for finding out suitable organism able to produce enzymes for the bioconversion of lignocellulosic biomass into utilizable materials. Promising microorganisms are aerobic bacteria, e.g. *Thermonospora* sp. (Moreira *et al.*, 1981), aerobic fungi, e.g. *Trichoderma* sp. (Mandels and Reese,

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was comprised of 14 to 18 polypeptides (Lamed *et al.*, 1987; Lamed and Bayer, 1988). Cellulosomes could act at some distance from the cell and were found in clusters covering the whole of the residual cellulose (Mayer *et al.*, 1987). The attachment between the enzyme complex and cellulose substrate was mediated by a non-cellulolytic binding factor (Wu *et al.*, 1988). Wilson and Wood (1992) observed that a minor component (4% of the total extracellular protein) of the cellulolytic enzymes of *N. frontalis* was present in a cellulosome like structure which was secreted there and got adsorbed to cellulose. Hence, the mechanism of cellulose degradation by anaerobic fungus seemed to be similar to that of *C. thermocellum*, although the molecular weight of the complex was much lower (750 to 1000 kDa vs. 2 x 10^6 kDa) from its bacterial counterpart (Wilson and Wood, 1992).

2.16.1.1 Purification of endoglucanase

Barichievich and Calza (1990) made first attempt to separate the supernatant protein and cellulase secreted by *Neocallimastix* strain EB188. Molecular weight of protein ranged between 19,000 to 1,16,000. Comparatively, the fungus grown over cellulose medium, secreted fewer low molecular weight proteins and more of high molecular weight proteins. Isoelectric points of cellulase varied from 3.7 to 8.3 and possessed maximum activities at pH range of 5.9-6.5.

Several forms of CMCase were separated by gel filtration chromatography, native polyacrylamide gel electrophoresis and isoelectric focussing (Wood *et al.*, 1986; Barichevich and Calza, 1990). The multiple forms of CMCase might represent distinct enzymes or proteolytic degradative products or those resulted from post-translational modification including glycosylation. Wallace and Joblin (1985) reported the presence of proteolytic activity in anaerobic fungus and this might contribute the proteolytic modification of cellulase. By using hydroxylapatite chromatography, Li and Calza (1991a) separated seven well defined CMCase peaks. The recovery of CMCase was 71.2 per cent and 68 per cent of total activity resided on two peaks, viz. C3 and C4 while minor percentage of activities were found among rest three peaks, viz. C4, C5 and C7. The CMCase specific activity of various peaks ranged from 0.77 to 15.66 IU/mg protein.

Most microorganisms produce isoenzymes. Differences in enzyme activities found after growth on the different substrates could be the result of variations in isoenzymes synthesized. The molecular weight of different isoenzymes (CMCase) were 215, 150, 128, 110 and 55 kDa. The dark bands which appeared within some of the clearing zones during destaining, was probably due to the presence of acid protein or the formation of an acid product by the enzymes. But during growth over lactose, two additional endoglucanase activity bands (MW 120 and 200 kDa) were also observed (Teunissen *et al.*, 1992).

The crystalline cellulose solubilizing factor (CCSF) was isolated from the extracellular cellulase of anaerobic rumen fungus *Neocallimastix frontalis* (Wilson and Wood, 1992). This factor was obtained by adsorption on cellulose. The CCSF showed endoglucanase and ß-glucosidase activity and effected the extensive hydrolysis of crystalline cellulose in the form of cotton fibre. The specific activity of CCSF (2.6 IU/mg protein) in degrading cotton fibre was much higher than any other cellulase or cellulase component so far. From SDS-PAGE it was confirmed that the activity of CCSF resided in a high molecular mass complex of 670 kDa.

2.16.1.2 Purification of avicelase

The activity of avicelase in the culture supernatant of *Neocallimastix frontalis* was very low (< 2 U/ml). Failure of solubilizing agents to release exoglucanase from fungal rhizoid, together with their low activities in culture fluid, suggested that these enzymes were produced in low quantities (Mountfort and Asher, 1985). The low avicelase (0.006 U/ml) activity was also reported by Pearce and Bauchop (1985). By hydroxylapatite chromatography, Li and Calza (1991a) were able to separate six major peaks for avicelase activity from the culture supernatant of *Neocallimastix frontalis*. The recovery of avicelase from the column was 76 per cent.

2.16.1.3 Purification of ß-glucosidase

ß-glucosidase is present in plants, fungi and bacteria. Considerable attention has been diverted towards ß-glucosidase (EC 3.2.1.21) primarily because of its involvement in the biological conversion of cellulose materials at terminal step. This enzyme brought about the hydrolysis of various compounds having ß-D-glucosidic linkages and played a vital role in large scale saccharification of cellulose, by eliminating the cellobiose, which is a potent inhibitor of both exo as well as endoglucanase (Shewale, 1982; Chen *et al.*, 1994).

The ß-glucosidase of *N. frontalis* occurred in several isoenzyme forms (Li and Calza, 1991a). They were able to separate four activity peaks for ß-glucosidase through hydroxylapatite column chromatography. On native PAGE, crude enzymes were present in three active bands. Such enzyme peaks might represent distinct enzymes, proteolytic degradative products of single enzyme or enzymes with different degree post-translational modification (i.e. glycosylation). The subsequent steps of purification (viz. gel filtration, ion-exchange chromatography, native PAGE). The molecular weight of purified enzyme was 1,25,000 daltons. Isoelectric focussing point was 7.10. This enzyme readily hydrolyzed p-nitrophenyl-ß-D-glucopyranoside and cellobiose only. Activity was inhibited by glucose, glucono-δ-lactone, Ag^{2+}, Hg^{2+}, Cu^{2+} etc. but stimulated by dithiothreitol. The km and V_{max} values were 2.55 mM and 5.02 IU/mg protein, respectively. Optimum temperature and optimum pH were 45°C and 5.5 to 7.0, respectively.

Several types or multiple forms of ß-glucosidase were detected in the culture supernatant of polycentric anaerobic fungus *Orpinomyces* sp. strain PC2 (Chen *et al.*, 1994). Because of aggregation of several hydrolytic enzymes, purification of the ß-glucosidase from another polycentric anaerobic fungus (*Orpinomyces* sp. strain PC-2) was cumbersome and low yielding (Li *et al.*, 1992). In microorganisms, different ß-glucosidases are encoded by separate genes, but multiple forms of the enzymes having different pI values, may also be due to differences in charge or in contents of carbohydrates (Shewale, 1982).

The purified ß-glucosidase from *Orpinomyces* sp. PC-2 was a glycoprotein containing 8.5 per cent carbohydrate (Chen *et al.*, 1994). The molecular weight of the enzyme was 86,000 and consisted of a single polypeptide. The enzyme was strongly inhibited by a sulfhydryl reagent (p-chloromercuribenzoate) and sulfhydral oxidant metals (Ag and Hg). The activity of the enzyme was stimulated by several bivalent ions, including Mn, Co and Ni. The enzyme was comprised of 45 cysteine residues suggesting the important role of their group within the enzyme.

Teunissen *et al.* (1992) purified an extracellular ß-glucosidase from anaerobic monocentric fungus. The molecular weight of the enzyme of was 45,000 daltons. pH and temperature optimum were close to physiological range of the rumen. The Km value of the enzyme against cellobiose was 0.05 mM. The enzyme was found to be inhibited by cellobiose, the substrate itself. Inhibition of high substrate concentrations resulted from the formation of transfer products, which was also observed with the cellobiase from *N. frontalis* EB188 (Li and Calza, 1991b).

Cellobiose is one of the major product from the hydrolysis of cellulose by endoglucanase and exoglucanase. But the hydrolysis is supposed to be inhibited by cellobiose. The ß-glucosidase released by anaerobic fungus were extracellular in nature and released into the medium (Li and Calza, 1991a). In contrast, the ß-glucosidase of the anaerobic rumen bacteria, viz. *Ruminococcus albus* (Ohmiya *et al.*, 1985) and *Fibrobactor succinogenes* (Groleau and Forsberg, 1981) were intracellular in nature. Therefore, it is possible that in the rumen the fungal ß-glucosidase play a vital and critical role by eliminating cellobiose from the rumen fluid and thereby relieve the inhibition caused by the cellobiose produced in the process of cellulose hydrolysis.

Xue *et al.* (1992a,b) made a cDNA expression library of *N. patriciarum* in *E. coli* and demonstrated the existence of two types of genes that encode for cellulase and other polysaccharide hydrolases on the anaerobic fungal genome. One type was found in multiple copies (cel A, cel B andcel C) coding for cellulase, with high activities towards crystalline and amorphous cellulose (cel A) and carboxymethyl cellulose (cel B and cel C). Cel A, cel B and cel C were responsible for encoding one cellobiohydrolase and two endoglucanase, respectively and their expression was induced by cellulose. The enzyme encoded by cel B contained a fully functional catalytic domain that had homology with some bacterial endoglucanase. An absence of introns from the cel B gene and the homology between the catalytic domain of cel B and a number of the endoglucanase from anaerobic rumen bacteria could be regarded as evidence that the evolution of cellulase might involve gene transfer between prokaryotes and eukaryotes (Zhu *et al.*, 1994).

2.16.1.4 Cloning of anaerobic fungal cellulase

Another type of gene (cel D) was found as a single copy that encoded for a multifunctional polysaccharide hydrolase with three catalytic domains, each domain possessing endoglucanase, cellobiohydrolase and xylanase activities. The enzyme encoded by cel D gene also possessed cellulose binding affinity (Xue *et al.*, 1992a). This enzyme was constitutively expressed and was not affected by the presence of cellulose in the culture media. Its characterization confirmed the earlier report of Lowe *et al.* (1987c), who observed some constitutive xylanase production and Barichievich and Calza (1990), who detected low levels of cellulase activity in glucose grown cultures.

2.16.2 XYLANASE

Hemicellulose is the second most abundant polysaccharide in plant cell walls, constituting up to 40 per cent of the total carbohydrate fraction. Xylan is the major component of hemicellulose complex and it is a highly branched polymer in which the xylopyranoside units are substituted with acetyl, arabinosyl and glucoronosyl residues depending on the source (Aspinall, 1983). Hemicellulose associated with lignin surrounds cellulose fibrils and constitutes a 'barrier', restricting the cellulose hydrolysis. Xylan degradation brought about by the combined action of endo-1, 4-ß-D-xylanase, ß-xylobiase, ß-xylosidase and enzymes responsible for cleaving the side groups. Some or all of these enzymes were reported to be secreted in culture fluid of anaerobic fungus grown either on pure xylan or natural substrates (Orpin and Letcher, 1979; Pearce and Bauchop, 1985; Lowe *et al.*, 1987c; Williams and Orpin, 1987a, 1987b; Mountfort and Asher, 1989; Teunissen *et al.*, 1991).

Xylanase (1,4-ß-D-xylanohydrolase, EC 3.2.1.8) was released principally into the culture fluid by *Neocallimastix frontalis* (Mountfort and Asher, 1989). The pH and temperature optima were 5.5 and 55°C, respectively. Though xylan was the most effective inducer of xylanase production but xylanase were also produced by anaerobic fungi even when grown on substrates like wheat, straw, cellulose, cellobiose, glucose or xylose (Lowe *et al.*, 1987c). This suggested that a basal level of xylanase was constantly produced by anaerobic fungi and production was further increased through the presence of xylan.

Garacia-Campayo and Wood (1993) purified a ß-D-xylosidase from *N. frontalis*. The enzyme had a pI of 4.6 and was a dimer of two polypeptide subunits of molecular masses of 83 and 53 kDa, respectively. The optimum pH and temperature were 6.4 and 37°C, respectively. The enzyme showed its activity against xylobiose and xylo-oligosaccharides and was competitively inhibited by D-xylose. The enzyme was inhibited by Cu^{2+}, Ag^{2+}, Zn^{2+}, EDTA, SDS and stimulated by Ca^{2+} and Mg^{2+}.

Gomez De Segura and Fevre (1993) purified two ß-endoxylanase from culture supernatant of *N. frontalis* by serial steps of purification, viz. ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. Xyalanase I was a non-glycosylated protein with an apparent molecular mass of 45 kDa. Xyalanse II was a glycoprotein with apparent molecular mass of 70 kDa. The pH optima of these enzymes were 5.5 and 6.0, respectively and the temperature optima was 55°C for both the enzymes. Thin layered chromatography of xylan hydrolysates indicated their endo mode of action. Antibodies raised against each purified protein exhibited no cross reaction and confirmed their biochemical specificities. Both enzymes exhibited carboxymethyl cellulase activity and xylanase I was absorbed on crystalline cellulase, indicating that these enzymes might belong to the family of ß-1, 4-glycanase.

A recombinant xy–lanse (xyl A) was purified from *E. coli* harbouring xyl A from cDNA library, constructed from mRNA of *M. patriciarum* (Gilbert *et al.*, 1992). This enzyme hydrolyzed oat spelt xylan to xylobiose and xylose but was not effective cellulosic substrates and was modular in construction with a distinct catalytic domain. Comparison of sequences for the enzyme of *N. patriciarum* and some bacterial xylanase revealed significant homology suggestive of a common evolutionary origin and the possibility of horizontal transfer of genes between rumen prokaryotes and lower eukaryotes (Gilbert *et al.*, 1992; Gilbert and Hazlewood, 1993).

Tamblyn Lee *et al.* (1993) cloned an endo acting ß-1,4-xyalanase of *N. patriciarum* into *E. coli* with phage and plasmid vectors. The xylanase activity of the recombinant enzyme was expressed in the periplasmic space of the host probably via its own promotor. This indicated that the *E. coli* synthetic system could recognize the promotor of *N. frontalis*. The temperature and pH optima of periplasmic enzymes were 40°C and 6.2, respectively. Besides xyalanse activity, it also showed activity against carboxymethyl cellulose. The catalytic affinity for xylan did not appear to be affected by the expression in *E. coli* because the km value for the recombinant enzyme against oat spelt xylan was similar to that of other xylanase from aerobic fungi. During electrophoresis, Zymograms, the periplasmic enzyme, revealed three bands of activity with a molecular weight of 48, 51 and 58 kDa.

2.16.3 PECTIN DEGRADING ENZYMES

Pectin is an important polymer of plant biomass. Like phenolic acids, pectin forms ester and ether linkages with lignin and hemicellulose and possibly plays an important role in binding lignin to hemicellulose (Jeffries, 1990). Pectin degradation therefore, causes, breaking of bonds between lignin and hemicellulose and subsequently makes the path towards the inaccessible nutrients.

Only a few anaerobic fungi possessed the ability to hydrolyse this polymer. There was substantial loss (20 to 40%) of pectin while anaerobic fungi was allowed to grow over wheat straw (Orpin, 1983/84). Williams and Orpin (1987a) also detected very low levels of pectin degrading enzymes from culture supernatant of *N. frontalis* and *N. patriciarum*. Contrary to that, Phillips and Gordon (1988) observed that neither pectin nor its breakdown products (viz. D-galactouronic acid and polygalactouronic acid) were fermented by a range of anaerobic fungi. Secretion of pectinolytic enzymes by *Neocallimastix* sp. was also reported by Gordon and Phillips (1992).

Neocallimastix isolate from feces of water buffalo were reported to grow over pre-washed apple pectin and polygalactouronate as a sole source of energy (Lawrence, 1993).

2.16.4 ESTERASE

The hemicellulose fraction of forages and cereals is composed mainly of xylan. Xylans are extensively substituted with acetyl, uronyl and arabinosyl residues. In grasses, 40 to 50 per cent of the arabinosyl residues are substituted at the O-5 position and a significant portion of these arabinosyl groups are ester linked to p-coumaroyl and feruloyl moieties (Chesson and Forsberg, 1988; Hartley and Ford, 1989). There is an evidence that these esterified phenolic groups dimerize *in vivo* either by oxidative coupling, forming dehydrodiferulic acid or by photodimerization in sun-light forming a series of substituted truxillic and truxinic acid cross links (Hartley and Jones, 1976; Hartley *et al.*, 1990). There are also evidences that feruloyl and p-coumeroyl groups form bonds between the xylan and lignin (Scalbert *et al.*, 1985; Chesson and Forsberg, 1988). Hindrance of plant cell wall biodegradation appears to be associated with *in situ* structural relationship between different types and quantities of cross-linking bonds between cell wall polymers, rather than to any one specific cell wall component (Ford and Elliot, 1987; Forsberg and Cheng, 1990). The extent of cross-linking by ferulic and p-coumeric acid dimers appears to correlate best with low biodegradability (Hartely and Ford, 1989; Forsberg and Cheng, 1990). Furthermore, the esterification of feruloyl and p-coumerol groups to arabinoxylans has been shown to limit the release of cell wall pentoses (Hespell and O'Bryan, 1992).

Trans-feruloyl and trans-p-coumeroyl esterase were detected from the culture filtrates of both monocentric and polycentric fungus (Borneman *et al.*, 1990). These enzymes were capable to release ferulic and p-coumeric acids from their methyl esters as well as from coastal bermuda grass. Another interesting finding was that these enzymes exhibited greater esterase activities against methyl ferulate and p-coumerate rather than p-coumaroyl groups of coastal bermuda grass. The monocentric isolate MC-2 had the highest esterase activity.

Borneman *et al.* (1991) purified an extracellular p-coumaroyl esterase from *Neocallimastix* strain MC-2. The enzyme was purified 121 fold from culture supernatant through ultrafiltration, column chromatography on Q-Sepharose and hydroxylapatite, isoelectric focussing and gel filtration. The native enzyme had an apparent mass of 11 kDa under non-denaturing conditions and 5.8 kDA under denaturing conditions. This suggested that the enzyme existed in dimeric form. The isoelectric point was 9.7 and pH optima was 7.2. The purified esterase released p-coumaroyl groups from finely, but not coarsely ground plant cell walls and this activity was enhanced by the addition of xylanase and other cell wall degrading enzymes.

Borneman *et al.* (1992) purified two extracellular feruloyl esterases (FAE-I and FAE-II) from the culture supernatant of anaerobic fungus *Neocallimastix* strain MC-2. The molecular weight of FAE-I and FAE-II were 69 and 24 kDa, respectively in denaturing as well as undenaturing conditions. Apparent Km and maximum rate of hydrolysis with FAXX were 31.9 μm and 2.9 μmol min^{-1} mg^{-1} for FAE-I and 9.6 μm and 11.4 μmol min^{-1} mg^{-1} for FAE-II. FAE-II was specific for O-{5-O-[(E)-feruloyl]-α-L-arabinofuranosyl}-(1→3)-O-ß-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) but FAE-I hydrolyzed both FAXX and DAXX.

2.16.5 LIGNIN DEGRADING ENZYMES

It is unlikely to secrete enzymes for lignin degradation by anaerobic fungi (Gordon and Ashes, 1984; Windham and Akin, 1984; Theodorou *et al.*, 1989). But it is common to secrete lignin degrading enzymes by aerobic fungi, viz. *Trichoderma mesophilia* (Zimmerman and Broda, 1989). However, there were some reports of lignin solubilization by the anaerobic fungi (Orpin, 1981; Akin *et al.*, 1983). In lignin biodegradation process, there is involvement of molecular oxygen, but rumen is completely anaerobic, so it should not expect existence of lignin degrading enzymes in the anaerobic rumen eco-system.

2.16.6 STARCH DEGRADING ENZYMES

The widespread investigation of starch degrading enzymes has mainly been related to their application in industry, such as conversion of starch into different sugars (Bareford, 1976; Fogarty and Kelly, 1979; Fogarty and Kelly, 1980). It is found that the most effective amylases are those which are relatively active at high temperature and thermostable too. Amylase with these characteristics were reported to be produced by a few mesophilic organisms (Saito, 1973; Fogarty and Kelly, 1980; Medda and Chandra, 1980; Krishnan and Chandra, 1983).

Many anaerobic fungi can utilize and grow over starch as a sole source of energy (Williams and Orpin, 1987a, 1987b; Phillips and Gordon, 1988). Mountfort and Asher (1988) observed that *N. frontalis* mostly secreted α-amylase into their culture supernatant. Cellulose, cellobiose, glucose, xylan and xylose were less effective substrates to induce amylase secretion while starch and maltose were equally effective to induce the amylase secretion by anaerobic fungi. Activity was upto 4.4 U ml^{-1} while aerobic fungus was grown over 2.5 mg of starch ml^{-1}. The pH and temperature optima were 5.5 and 55°C. The anaerobic fungal amylase was stable even at 60°C in the presence of low concentration of starch. This suggested their suitability for industrial application. McAllister *et al.*(1993) investigated *Orpinomyces joyonii, N. patriciarum* and *Piromyces communis* for their ability to degrade cereal starch and found that all species degraded corn starch more readily than from barley or wheat. This fungal amylolytic activity could be important in high yielding animals where a major part of the diet comprised of cereal grains like barley, oat, maize.

2.16.7 PROTEOLYTIC ENZYMES

Because of the active and vital role of anaerobic fungus on fiber degradation, proteolytic activity of fungus received very little attention. A high metalloprotease activity having trypsin like specificity was detected by Wallace and Joblin (1985) from the *N. fronatalis*. The protease activity of anaerobic fungus was comparable to that of rumen anaerobic bacteria. Unlike rumen cellulolytic bacteria, the proteolytic activity of fibre degrading anaerobic fungi removed the protein layer of fibre and allowed the fibre degrading enzymes to avail the inaccessible nutrient hidden within the fibre fraction. The extracellular metalloprotease, cysteine protease, serine protease of anaerobic fungus were found to be active between pH 6.5 to 10.5, with an optimal range nearer towards neutral to alkaline side.

2.17 CATABOLITE REGULATION

The cellulase, hemicellulase, amylase and other glycosidase activities of anaerobic fungi were subjected to catabolite regulation (Mountfort and Asher, 1983, 1985, 1988, 1989; Wallace and Joblin, 1985; Morrison *et al.*, 1990). Presence of monosaccharides in anaerobic fungal cultures grown over cellulose, xylan or starch resulted in reduced or zero production of polymer degrading enzymes (Mountfort and Asher, 1985, 1988). Xylose and arabinose played a regulatory role in xyalanse production by *N. frontalis* (Mountfort and Asher, 1989). Like cellulases and hemicellulase, the production of starch degrading enzymes was also regulated by the presence of monomeric sugar (Mountfort and Asher, 1988). Induction of extracellular cellulase was also observed in *N. frontalis* by switching pre-grown cultures from glucose to cellulosic substrates (Barichievich and Calza, 1990).

Besides catabolic regulation, enzyme production by anaerobic fungi was also dependent on *de novo* protein synthesis and protein glycosylation (Calza, 1991a, 1991b; Li and Calza, 1991b). Glycosylation inhibitor, viz. tunicamycin and 2-deoxy-D-glucose inhibited the secretion of protein and cellulase by *N. frontalis* EB 188 (Li and Calza, 1991b). Cellulase synthesized in presence of inhibitors possessed altered catalytic properties. Electrophoresis experiments measuring protein molecular masses and pkl suggested that a different set of proteins were synthesized by anaerobic fungus. Calza (1991b) concluded that glycosylation played an important role in the secretion, catalytic activity and stability of cellulase enzymes of anaerobic fungus.

The action of fibre degrading enzymes were also subject to control by surface of the substrates. The attachment and cellulose digestion by anaerobic fungi were completely inhibited by the surfactant like methylcellulose (Cheng *et al.*, 1991). Although the addition of methylcellulose did not affect the growth of anaerobic fungi, but further cellulolysis was inhibited by the above surfactant. Like bacterial cellulolysis, anaerobic fungi required the spatial juxtaposition with its attacking substrates (Cheng *et al.*, 1991).

2.18 FERMENTATION END PRODUCTS OF ANAEROBIC FUNGI

Until the mid-1970, it was thought that rumen ecosystem was dominated only by bacteria and protozoa. However, this assumption was challenged by Orpin in 1975, who first reported that anaerobic chytridiomycete fungus were also permanent and important member of rumen eco-club along with bacteria, protozoa, yeast and phage. The fermentation end products of these anaerobic fungi were governed by several factors, viz., nature of substrates, presence of other microbes and so on (Theodorou *et al.*, 1996).

During growth on xylan, reducing sugars, including arabinose and xylose were rapidly accumulated in the medium (Lowe *et al.*, 1987c). Xylose and other reducing sugars were subsequently used for growth of anaerobic fungi, leaving only arabinose. During growth over wheat straw, wheat straw-holocellulose or cellulose, there was also accumulation of reducing sugars but at lower rate. The fermentation by *Neocallimastix* sp. over glucose and xylose resulted in the production of formate, acetate, lactate and ethanol (Lowe *et al.*, 1987b). In a study with five anaerobic fungi, Borneman *et al.* (1989) concluded that accumulation of fermentation products was concurrent with substrate utilization. The major fermentation products for all isolates were formate, acetate, D(-) lactate, L(+) lactate, ethanol, carbon dioxide and hydrogen. Monocentric isolates produced a greater ratio of oxidized to reduced products when grown on glucose or coastal bermuda grass than did the polycentric isolates, which produced a nearly equal ratio of these products.

When grown in the presence of methanogenic bacteria, the fermentation profile of anaerobic fungus was shifted away from electron sink products such as ethanol and lactate and towards the more reduced products such as acetate and formate (Theodorou *et al.*, 1996). In methanogenic co-culture of rumen fungi, acetate was the major product and carbon dioxide production increased, whereas lactate and ethanol decreased. Substantial amounts of methane were also produced (Bauchop and Mountfort, 1981). In addition to changes in fermentation profile, methanogenic co-culture showed significant increase in fungal biomass production also. This could be due to removal of fermentation inhibitory intermediates, viz. ethanol, formate and lactate (Theodorou *et al.*,1988; Joblin and Naylor, 1993).

Chapter 3

Materials and Methods

3. MATERIALS AND METHODS

In the mid 1970s, it was confirmed that the rumen organisms which were earlier identified as zooflagellates, were actually of fungal origin (Orpin, 1975). Later on, several aspects of rumen fungi have been reported including their obligately anaerobic nature, presence of chitin in the cell walls, colonization over plant fibre, stages of life cycle, secretion of different enzymes etc. (Orpin, 1975,1977a,1977b; Theodorou *et al.*, 1996; Samanta *et al.*, 1997). Rumen fungi are able to grow on a rumen simulating media, indicating that pH of 6.5 to 6.7, temperature around 39°C and nutrient requirements of the fungi are met from the media normally used for rumen bacteria. The semi-synthetic basal medium of Caldwell and Bryant (1966), in which yeast extract, tryptone, hemin and a mixture of VFAs replaced rumen fluid, has also supported a variety of anaerobic fungi. However, absolute requirements for growth have not been determined for most of the anaerobic fungi. While reviewing the nutritional requirement for *N. patriciarum*, Orpin (1989) reported that heme, D-biotin and thiamin or its precursors, sulfur were required in reduced form while nitrogen requirement was met with ammonium ions or amino acids. Based on the above information, following techniques and methodologies were followed to study the anaerobic fungi from cattle and buffalo for fulfilling the set objectives.

3.1 DESCRIPTION OF THE EXPERIMENTAL ANIMALS

3.1.1 CATTLE

Three crossbred (Sahiwal x Holstein Friesian) male calves of 2 to 2.5 years age were procured from NDRI farm and fitted with rumen fistula. The average weight of the animals were 280 to 300 kg. Animals were housed in the well ventilated individual pens keeping in mind the optimum comfort required by the animal. Before starting the experimental feeding, all the animals were dewormed and vaccinated against prevalent diseases in order to keep them free from infection during the experiment.

3.1.2 BUFFALO

Three Murrah male buffaloes, aged about 2 years were procured from the Institute herd. The average weight of the animals was 250 kg. The animals were subjected to rumen fistulation and were housed in the well ventilated individual pens by providing optimum comfort. Before starting the experimental feeding, all buffaloes were dewormed and vaccinated against prevalent diseases during that season.

3.2 DIETARY COMBINATIONS USED FOR FEEDING ANIMALS

The animals (3 rumen fistulated cattle and 3 rumen fistulated buffaloes) were maintained on three dietary regimes with different roughage and concentrate ratios. The details of the diets are given on Table 3.1.

Table 3.1 Feeding schedule to experimental animals

Dietary component	*Dietary combinations*		
	T1	*T2*	*T3*
Wheat straw (parts)	50	0	50
Concentrate (parts)	50	50	0
Green fodder (parts)	0	50	50
Roughage:concentrate	50:50	50:50	100:0

In case of T1, all roughage source was from wheat straw and there was no green fodder in the ration. The roughage source of T2 was only from green fodder and there was no wheat straw at al. But in case of T3, 50 parts were from green fodder and rest 50 parts from wheat straw. There was no concentrate at al.

3.3 COMPOSITION OF THE CONCENTRATE MIXTURE

The individual ingredients of the concentrate mixture have been given in Table 3.2.

Table 3.2 Proportion of different ingredients of concentrate mixture

Sl. No.	*Ingredients*	*Part*
1.	Maize	45
2.	Wheat bran	22
3.	Groundnut cake	30
4.	Mineral mixture	2
5.	Salt	1

3.4 SAMPLING TIME

Rumen liquor was collected at 0, 1, 2, 3 and 4 hours of post feeding for counting the fungal population.

3.5 SAMPLING PROCEDURE

Rumen liquor was collected by plastic tube through permanent rumen fistula and brought in a pre-gassed (using carbon dioxide) and autoclaved flat bottom flask of 250 ml capacity. The flask containing rumen liquor was kept in a thermostatic bucket containing water at 39 ± 1° C.

3.6 ENUMERATION OF ANAEROBIC FUNGAL POPULATION

Hungate roll tube technique (Hungate, 1969), as adopted by Joblin (1981) for enumeration of anaerobic fungi was followed for counting rumen as well as fecal anaerobic fungal population. The carbon dioxide gas was first passed through heated (350°C) copper column of Biosystem Gassing Manifold to make the gas free from oxygen. Under heating condition, copper reacts with oxygen and the manifold system supplies the oxygen free carbon dioxide during the culturing of anaerobic fungi. The composition of the medium (Joblin, 1981) has been presented in Table 3.3.

Table 3.3 Anaerobic media for enumerating fungal population (Joblin, 1981)

Ingredients	*Quantity*
Salt solution A	17 ml
Salt solution B	17 ml
Clarified rumen fluid	15 ml
Yeast extract	50 mg
Tryptone	100 mg
Cellobiose	200 mg
0.1% Resazurin solution	0.3 ml
0.05% Hemin solution	0.2 ml
5% Sodium bicarbonate	5.0 ml
5% Cysteine hydrochloride	2.0 ml
Distilled water	43.6 ml
Agar	2.0 g

3.6.1 COMPOSITION OF SALT SOLUTIONS

The composition of salt solutions are as par Table 3.4 and 3.5.

Table 3.4 Composition of salt solution A (Bauchop and Mountfort, 1981)

Salt	*Quantity (wt/vol)*
KH_2PO_4	0.3%
NaCl	0.6%
$[NH_4]_2SO_4$	0.3%
$CaCl_2$	0.03%
$MgSO_4$	0.03%

Table 3.5 Composition of salt solution B (Bauchop and Mountfort, 1981)

Salt	*Quantity (wt/vol)*
K_2HPO_4	0.3%

3.6.2 CLARIFIED RUMEN FLUID (CRF) FOR THE MEDIUM

The freshly collected rumen liquor was strained through double layer of muslin cloth and clarified through centrifugation at 12,000 rpm for 30 minutes and kept at -5° C. Before adding to the medium, the rumen liquor was thawed and again centrifuged to remove any sedimented material. The clarified rumen fluid was used to provide unidentified growth factor to the anaerobic fungus.

3.6.3 0.05% HEMIN SOLUTION

0.05 g hemin powder was dissolved in a solution of 1:1 ethanol and 0.05 M sodium hydroxide solution.

3.6.4 ANAEROBIC MEDIA PREPARATION

With the exception of reducing agents (sodium bicarbonate and cysteine hydrochloride), al: the quantified ingredients of medium were taken in 250 ml high necked, flat bottom flask. The pH was adjusted within the range of 6.6 to 6.8. The medium was boiled to free it from dissolved oxygen and cooled under the flow of carbon dioxide, supplied through biosystem gassing manifold. Before solidification of media under continuous carbon dioxide flow, the quantified reducing agents were added. The flask containing the media was closed by rubber cork and tied through wire and autoclaved. The colourless or slight yellowish appearance of media was indicative of perfectly anaerobic condition while any pinkish appearance was indicative of aerobic or imperfect anaerobic condition.

3.6.5 PREPARATION OF ANAEROBIC DILUENT

The composition of the anaerobic diluent has been presented in Table 3.6.

Table 3.6 Composition of anaerobic diluent (Bryant and Burkey, 1953)

Ingredients	*Quantity*
Salt solution A	5.0 ml
Salt solution B	5.0 ml
5% Cysteine hydrochloride	4.0 ml
5% Sodium carbonate	10.0 ml
0.1% Resazurin	0.2 ml
Distilled water	175.8 ml

By following the anaerobic media preparation technique, 9 ml of anaerobic diluent was dispensed in pre-gassed 20 ml glass vial, sealed and autoclaved. The anaerobic diluent was used for dilution of rumen liquor at the time of anaerobic fungal culture.

3.6.6 ANTIBIOTIC SOLUTION

A. Penicillin stock solution

The stock solution for sodium salt of benzyl penicillin was prepared, having a concentration of 2×10^4 IU per ml and sterilized through membrane filtration (0.22 μm pore diameter). This was gassed through oxygen free carbon dioxide and 0.25 ml was added in each roll tubes.

B. Streptomycin sulfate stock solution

Streptomycin stock was prepared at a concentration of 2 mg per ml and sterilized through membrane filter (0.22 μm pore diameter). This was gassed through oxygen free carbon dioxide before use and 0.25 ml was dispensed into the roll tubes.

3.6.7 PROCESSING OF RUMEN LIQUOR

Freshly collected rumen liquor (either from cattle or buffalo) was used without straining as a source of inoculum, and stirred for 5 to 7 minutes on a magnetic stirrer. The stirring was performed with a continuous flow of carbon dioxide in front of the flame. Then 1 ml of stirred rumen liquor was dispensed in 20 ml glass vial containing 9 ml of anaerobic diluent to get requisite dilution. This gave a dilution of 1:10. The same procedure was again followed for making subsequent dilutions.

3.6.8 PROCESSING OF FECAL SAMPLE

One gram of freshly collected feces of either cattle or buffalo was stirred for 5 to 7 minutes in a fixed volume of anaerobic diluent with a continuous flow of carbon dioxide in front of the flame. This was used as a source of inoculum for the enumeration of fecal anaerobic fungus.

3.6.9 DISPENSING OF ANAEROBIC MEDIA

The sterile roll tubes (7.5 x 1.5 cm) were gassed with oxygen free carbon dioxide for 2 minutes under aseptic conditions. Then the molten media (4 ml) was dispensed in each tube and kept in water bath at 45° C.

3.6.10 INOCULATION INTO ROLL TUBES

The inoculum (0.5 ml) from different sources at desired level of dilution was added in roll tubes containing molten media and antibiotic solution. The tubes were then rolled over platform of crushed ice to solidify the media uniformly as a thin film on the inner wall of the roll tube. The tubes were kept in incubator at a temperature of 39 ± 1° C on inverted position. During incubation, the roll tubes were regularly inspected for appearance of colonies visible either through naked eye or through an inverted microscope. The number of colonies were then counted and characterized.

3.7 ISOLATION OF ANAEROBIC FUNGUS

The anaerobic fungal colonies which appeared in roll tubes were transferred to a media containing 0.3 per cent agar, or in Joblin's media, where cellobiose was replaced by 1 per cent wheat straw, in the presence of the recommended dose of antibiotics.

3.8 CULTURE PURITY

The purity of the culture was examined regularly under microscope and confirmed by reisolation on solid media until single type of colony was obtained. The culture were also checked for growth under aerobic conditions. The pure cultures grown only under strict anaerobic conditions were maintained subsequently.

3.9 MAINTENANCE OF FUNGAL ISOLATES

The anaerobic fungal isolates were maintained in 1 per cent wheat straw containing media or 0.2 per cent cellobiose soft agar media (0.3% agar) in the presence of recommended dose of antibiotics. It was done in 250 ml flat bottom, high necked flask. In each case subculturing was done by transferring the inoculum at 5% level.

3.10 IDENTIFICATION OF ANAEROBIC FUNGUS

Identification of the fungus was done upto the genus level, based on morphological features of zoospores, sporangia and nature of the rhizomycelia (Trinci *et al.*, 1994; Theodorou *et al.*, 1996).

3.11 SUBSTRATE UTILIZATION BY FUNGAL ISOLATE

The fungal isolates were tested for their ability to grow on different energy source, viz. cellobiose, cellulose, xylan, Whatman filter paper and wheat straw.

3.12 ANALYSIS OF THE METABOLIC PRODUCTS OF ANAEROBIC FUNGUS

3.12.1 GAS PRODUCTION

The isolate was first grown in a sealed glass vial containing 5 ml cellobiose soft agar at 39° C. During course of incubation the gas production was visually assessed and isolates were grouped into profused (+ + + +), moderate (+ + +), small quantity (+ +) and negligible (+) gas producer.

3.12.2 FRACTIONATION OF VFAs

The fungal isolates were grown on 4 ml of cellobiose soft agar medium. Following inoculation, one tube was taken from the incubator periodically at 24 hours interval, and the media was mixed with 1 ml of 20% metaphosphoric acid (prepared in 5 N H_2SO_4). This was kept for overnight at room temperature and centrifuged at 4,000 rpm for 30 minutes (Erwin *et al.*, 1961). The supernatant obtained after centrifugation was subjected to VFA analysis on Nucon Gas Chromatograph Series 5500 fitted with flame ionization detector. The column was chromosorb 101. Standard VFA mixture containing acetate, propionate and butyrate in the ratio of 70:20:10 were injected to the GLC before loading the sample.

Calculation

Area of the peak = 1/2 x height x width of the base

Area of the peak for acetate, propionate and butyrate were calculated and compared with the area of the peaks of standard and were presented accordingly.

3.13 LIGHT MICROSCOPY

Pieces of wheat straw either from rumen or from culture tubes were routinely examined under microscope without staining or by using lactophenol cotton blue as the stain. Similarly, fungal zoospore, sporangia and rhizomycelia were observed under microscope with or without staining.

For counting and motility of the zoospore, a hemocytometer was used. The measurement of zoospore, sporangia and rhizomycelia were also done with the help of stage and occulometer under the microscope.

3.14 AMINO ACID COMPOSITION OF FUNGAL ISOLATE

The isolate was grown in 1 litre of cellobiose soft agar for five days. The fungal biomass was collected by filtering through a muslin cloth. The residue left was given washings by normal saline in order to remove traces of media. Fungal cells were then kept in an oven (60-70° C) for 72 hours to dry the cells completely. Subsequently, cells were powdered in pestle-mortar and kept for amino acid analysis by using high performance liquid chromatography (HPLC).

3.14.1 PREPARATION OF PROTEIN HYDROLYSATE

A known quantity (200 mg) of the dried fungal biomass was taken in a 20 ml glass vial. 10 ml of 6 N hydrochloric acid was added to the vial. After passing nitrogen gas for 1 to 2 minutes, the vial was sealed by aluminium cap. The sealed vial was kept in the oven at 110° C for 20 hours (Roach and Gehrke, 1970) to complete the hydrolysis of the fungal proteins.

3.4.12 INJECTION OF SAMPLE AND ANALYSIS

After 20 hours, the protein hydrolysate was filtered through Whatman filter paper (No. 40) and the volume was made upto 15 ml with distilled water. The diluted sample was again filtered through millipore filter (0.22 μm). 10 μl of above filtered sample was taken in a capillary tube and dried under vacuum for 45 minutes. 30 μl of solution containing water, methanol and triethylamine in the ratio of 2:2:1 was also added in the capillary tube and the contents were again dried under vacuum for 45 minutes. 30 μl of water, methanol and triethylamine (2:2:1) solution was again added and dried under vacuum for 45 minutes. Then 200 μl of dilutor (0-phthal-aldehyde/2 mercaptoethanol) was added and incubated for 1 minute at 39° C for the reaction to take place to cause fluorescence. About 20 μl of the above diluted sample was injected for analysis in high performance liquid chromatography (HPLC). Standard of known concentration of each amino acid was run simultaneously to know the concentration of unknown amino acids.

3.15 SCREENING OF ANAEROBIC FUNGAL ISOLATES FOR THE PRODUCTION OF ENDOGLUCANASE ACTIVITY

The different fungal isolates were anaerobically grown over cellobiose soft agar in sugar tubes, capped by rubber corks. These were incubated at 39 ± 1° C for a period of four days after which the supernatant containing enzymes were harvested by centrifugation at a speed of 12,000 rpm for 30 minutes at 4°C. The supernatant obtained was used for endoglucanase assay as well as congo red assay for their ability to produce yellow zone of clearance in CMC agar.

3.15.1 CONGO RED ASSAY

The method described by Beguin (1983) was followed to confirm the endoglucanase activity of different fungal isolates. Congo red bound strongly to polysaccharides containing ß-1, 4-glycosidic linkage and provided the basis for the highly sensitive and qualitative test for detecting cellulase activity.

The carboxymethyl cellulose/xylan (1%) containing 1.8 per cent agar was dispensed around 25 to 30 ml in each sterilized petri plates. After solidification, wells were made by standard sized well borer in front of flame. Then about 100 to 200 µl of enzyme preparation was poured in each well and incubated at 39° C for overnight. The petri plates were flooded with 0.1 per cent congo red solution and kept for 30 minutes. The staining solution was drained out. The plate was destained by 1 M sodium chloride solution and looked out for the appearance of any yellow zone of clearance, which was indicative of the endoglucanase/xylanase activity.

3.15.2 QUANTIFICATION OF ENDOGLUCANASE ACTIVITY

Endoglucanase was assayed by the method of Mountfort and Asher (1985).

3.16 TIME OPTIMIZATION FOR HARVESTING THE ENZYME

Different fungal isolates were grown in 200 ml soft agar media under strict anaerobic condition. The inoculum was used at 5% level. Two antibiotics, viz. Penicillin and Streptomycin were used to keep the fungal isolate free from any bacterial contamination. Following inoculation, culture samples were withdrawn periodically at 24 hours interval by following aseptic and anaerobic condition during the total growth period of 8 to 9 days. These samples were then subsequently used for harvesting the endoglucanase.

3.17 HARVESTING OF ENZYMES

The culture supernatant thus withdrawn were taken in 25 ml clean, autoclaved centrifuge tube and spinned in refrigerated centrifuge at a speed of 12,000 rpm for 30 minutes at 4°C. The supernatant obtained was kept for different enzyme assays and protein estimation. The pellet formed was also subjected to sonication for enzyme assay.

3.18 DISRUPTION OF FUNGAL RHIZOMYCELIA AND SPORANGIA

The cell pellet obtained after centrifugation was thoroughly washed with 0.1 M citrate phosphate buffer (pH 6.0) to remove the left over agar and other media ingredients. The fungal cell including rhizomycelia and sporangia free from any media components were subject to sonication, four times for 15 seconds each, with a time interval of 2 minutes under ice covering (Malik, 1996). The sonicated sample was then centrifuged at a speed of 12,000 rpm for 30 to 40 minutes at 4°C. The supernatant was collected and tested for enzyme activity.

3.19 PARTIAL PURIFICATION OF ENDOGLUCANASE

3.19.1 SOURCE OF ENZYME

The fungal isolate having maximum endoglucanase activity was grown in 500 ml flat bottom, high necked anaerobic flask containing 200 ml cellobiose soft agar. The inoculum was used at 5% level and allowed to grow at 39 ± 1°C for a period of four days in the presence of penicillin and streptomycin. After the growth of fungus for a stipulated period, the enzyme was harvested from the medium by centrifugation at a speed of 12,000 rpm for 30 to 40 minutes at 4° C. The supernatant containing the enzyme was preserved in clean, autoclaved flask at -20° C following the addition of PMSF at a level of 0.2 mM.

3.19.2 AMMONIUM SULFATE PRECIPITATION

The first test was done in order to find out the optimum concentration of ammonium sulfate to precipitate the protein present in the supernatant. For this, 20 ml of the supernatant containing the enzyme was tried to precipitate out the protein at ammonium sulfate level of 40% , 60% and 80%. Measured quantity of solid ammonium sulfate was added gradually with slow stirring and tried to complete this step within 10 to 15 minutes under the at low temperature. This was kept at 4°C for either 2 hours or overnight. The above material was then centrifuged at 12,000 rpm for 30 to 40 minutes at 4°C. The supernatant was kept for enzyme and protein assay. The pellet so obtained was dissolved in minimum volume of either 20 mM sodium phosphate buffer (pH 6.5) or 20 mM Tris-HCl buffer (pH 7.5) and subjected to enzyme and protein assay. The level of ammonium sulfate so determined was used in subsequent experiment to precipitate out the enzyme protein on large scale. All steps as described above were followed for large volume of enzyme also.

3.19.3 DIALYSIS

The protein (enzyme) obtained by ammonium sulfate precipitation was dialyzed overnight against 20 mM sodium phosphate buffer (pH 6.5) or 20 mM Tris HCl buffer (pH 7.7). The dialyzed sample was tested for different glycosidase activities.

3.19.4 ION EXCHANGE CHROMATOGRAPHY

The dialysed sample was then tried for further purification through DEAE Sepharose as well as hydroxylapatite column chromatography.

3.19.4.1 DEAE Sepharose ion exchange chromatography

The DEAE Sepharose 2B (Sigma) was available in 20 per cent alcoholic suspension. Before packing the column, the gel was subjected to several steps of washings.

A. GEL PREPARATION AND COLUMN PACKING

(a) 1 x vt. (total bed volume) 1 M sodium acetate (pH adjusted at 3.0 with diluted HCl) for 5 minutes at 4°C.

(b) 1.5 x vt. 0.5 M NaOH. The gel was kept in 0.5 M sodium hydroxide solution overnight at 4°C.

(c) 1.5 x vt. 1 M sodium acetate (pH 3.0) for 5 minutes at 4°C.

(d) The gel was then equilibrated with the 20 mM sodium phosphate buffer, pH 6.5 (starting buffer). The gel was washed with this buffer till the pH was brought to 6.5. The equilibrated DEAE Sepharose-2B matrix was then packed into the column (2.2 x 12 cm) and kept under cold conditions (4°C).

B. SAMPLE LOADING AND ELUTION

The dialysed enzyme sample was layered onto the column and unbound proteins were eluted with 1 bed volume (20 ml) of 20 mM sodium phosphate buffer at pH 6.5, with a flow rate of 30 ml h^{-1}. The bound proteins were eluted by using a linear gradient of 200 ml of 20 mM sodium phosphate buffer, pH 6.5 and 200 ml of 500 mM sodium chloride prepared in 20 mM sodium phosphate buffer, pH 6.5. Strongly bound proteins were attempted to elute through 1 M sodium chloride prepared in 20 mM sodium phosphate buffer, pH 6.5. Fractions of 5 ml each were collected. The flow rate was

50 ml h^{-1}. All the fractions were kept under ice-cold jacket for further analysis of protein and enzyme activity.

C. TESTING OF DEAE SEPHAROSE ANION EXCHANGER FOR THEIR ABILITY TO BIND WITH ENDOGLUCANASE

One ml of fresh DEAE Sepharose anion exchanger was taken in 15 ml centrifuge tube and equilibrated with 20 mM sodium phosphate buffer (pH 6.5) by several changes (4 to 5 times). Then 0.5 ml of dialysed enzyme was added to the anion exchanger and kept at 4° C for 5 minutes and centrifuged at 5,000 rpm for 10 minutes. The supernatant was designated as S1. The pellet was dissolved in 2 ml of 20 mM sodium phosphate buffer (pH 6.5). Centrifuged at 5000 rpm for 10 minutes. The supernatant was designated as S2. The pellet was dissolved in 2 ml of 20 mM sodium phosphate buffer. Centrifuged at 5000 rpm for 10 minutes. Designated the supernatant as S3. Dissolved the pellet in 2 ml of 1 M sodium chloride prepared in 20 mM sodium phosphate buffer. Centrifuged at 5000 rpm for 10 minutes. Designated the supernatant as S4. Dissolved the pellet in 2 ml of 1 M sodium chloride prepared in 20 mM sodium phosphate buffer. Centrifuged at 5000 rpm for 10 minutes. Designated the supernatant as S5. The pellet was termed as DEAE Sepharose protein complex (DEAE SPC). The different supernatant fractions, viz. S1, S2, S3, S4, S5 and DEAE SPC were tested for endoglucanase assay.

3.19.4.2 Hydroxylapatite column chromatography

Hydroxylapatite matrix (Sigma) was first washed with 5 mM potassium phosphate buffer, pH 6.5 for several times under cold condition. Then the anion exchanger was packed into small glass column (1.5 x 10 cm) and kept under cold condition. The dialysed enzyme (6 ml) was layered onto the column and unbound proteins were eluted with 50 ml of 5 mM of potassium phosphate buffer, pH 6.5 with a flow rate of 5 ml h^{-1} (Li and Calza, 1991a). The bound proteins were eluted with a linear gradient of 60 ml of 5 mM potassium phosphate buffer and 60 ml of 450 mM potassium phosphate buffer. Strongly bound proteins were eluted with 50 ml of 1 M potassium phosphate buffer (pH 6.5). Fractions of 3.5 ml each were collected and kept at 4°C for enzyme and protein assay.

3.19.5 FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC) OF FUNGAL ENZYMES

The dialysed enzyme was further attempted to purify through FPLC on Superose 12 gel (cross linked agarose)/mono-Q HR 5/5 column designed with FPLC system (Pharmacia).

3.19.5.1 Description of Superose 12 gel

The superose 12 gel was available in 1 per cent sodium azide. The fractionation range varied from 1000 to 3 x 10^5 Dalton with functional pH range from 1 to 14. Workable temperature ranged from 4 to 40°C. Size of the column was 25 ml. The gel was first washed and equilibrated with 50 mM sodium phosphate buffer (pH 6.5) containing 150 mM sodium chloride and 0.2 mM PMSF. The equilibration was performed at a flow rate of 0.4 ml per minute and the back pressure was maintained below 2 MPa. Two column volumes, i.e., 50 ml of the above mentioned de-gassed phosphate buffer was passed through the column before loading the enzyme sample. The seven way, 3 position valve was fixed on load position at the time of washing or equilibration.

3.19.5.1.A Fraction collector

The fraction collector (Redifrac, Pharmacia) was connected with the FPLC system before loading the sample. The fraction of 1 ml each was collected by setting the flow rate at 0.5 ml per minute.

3.19.5.1.B Recorder

The recorder was connected with the FPLC and the recorder ink pen was set at the uniform base line after setting the sensitivity at 0.1. The chart speed was 2 mm per minute.

3.19.5.1.C Sample preparation

Before loading on the FPLC system, the sample was centrifuged at 6,000 rpm for 10 minutes to settle down the precipitated proteins. This was needed for protecting the column from blocking.

3.19.5.1.D Sample application and elution

After all the settings (connection of FPLC with fraction collector and recorder, uniform flow rate, correct base line etc.) were over, the 7-way valve was shifted from the load position to the inject position. Then 200 μl of the dialysed enzyme sample was injected into the injector loop through the help of a syringe (Malik, 1996). Following loading of the sample, the 7-way valve was shifted back to the load position. Immediately, fraction collector and recorder were started. Elution of the sample was performed at the flow rate of 0.5 ml per minute. The chart speed was set at 2 mm per minute. One ml of each fraction was collected and endoglucanase activity in each fraction was assayed. Protein profile was recorded by the recorder.

After the run, the column was washed with two bed volume (50 ml) of elution buffer and subsequently stored in 1 per cent sodium azide.

3.19.5.2 Description of mono-Q HR 5/5 column

This anion exchanger gel was available in 20 per cent ethanol-water solution with sulfate as counter ion. The mono-Q is a strong anion exchanger (particle size 10 μm) based on a beaded hydrophilic resin with one of the narrowest particle size. The charged group on the gel is $-CH_2-N^+-(CH_3)_3$. Separation limit of the substances is with molecular weight upto 10^7. The column size was 1 ml. The column was first washed with 5 ml of start buffer (20 mM Tris HCl buffer, pH 7.7). Then it was washed with 0.4 M NaCl prepared in 20 mM Tris HCl buffer (pH 7.7). Again it was equilibrated with 5 ml of start buffer. The flow rate and back pressure were restricted at 1 ml per minute and less than 5 MPa, respectively. The seven way, 3-position valve was fixed at the load position during washing as well as during equilibrating the column.

3.19.5.2.A Fraction collector

As described in section 3.19.5.1.A

3.19.5.2.B Recorder

As described in section 3.19.5.1.B

3.19.5.2.C Sample preparation

As described in 3.19.5.1.C

3.19.5.2.D Sample application and elution

The following programme (Table 3.7) was applied for gradient elution of protein on mono-Q column.

Table 3.7 The programme for gradient elution of protein on mono-Q column

Time (minute)	*Percentage of B*
0	0
7	0
25	75
25	100
30	100
30	0
35	0

where, B was the 0.4 M sodium chloride dissolved in 20 mM Tris HCl buffer (pH 7.7) and A was the 20 mM Tris HCl buffer (pH 7.7).

After all the settings were over, the seven way valve was shifted from the load position to the inject position. Then 500/1000 μl of the dialysed enzyme sample was injected into the injector loop with the help of a syringe. After loading of the sample, the 7 way valve was shifted back to the load position. The fraction collector and recorder were started immediately. Elution of the sample was continued at a flow rate of 0.5 ml per minute. 1 ml of each fraction was collected and endoglucanase/ xylanase activity was assayed. Protein profile was recorded by the recorder. After the each run, the column was washed with 20 mM Tris HCl buffer (pH 7.7) until the back pressure come down at 2 MPa (while flow rate is 1 ml per minute).

3.20 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE in slab gel was performed as per the method of Laemmli (1970).

3.20.1 STOCK ACRYLAMIDE/BIS-ACRYLAMIDE (30%)

29.2 g of acrylamide and 0.8 g of bis-acrylamide were dissolved in 100 ml of distilled water. The solution was filtered by Whatman filter paper No. 1 and stored at 4°C in dark bottle.

3.20.2 1.5 M TRIS-HCl (pH 8.8)

18.15 g of Tris was dissolved in 80 ml of distilled water and adjusted the pH at 8.8 with 1 N HCl and volume was made 100 ml with distilled water and kept at 4°C.

3.20.3 0.5 M TRIS-HCl (pH 6.8)

6 g of Tris was dissolved in 80 ml of distilled water and the pH was set at 6.8 with 1 N HCl. Total volume was made upto 100 ml with distilled water and stored at 4°C.

3.20.4 10% SDS

10 g of sodium dodecyl sulfate was dissolved in 100 ml of distilled water.

3.20.5 ELECTRODE BUFFER (5X, pH 8.3)

9 g of Tris, 43.2 g of glycine and 3 g of sodium dodecyl sulfate were dissolved in 600 ml of distilled water. If chemicals are perfect and weighed correctly, the pH

automatically reaches the value of 8.3 ± 0.2. As per recommendation of the method, the pH should not be adjusted either with alkali or acid. Before running of the gel, the electrode buffer was diluted five times with distilled water and used in chilled condition only.

3.20.6 STACKING GEL (10 ml)

It was prepared with the following solutions as in Table 3.8.

Table 3.8 Composition of stacking gel

Components	*Quantity*
Distilled water	6.14 ml
30% acrylamide mixture	1.30 ml
0.5 M Tris-HCl (pH 6.8)	2.50 ml
10% ammonium persulphate	50 µl
TEMED	10 µl

3.20.7 SEPARATING GEL (8%, 10 ml)

It was prepared with the following solutions as given in Table 3.9.

Table 3.9 Composition of separating gel

Components	*Quantity*
Distilled water	4.895 ml
30% acrylamide mixture	2.5 ml
1.5 M Tris HCl (pH 8.8)	2.5 ml
10% ammonium persulphate	100 µl
TEMED	5 µl
Carboxymethyl cellulose or xylan (0.1%)	10 mg

Carboxymethyl cellulose was first dissolved in 1.5 M Tris HCl (pH 8.8). In case of xylan, 10 mg was first weighted and dissolved in 1.5 M Tris HCl (8.8) by slight heating.

3.20.8 GEL PREPARATION

Immediately after adding the measured quantity of ammonium per sulfate and TEMED, the separating gel was poured into thé gel cassette with 1 mm spacer. Water was layered at appropriate time. Appearance of interface within 5 to 10 minutes indicated the completion of polymerization of the gel. Water was carefully removed. After addition of fixed quantity of 10% ammonium per sulfate and TEMED, immediately the stacking gel was layered over the polymerized separating gel. The comb was then placed into the stacking gel for making the wells. Comb was taken out after polymerization of the gel.

3.20.9 PREPARATION OF MOLECULAR WEIGHT MARKER

The SDS-PAGE molecular weight standards, high range and low range were used. Following steps were followed for preparing molecular weight markers.

3.20.9.1 Dilution of molecular weight standards

The standards were diluted 1:20 in SDS reducing sample buffer. The same was boiled for 3-5 minutes at 95°C. Cooled and 5 μl was loaded into the gel.

3.20.9.2 SDS reducing sample buffer

It was prepared immediately before use

ß-mercaptoethanol	25 μl
Stock sample buffer	475 μl

3.20.9.3 Stock sample buffer

It was prepared with the following solutions as described in Table 3.10.

Table 3.10 Composition of the stock sample buffer

Solutions	*Quantity*
0.5 M Tris HCl (pH 6.8)	1.2 ml
Glycerol	1.0 ml
10% (w/v) SDS	2.0 ml
0.1% (w/v) Bromophenol blue	0.5 ml
Distilled water	4.8 ml

3.20.9.4 Protein molecular weight marker (low range)

The SDS-PAGE Low range molecular weight standards are given in Table 3.11.

Table 3.11 The low range SDS-PAGE molecular weight standards

Protein	*Molecular weight (daltons)*
Bovine serum albumin	66,000
ß-amylase	50,000
Alcohol dehydrogenase	38,000
Carbonic anhydrase	31,000
Cytochrome C	12,400

3.20.9.5 Protein molecular weight markers (high range)

The higher range of protein molecular weight standards are given in Table 3.12.

Table 3.12 The high range SDS-PAGE molecular weight standards

Protein	*Molecular weight (daltons)*
Mycsin	200,000
ß-galactosidase	116,250
Phosphorylase b	97,400
Serum albumin	66,200
Ovalbumin	45,000

3.20.10 SAMPLE PREPARATION

Enzyme sample for observing protein bands and activity bands (zymograms) were processed in different ways. Following sample buffers were tried:

Sample buffer A

Glycerol	1.6 ml
10% SDS	3.2 ml
0.5 M Tris HCl (pH 6.8)	1.0 ml
Distilled water	1.4 ml

Sample buffer B

Sample buffer A	0.9 ml
ß-mercaptoethanol	0.1 ml

Sample buffer C

Sample buffer A	0.98 ml
10 mM DTT	0.02 ml

3.20.10.1 Protein bands

Before loading in gel, the enzyme solution was mixed with twice its volume of sample buffer B. One μl 0.1 per cent (w/v) bromophenol blue was added. The mixture was heated on boiling water bath for 5 minutes. Cooled and 30 μl was loaded onto the gel.

3.20.10.2 Activity bands (zymogram)

The enzyme sample was mixed with twice its volume of sample buffer C. One μl 0.1 per cent (w/v) bromophenol blue was added. It was now ready for loading into the gel.

3.20.11 ELECTROPHORESIS

The electrode buffer was properly diluted, chilled and poured into the electrophoretic unit (Startagene/Bio-Rad). For detecting the protein bands, 20 to 30 μl

of protein was loaded. For zymogram, 3 to 5 μl of sample was loaded in different wells in the gel containing either carboxymethyl cellulose or xylan. Then electrophoresis was carried out at 4°C at a constant current of 25 mA. Electrophoresis was continued till the dye reached at the bottom of the gel.

3.20.12 PROCESSING OF GEL AFTER ELECTROPHORESIS

After completion of electrophoresis, gels were removed from electrophoretic unit and cut into vertical pieces. One fragment was kept for detection of protein bands either by Coomassie Brilliant Blue or by silver staining method. Another fragment was kept for detection of enzyme activity bands.

3.20.12.1 Staining with Coomassie Brilliant Blue

The staining solution was prepared with Coomassie Brilliant Blue R-250 0.25 per cent (v/v), methanol 25 per cent (v/v) and glacial acetic acid 10 per cent (v/v). The cut fragment was flooded with above staining solution and kept on rocking floor (3 revolutions per minute) for 4 h (Wood and McCrae, 1977).

3.20.12.2 Destaining of the gel

Following completion of staining for 4 h, the gel was transferred to the destaining solution and kept on rocking floor (3 revolutions per minute) for overnight with frequent changing of destaining solution. The destaining solution was prepared with 5 per cent (v/v) methanol and 7.5 per cent (v/v) glacial acetic acid. Destaining was continued till the appearance of protein bands.

3.20.12.3 Visualization of bands by silver staining

The ammonical silver staining method described by Harlow and Lane (1988) was followed. The steps were as follows:

1. After running the SDS-PAGE, the gel fragment was placed in 5 gel volume of 50 per cent ethanol, 10 per cent glacial acetic acid for overnight with shaking at room temperature. Solution was discarded and gel was rinsed with water.

2. Gel was placed in 5 gel volume of 20 per cent ethanol for 30 minutes with continuous shaking. This step was repeated for two times.

3. After removing ethanol solution, the gel was placed in 5 gel volume of 5 per cent glutarladehyde (prepared in deionized water) at room temperature for 30 minutes with continuous shaking.

4. Following removal from glutaraldehyde solution, the gel was washed with deionized water and kept on 5 gel volume of 20% ethanol with continuous shaking for 20 minutes. This ethanol washing step was repeated two times more.

5. After completion of ethanol wash, the gel was rinsed with deionized water and incubated in 5 gel volume of water for 10 minutes with continuous shaking.

6. After the water was removed, four gel volume of a freshly prepared ammonia/silver solution were added. 100 ml of this solution was prepared as follows: 1.4 ml of 14.8 M NH_4OH (concentrated ammonia) was added to 100 ml of distilled water. 190 μl of 10 N NaOH was added to this. This mixture was vortexed and 1 ml of freshly prepared silver nitrate solution was added (0.8 g of $AgNO_3$ was dissolved in 1 ml of distilled water) drop by drop. Initially a precipitate appeared which dissolved gradually. The gel was incubated for 30 minutes at room temperature with continuous shaking.

7. After removal of ammonia-silver solution, the gel was rinsed with deionized water (5-6 changes) over a period of 20 minutes.

8. When the gel was properly washed, 5 gel volume of freshly prepared 0.005 per cent citric acid, 0.019 per cent formaldehyde (diluted from commercial 37% solution) were added to the gel. This developer was removed when the bands appeared and the reaction was stopped by incubating the gel in solution containing 10 per cent glacial acetic acid and 20 per cent ethanol.

3.20.12.4 Measurement of relative mobility

The relative mobility (Rf) of each protein band was calculated by the following formula:

$$\text{Rf} = \frac{\text{distance migrated by the protein band}}{\text{distance migrated by the dye}}$$

3.20.13 MOLECULAR WEIGHT DETERMINATION FROM SDS-PAGE

The SDS-PAGE molecular weight standards were run on the gel along with each run of enzyme sample. Log of molecular weight of standard proteins were plotted against their relative mobilities (Rf). The relative mobilities of the enzyme samples were determined and molecular weight was calculated accordingly.

3.21 ENDOGLUCANASE ACTIVITY

Endoglucanase activity was measured by estimating the reducing sugars released from carboxymethyl cellulose (CMC). The method of Nelson, modified by Somogyi (1952) was used for the measurement of reducing sugars. The reagents were prepared as follows.

3.21.1 SOMOGYI REAGENT I

36 g of sodium sulfate was dissolved in 125 ml of boiled distilled water. To this, 3 g of Rochelle's salt (sodium-potassium tartarate), 6 g of anhydrous sodium carbonate and 4 g of sodium carbonate were added. Final volume was made upto 200 ml with boiled distilled water and stored at room temperature.

3.21.2 SOMOGYI REAGENT II

9 g of sodium sulfate was dissolved in 37.5 ml of boiled distilled water, to which 1 g of copper sulfate was added. Final volume was made upto 50 ml with boiled distilled water.

3.21.3 WORKING SOMOGYI REAGENT

Just before use, 4 volume of Somogyi reagent I was mixed with 1 volume of Somogyi reagent II to form the working Somogyi reagent.

3.21.4 NELSON REAGENT

10 g of ammonium molybdate (molybdic acid) was dissolved in 180 ml of distilled water. 8.4 ml of concentrated H_2SO_4 was added with proper stirring. To this, 1.2 g of sodium arsenate (dissolved in 10 ml of distilled water) was added. The reagent was stored at 37°C in amber coloured bottle for 1 to 2 days and then at room temperature.

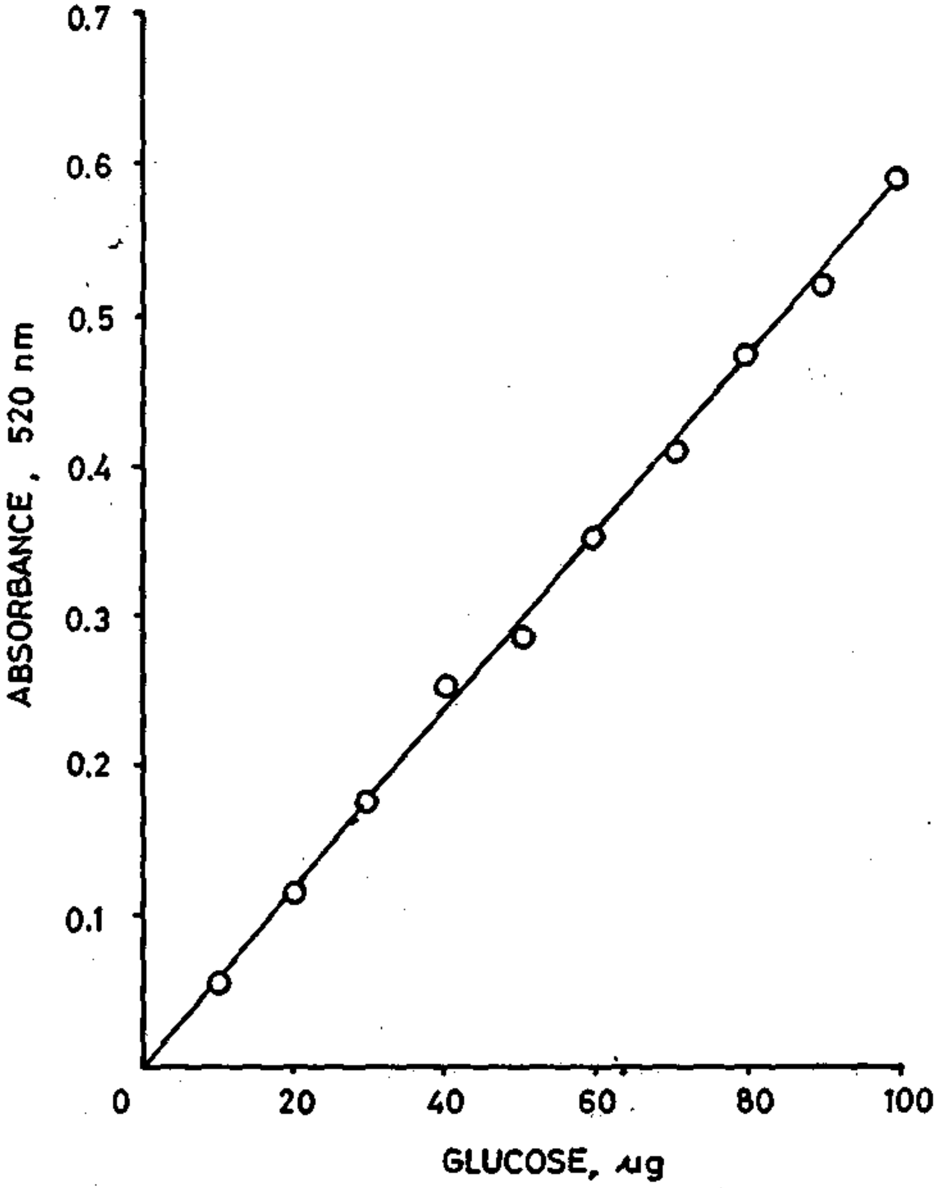
3.21.5 STANDARD CURVE

Appropriate volume of glucose stock solution (100 μg/ml) were taken in different tubes and volume was made upto 1 ml with distilled water. Then 1 ml of working Somogyi reagent was added and the tubes were placed in boiling water bath for 15 minutes. After taking out from the boiling water bath, the tubes were cooled. Then 1 ml of Nelson reagent and 2 ml of distilled water were added to each tube and absorbance was recorded at 520 nm. Standard curve was prepared by plotting the absorbance vs. concentration of glucose (Fig. 3.1).

3.21.6 ENDOGLUCANASE ACTIVITY

The method of Mountfort and Asher (1985) was followed for estimation of endoglucanase activity. The total assay system (2 ml) contained 1 ml of substrate (1% CMC dissolved in 0.1 M citrate phosphate buffer, pH 6.5), appropriate volume of enzyme and 0.1 M citrate phosphate buffer (pH 6.5). After 2 h incubation at 50°C in water bath, 1 ml working Somogyi reagent was added to each tube and boiled for 15 minutes in a boiling water bath. Then the tubes were cooled and 1 ml Nelson reagent and 1 ml distilled water were added. The resultant mixture was centrifuged at 3,500 rpm for 15 to 20 minutes and absorbance was read at 520 nm against a reagent blank containing 1 ml of 1 per cent CMC, 1 ml 0.1 M citrate phosphate buffer (pH 6.5), 1 ml

Fig. 3.1 Standard curve of glucose by Nelson and Somogyi method


0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE , 520 nm
0
20
40
60
80
100
GLUCOSE, µg

working Somogyi reagent, 1 ml Nelson reagent and 1 ml distilled water. Wherever required, enzyme blanks were also prepared by terminating reaction at zero time. This was done to substract the values of reducing sugars, if present in sample.

Enzyme unit (IU) was defined as the μmol of glucose released per minute. Specific activity was defined as units (IU) of activity per mg of protein.

3.21.7 ESTIMATION OF PROTEIN

The dye binding method of Bradford (1976) was followed for estimation of protein. Standard curve was prepared by using bovine serum albumin (Fig 3.2).

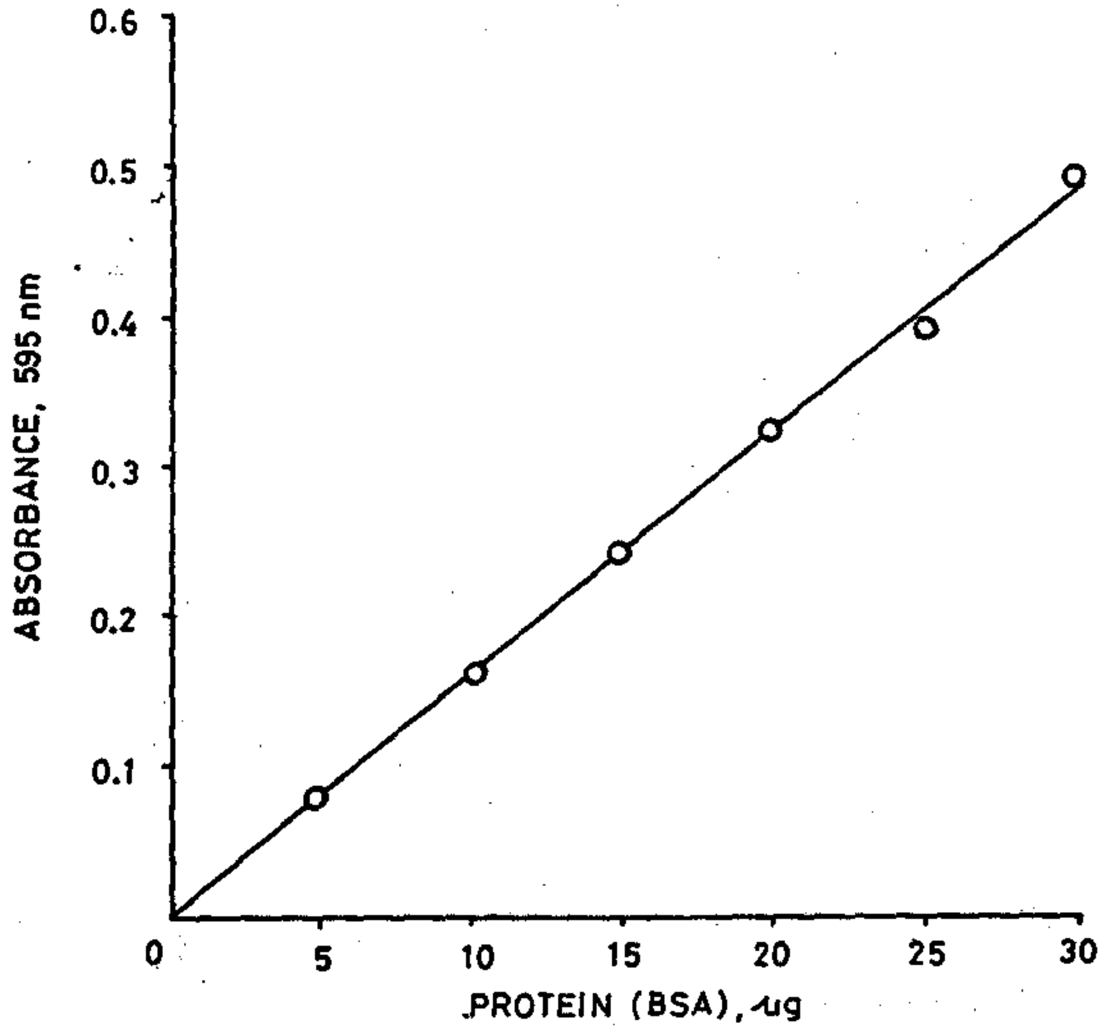
3.22 AVICELASE

Avicelase activity was based on the procedure described by Mountfort and Asher (1985). The total assay system (2 ml) contained 1 ml of substrate (1% avicel dissolved in 0.1 M citrate phosphate buffer, pH 6.5), appropriate volume of enzyme and 0.1 M citrate phosphate buffer, pH 6.5. After incubation at 50°C in water bath for specific time, 1 ml working Somogyi reagent was added and boiled for 15 minutes in a boiling water bath. Tubes were cooled, and 1 ml Nelson reagent and 1 ml water were added. The resultant mixture was centrifuged at 3,500 rpm for 15 to 20 minutes. Absorbance was read at 520 nm against a reagent blank. Wherever required, enzyme blank (control) were also prepared by terminating reaction at zero time. This was done to substract the values of reducing sugars, if present in sample. Enzyme unit (IU) of avicelase activity was defined as μmol of glucose equivalents released per minute. Specific activity was defined as units (IU) of activity per mg of protein.

3.23 CELLOBIASE ACTIVITY

Cellobiase was determined by measuring the glucose released from cellobiose (Saddler and Khan, 1980). The reaction mixture (1.5 ml) consisted of 1 ml substrate (0.4% cellobiose dissolved in 0.1 M citrate phosphate buffer (pH 6.5) and 0.5 ml of enzyme. The reaction mixture was incubated at 50°C for 2 h. The reaction was terminated by placing the tubes in boiling water bath for 15 minutes. After cooling, the

Fig.3.2 Standard curve for estimation of protein by dye binding method


ABSORBANCE, 595 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
30
PROTEIN (BSA), µg

released glucose was determined by glucose oxidase-peroxidase method (Bergmeyer and Bernt, 1974). Absorbance was read at 505 nm against a reagent blank. Wherever required, enzyme blanks (control) were also prepared by terminating reaction at zero time. This was done to substract the values of glucose, if present in sample.

Enzyme unit (IU) of cellobiase activity was defined as μmol of glucose equivalents released per minute. Specific activity was defined as units (IU) of activity per mg of protein.

3.23.1 STANDARD CURVE OF GLUCOSE BY GOD/POD METHOD

Appropriate volumes of glucose standard solution (1 μg/μl) were taken in different tubes and volume was made upto 1 ml with distilled water. Then 1.5 ml working reagent was added. Mixed well and allowed to stand for 30 minutes at room temperature (25 to 30°C). To this 0.5 ml water was added. The pink colour developed was read at 505 nm against the reagent blank. The standard curve was prepared by plotting the absorbance vs. glucose concentration (Fig. 3.3).

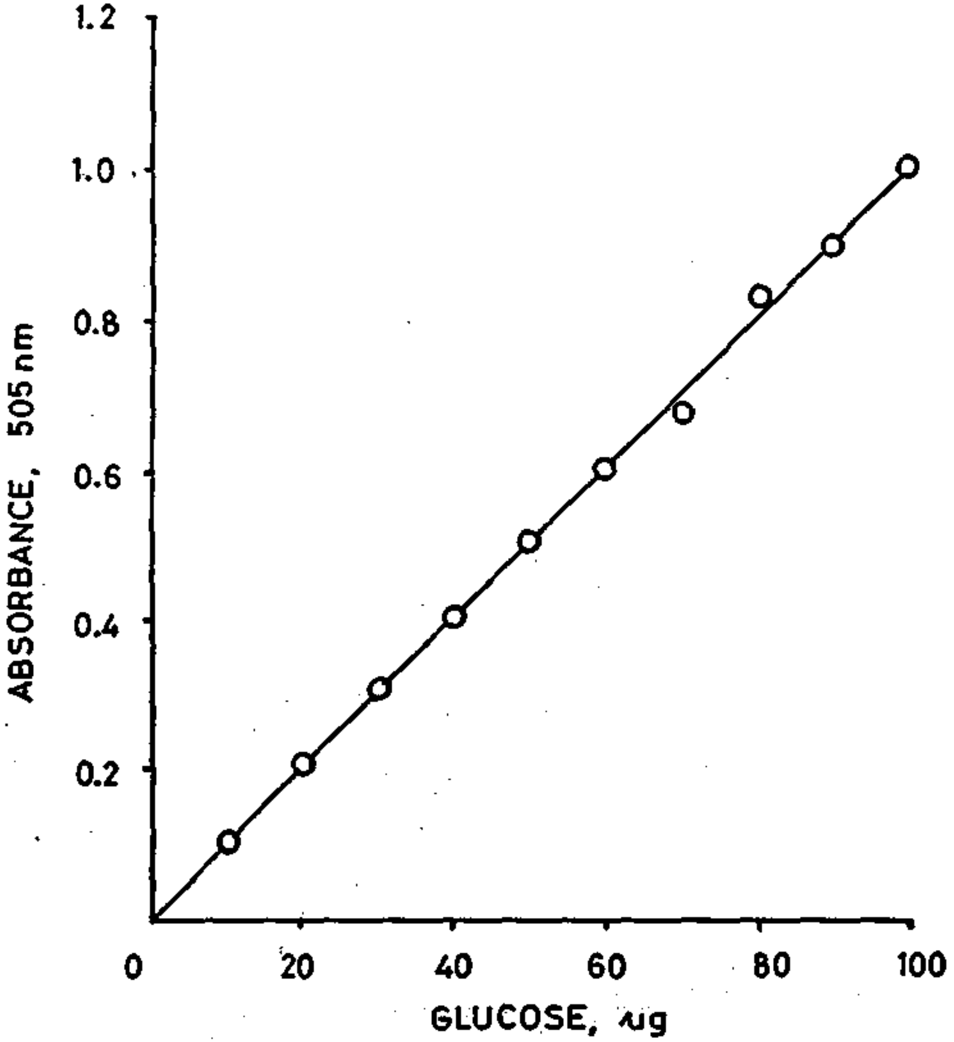
3.23.2 PREPARATION OF WORKING REAGENT

One tablet of glucose oxidase-peroxidase was dissolved in 50 ml of phenol solution. Mixed and dissolved the tablet thoroughly. Then 1.5 ml of this working reagent was used in each tube to develop the colour.

3.24 ASSAY OF XYLANASE

About 1 per cent (wt/vol) of the larch wood xylan was dissolved in 0.1 M citrate phosphate buffer (pH 6.5) by heating at 80°C until complete solubilisation of substrate. The reaction mixture (2 ml) comprised of 1 ml of substrate and appropriate volume of enzyme and 0.1 M citrate phosphate buffer (pH 6.5). The above reaction mixture was incubated at 50°C for 1 h. Then 1 ml working Somogyi reagent was added and boiled for 15 minutes in a boiling water bath. Tubes were cooled and 1 ml Nelson reagent and 1 ml water were added. The resultant mixture was centrifuged at 3,500 rpm for 15 to

Fig.3.3 Standard curve of glucose by GOD/POD method


1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE, 505 nm
0
20
40
60
80
100
GLUCOSE, µg

20 minutes. Absorbance was read at 520 nm against a reagent blank. Wherever required, enzyme blanks (control) were also prepared by terminating reaction at zero time. This was done to substract the values of reducing sugar, if present in sample.

Enzyme unit (IU) of xylanase activity was defined as μmol of xylose equivalents released per minute. The specific activity was expressed as units (IU) of activity per mg of protein.

3.24.1 STANDARD CURVE OF XYLOSE

Appropriate volume of xylose stock solution (100 μg/ml) were taken in different tubes and volume was made upto 1 ml with distilled water. Then 1 ml of working Somogyi reagent was added and the tubes were placed in boiling water bath for 15 minutes. After taking out from the boiling water bath the tubes were cooled. Then 1 ml of Nelson reagent and 2 ml of distilled water were added to each tube and absorbance was recorded at 520 nm. Standard curve was prepared by plotting the absorbance vs. concentration of xylose (Fig. 3.4).

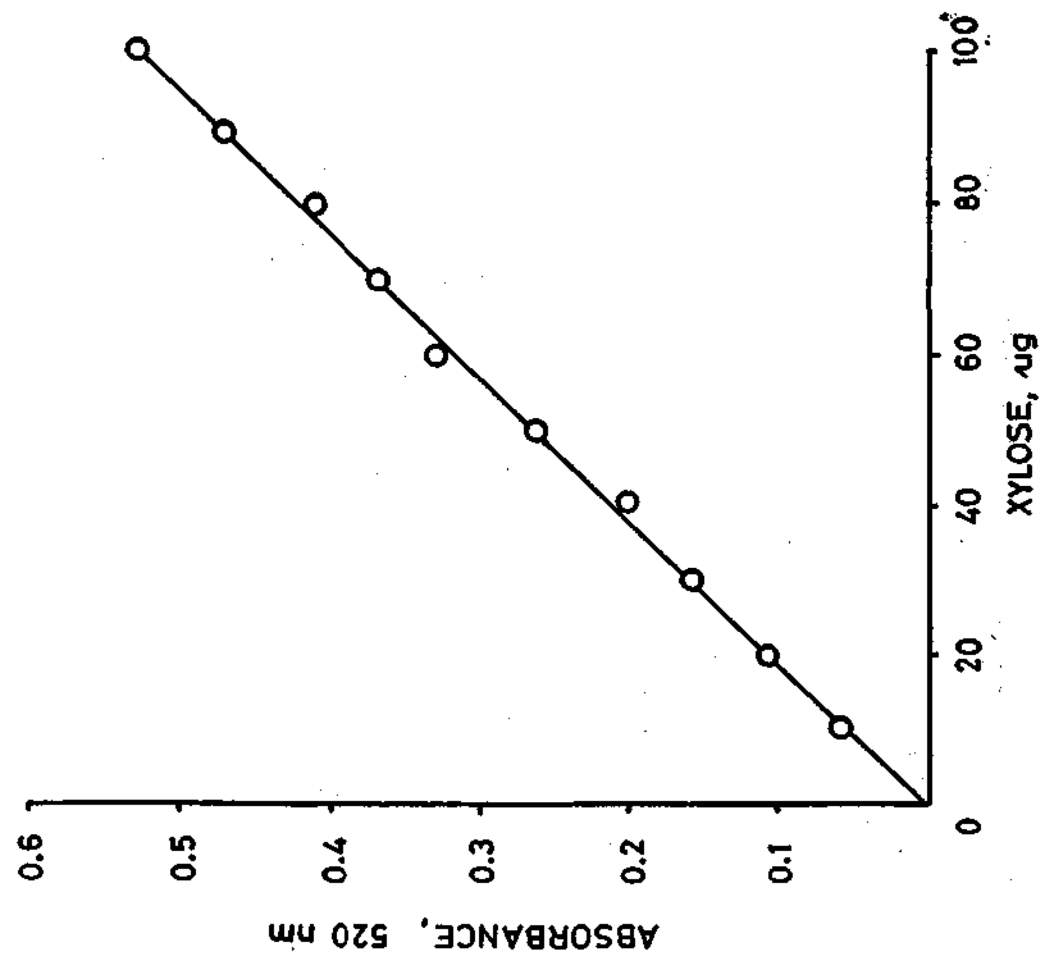
3.25 EFFECT OF pH ON ENZYME ACTIVITY

0.1 M citrate phosphate buffer with a pH range of 5.0 to 8.0 (5.0, 5.5, 6.0, 6.5, 7.0 and 8.0) were used for determination of optimum pH.

3.26 *IN VITRO* DIGESTION STUDIES

To specifically define the contribution of bacteria and fungi on wheat straw degradation, a series of IVDMD experiments were conducted in the presence of antibiotics (Windham and Akin, 1984). Antibiotics against bacteria, i.e., penicillin and stremptomycin and against fungi, i.e, cyclohexamide were used (Windham and Akin, 1984). *In vitro* incubation was done in 100 ml glass vial capped and sealed by rubber and aluminium cap respectively. Following treatments were applied to conduct these studies.

Fig.3.4 Standard curve of xylose by Nelson and Somogyi method


ABSORBANCE, 520 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
20
40
60
80
100
XYLOSE, ug

3.26.A Treatment zero (T0)

T0 comprised of 500 mg of milled wheat straw, 39 ml of McDougall's buffer, 10 ml of stirred and strained rumen liquor and 1 ml of distilled water. Activity was stopped at zero hour itself by the addition of a few drops of saturated mercuric chloride solution. This was the negative control.

3.26.B Treatment 1 (T1)

T1 comprised of 500 mg of milled wheat straw, 39 ml of McDougall's buffer, 10 ml of stirred and strained rumen liquor and 1 ml of distilled water. Incubation was done at 39 ± 1°C for 48 h. This reflected the activity of bacteria and fungi, i.e., positive control.

3.26.C Treatment 2 (T2)

T2 comprised of 500 mg of milled wheat straw, 39 ml of McDougall's buffer, 10 ml of stirred and strained rumen liquor and 1 ml of penicillin and streptomycin solution. The vials were incubated at 39 ± 1°C for 48 h. This reflected the activity of fungi only.

3.26.D Treatment 3 (T3)

T3 comprised of 500 mg of milled wheat straw, 39 ml of McDougall's buffer, 10 ml of stirred and strained rumen liquor and 1 ml of cyclohexamide solution. Incubated the vial at 39 ± 1°C for 48 h. This reflected the activity of bacteria only.

3.26.E TREATMENT 4 (T4)

T4 comprised of 500 mg of milled wheat straw, 39 ml of McDougall's buffer, 10 ml of stirred and strained rumen liquor and 1 ml of 48 h old fungal culture (B3 isolate). Incubated at 39 ± 1°C for 48 h. This reflected the probiotic effect, if any.In all above treatments, six replicates were taken. Following the stipulated period of incubation, the activity was stopped by addition of few drops of saturated mercuric chloride solution.

3.27.1 COMPOSITION OF McDOUGALL'S BUFFER

The composition of McDougall's buffer has been given in Table 3.13.

Table 3.13 Composition of McDougall's buffer (McDougall, 1948)

Component	*Quantity*
Sodium hydrogen carbonate	9.80 g
Anhydrous disodium hydrogen phosphate	3.71 g
Sodium chloride	0.47 g
Potassium chloride	0.57 g
Magnesium sulfate	0.06 g
Calcium chloride	0.04 g
Distilled water	1000 ml
pH adjusted at	6.6-6.8

3.27.2 ANTIBIOTIC SOLUTION

3.27.2.1 Against fungi

Cyclohexamide was used to inhibit fungal activity. Concentration was 5 mg per ml of distilled water. Filter sterilized by 0.2 μm filter and added 1 ml to each vial before addition of rumen liquor.

3.27.2.2 Against bacteria

Sodium salt of benzyl penicillin and streptomycin sulfate were included in one solution as follows:

Penicillin (1600 U/mg)	12.5 mg/ml
Streptomycin (650 U/mg)	2.0 mg/ml

The above antibiotic mixture was filter sterilized (0.2 μm) and used at the rate of 1 ml per vial before addition of rumen liquor.

3.27.3 COLLECTION AND PROCESSING OF RUMEN LIQUOR

Rumen liquor was collected from cattle and buffalo through permanent rumen fistula. It was agitated/stirred for a few minutes and strained through muslin cloth and used subsequently as source of inoculum.

3.27.4 DIET SCHEDULE OF ANIMAL

Fistulated cattle and buffalo were maintained on a diet comprising of concentrate 1 kg (composition described earlier) and green fodder *ad libitum.*

3.28 MEASUREMENT OF *IN VITRO* GAS PRODUCTION

The glass vials were made anaerobic by passing carbon dioxide to each vial. The vials were fitted with rubber corks and sealed with aluminium cap by the hand sealer. Following incubation at 39 ± 1°C, the gas production was monitored at 8 h interval for a period of 48 h by an electronic instrument Pressure Transducer manufactured by Bailey and Mackay Ltd., U.K. (Theodorou *et al.,* 1995).

3.29 DETERMINATION OF TOTAL VOLATILE FATTY ACIDS (TVFA)

TVFA concentration was estimated by the method of Barnett and Reid (1956). 2 ml of sample was taken into Markham's distillation apparatus. The sample was steam distilled with 2 ml of oxalate buffer containing 1 ml of 10 per cent potassium oxalate and 1 ml of 5 per cent oxalic acid. About 100 ml of distillate was collected and then titrated against standard (N/100) sodium hydroxide solution. Phenolphthalein was used as indicator which gave light pink colour at the end point.

Calculation

$$\text{TVFA (mM/100 ml SRL)} = \frac{\text{Volume of N/100 NaOH used} \times \text{Normality of NaOH}}{\text{Volume of sample taken}} \times 100$$

3.30 PARTITIONING OF VOLATILE FATTY ACIDS

The method of Erwin *et al.* (1961) was followed. The different volatile fatty acids present were partitioned into acetic, propionic and butyric acid on Nucon Gas Chromatograph Series 5500 fitted with dual flame ionization detector and a pair of stainless steel column packed with chromosorb 101 to serve as stationary phase.

4 ml of sample + 1 ml of 20 per cent metaphosphoric acid (prepared in 5 N H_2SO_4) were kept overnight and then centrifuged at 3500 to 4000 rpm for 15 to 20 minutes. The supernatant was used for fractionation of VFAs.

2 μl of this centrifuged sample was injected in gas chromatograph. A mixture of known quantities of acetic, propionic and butyric acid was also injected simultaneously as standard. Area of peaks were calculated according to following formula:

Area of peak = 1/2 x height x width of the base

Proportion of individual fatty acids

Individual VFA such as acetic, propionic and butyric acid proportion were calculated as follows:

$$\text{Proportion of individual fatty acid} = \frac{\text{Area covered by individual peak of fatty acid}}{\text{Total area of all fatty acids}} \times 100$$

3.31 ESTIMATION OF *IN VITRO* DRY MATTER DIGESTIBILITY (IVDMD)

After the incubation of substrates for 48 h, the sample was filtered through G1 glass crucible. Crucibles were dried in an oven for 48 h at 80°C and weighed. Weight of residue was recorded and dry matter digestibility was calculated by subtraction.

3.32 ESTIMATION OF *IN VITRO* ORGANIC MATTER DIGESTIBILITY (IVOMD)

After the incubation of substrates for 48 h at 39°C, samples were subjected to ashing on muffle furnace and IVOMD was calculated by subtraction.

Chapter 4

Results

4. RESULTS

The microbiology of the different segments of the digestive tract, specially the rumen is extremely complex due to the large number of organisms present, the shifting populations that result from changes in the diet of host animal and the interaction among the various microbial groups/species. In recent times, it is established that anaerobic fungi are also permanent and important member of rumen eco-club besides bacteria, protozoa and phage particles. Rumen fungi produce high levels of cellulase and hemicellulase and are particularly proficient in producing xylanases. The synthesis of these enzymes are also regulated by availability of substrates to the organisms. Rumen fungi extensively colonize the lignin containing tissues of forages and appear to be highly active in fibre degradation. Their population is generally high in the rumen of animals fed high fibrous diets than in those fed less fibrous diet. This indicates that the enzymes essential for hydrolysis of lignified forages are secreted by anaerobic fungi. The fibre degrading ability of fungi is more than the bacteria. Anaerobic fungi are unique among rumen microorganisms due to their penetrating ability to the cuticle layer of plant cell. Residues after incubation with fungi are physically weaker than those incubated with whole rumen fluid or with rumen bacteria, suggesting that fungi could alter the fibrous residues for easier mastication by the animal. But the only thing which puts rumen fungi in a disadvantageous position compared to bacteria is their small numerical strength.

4.1 ENUMERATION OF FUNGAL POPULATION

4.1.1 BUFFALO

Fungal population was enumerated simply by counting colonies, which appeared in roll tubes after 2-3 d of incubation at 39°C. The number of anaerobic fungi on different dietary combinations at different hours after feeding has been presented in Table 4.1. In case of T1, the average fungal populations in buffaloes were 1.10 x 10^3/ml, 2.42 x 10^3/ml, 1.41 x 10^3/ml, 1.22 x 10^3/ml and 1.21 x 10^3/ml at 0, 1, 2, 3 and 4 hours after feeding, respectively. In T2 treatment, the fungal populations were

Table 4.1 Total fungal population (number/ml) in buffalo rumen at different hours after feeding on different dietary regimes

Time after feeding (h)	***Dietary regime***		
	T1	***T2***	***T3***
0	1.10×10^3	1.21×10^3	2.48×10^3
1	2.42×10^3	2.56×10^3	5.41×10^3
2	1.41×10^3	1.52×10^3	2.90×10^3
3	1.22×10^3	1.39×10^3	2.56×10^3
4	1.21×10^3	1.36×10^3	2.51×10^3
Average	1.47×10^3	1.60×10^3	3.17×10^3

T1 = Wheat straw: concentrate::50:50

T2 = Oat fodder:concentrate::50:50

T3 = Oat fodder + wheat straw:concentrate::100:0

1.21×10^3/ml, 2.56×10^3/ml, 1.52×10^3/ml, 1.39×10^3/ml and 1.36×10^3/ml at 0, 1, 2, 3 and 4 hours after feeding, respectively. In T3 treatment, fungal populations were 2.48×10^3/ml, 5.41×10^3/ml, 2.90×10^3/ml, 2.56×10^3/ml and 2.51×10^3/ml at 0, 1, 2, 3 and 4 hours after feeding, respectively. The average values for three types of diets, irrespective of the intervals after feeding were 1.47×10^3/ml for T1, 1.60×10^3/ml for T2 and 3.17×10^3/ml for T3.

4.1.2 CATTLE

Total fungal population from cattle rumen was again enumerated by the roll tube method and the results are presented in Table 4.2. In case of T1, fungal populations were 1.32×10^3/ml, 2.57×10^3/ml, 1.69×10^3/ml, 1.41×10^3/ml and 1.39×10^3/ml at 0, 1, 2, 3 and 4 hours after feeding, respectively. In T2 animal fungal population were 1.29×10^3/ml, 2.67×10^3/ml 1.78×10^3/ml, 1.38×10^3/ml and 1.34×10^3/ml at 0, 1, 2, 3 and 4 h after feeding respectively. In T3 animal, fungal populations were 2.41×10^3/ml, 5.14×10^3/ml, 2.75×10^3/ml, 2.44×10^3/ml and 2.43×10^3/ml at 0, 1, 2, 3 and 4 hours after feeding, respectively. For different dietary combinations, the average values, irrespective of the sampling time, were 1.67×10^3/ml for T1, 1.69×10^3/ml for T2 and 3.03×10^3/ml for T3.

4.1.3 EFFECT OF BERSEEM AND LEUCAENA LEAF MEAL FEEDING ON FUNGAL POPULATION

A fistulated buffalo fed 20 kg berseem and 3 kg concentrate possessed a fungal population of 5.2×10^2/ml at 1 h after feeding. However, a buffalo fed 5 kg leucaena leaf meal and 1 kg concentrate had a fungal population of 5.80×10^2/ml at 1 h after feeding.

4.1.4 FUNGAL POPULATION IN FECAL SAMPLE

In the case of buffalo (T3), fecal fungal population was 6.5×10^3 to 6.8×10^3 per gram of fecal dry matter, while in case of cattle (T3), the value was 5.3×10^3 to 5.6×10^3 per gram of fecal dry matter.

Table 4.2 Total fungal population (number/ml) in cattle rumen at different hours after feeding on different dietary regimes

Time after feeding (h)	*Dietary regime*		
	T1	*T2*	*T3*
0	1.32 x 10^3	1.29 x 10^3	2.41 x 10^3
1	2.57 x 10^3	2.67 x 10^3	5.14 x 10^3
2	1.69 x 10^3	1.78 x 10^3	2.75 x 10^3
3	1.41 x 10^3	1.38 x 10^3	2.44 x 10^3
4	1.39 x 10^3	1.34 x 10^3	2.43 x 10^3
Average	1.67 x 10^3	1.69 x 10^3	3.03 x 10^3

T1 = Wheat straw: concentrate::50:50

T2 = Oat fodder:concentrate::50:50

T3 = Oat fodder + wheat straw:concentrate::100:0

4.2 TYPES OF FUNGAL COLONIES

After the addition of rumen inoculum into the roll tubes containing cellobiose agar medium, fungal colonies appeared in different forms. Evaluation of the agar surface of the roll tubes at specific times showed variations due to growth pattern of different species of fungus. These growth patterns were classified into three categories.

4.2.1 TYPE A

Colonies were completely circular (Plate 1). Diameter varied from 2 to 10 mm. Within the colony, three well demarcated zones were visible. A completely opaque zone was visible at the centre of fungal colony. The opaqueness of the area was possibly due to aggregation of sporangia. The second was less dense zone - formed due to the spreading of thick rhizomycelia. The third one was more or less transparent zone - formed by the finely branched thinner rhizomycelia. This type comprised of 60% of the total colonies.

4.2.2 TYPE B

Colonies were bigger in size with irregular boundaries (Plate 2). Diameter ranged from 10 to 15 mm. No well demarcated zones were visible. Total area of colony was transparent. This type comprised of 15% of the total colonies.

4.2.3 TYPE *C*

Colonies were small (Plate 3). Diameter ranged from 0.5 to 2 mm. Two zones were visible - central zone was completely opaque and outer rhizomycelia zone was transparent. It comprised 25% of total colonies in the roll tubes.

4.3 TYPES OF ANAEROBIC FUNGI PRESENT IN CATTLE AND BUFFALO RUMEN

Both polycentric and monocentric types of fungi were found to be present within the rumen of Indian cattle and buffaloes. Fungal colonies which appeared on cellobiose roll tubes were directly scrapped and seen under microscope with or without

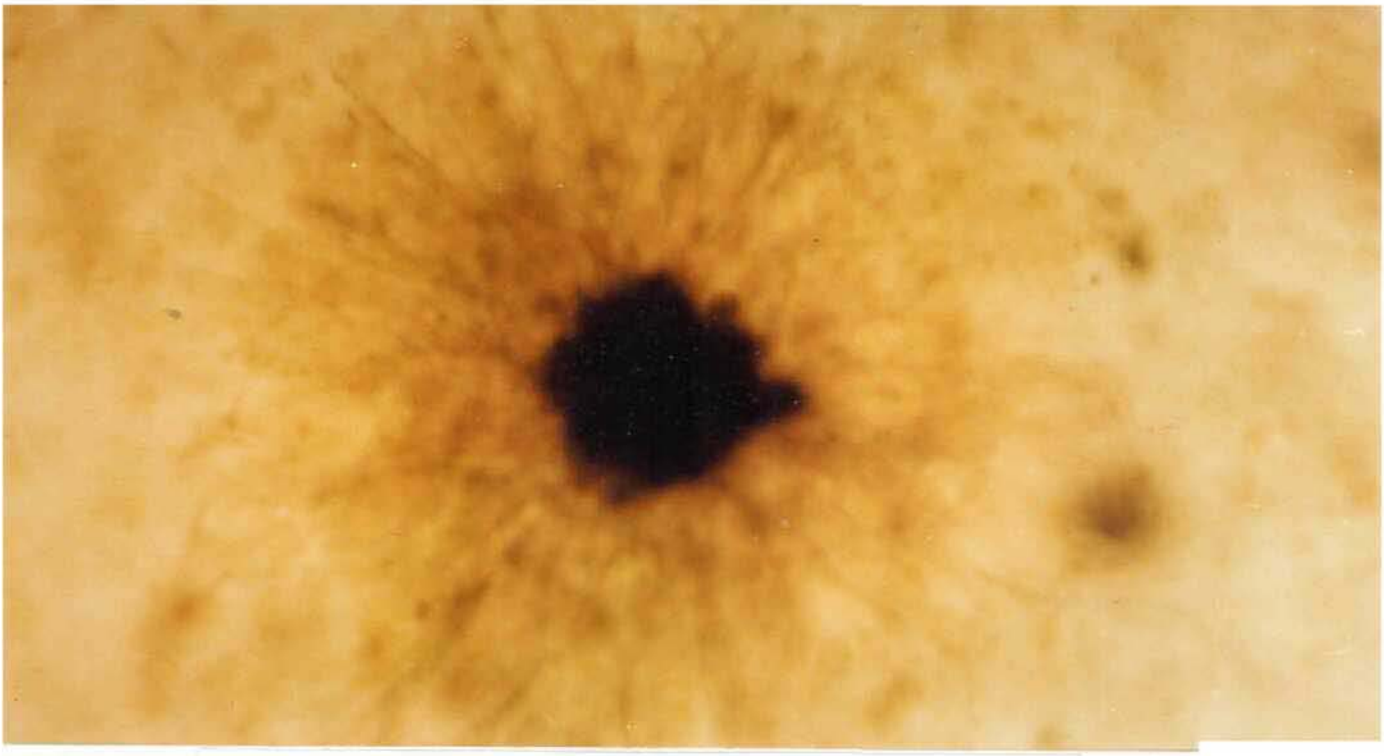


Plate 1: Type A colony under zoom microscope

Plate 2: Type B colony under Zoom microscope

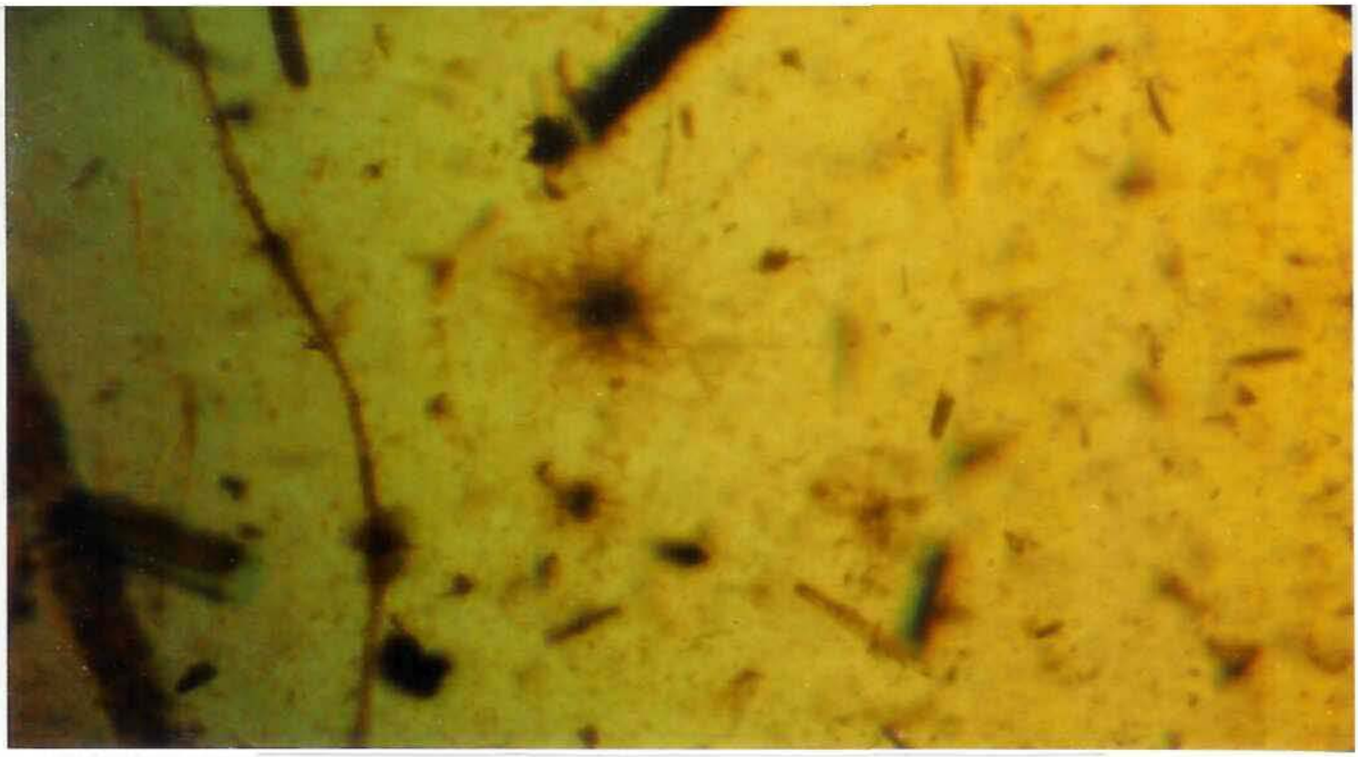


Plate C: Type 3 colony under zoom microscope

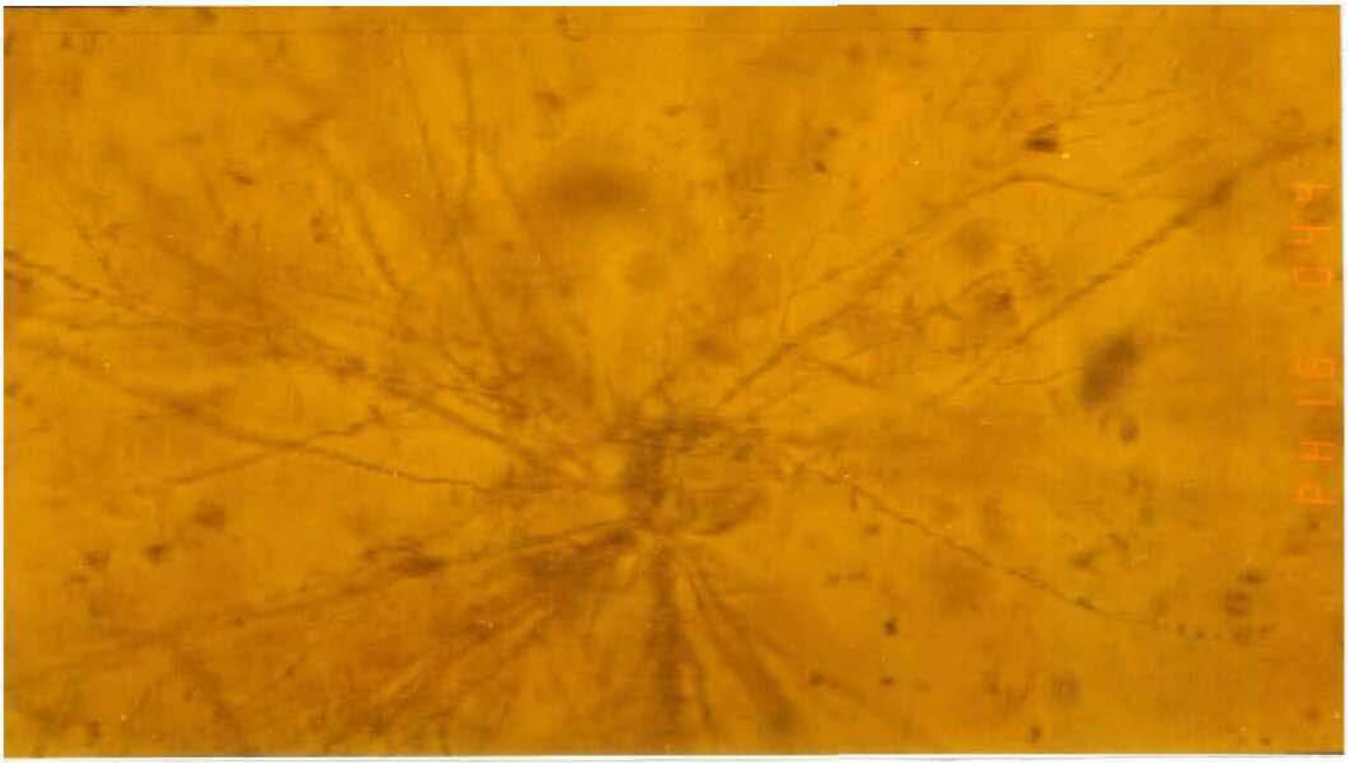


Plate 4: Monocentric fungus. Round sporangium with extensively branched rhizomycelia

lactophenol cotton blue stain. Monocentric were those where single sporangium developed from the thallus (Plate 4). Sporangia were round shaped. There were extensively branched rhizomycelia. Initially rhizoids were thick and subsequently became thinner to form extensively branched mycelial network (Plate 5).

Polycentric fungus were those where more than one sporangia developed from a single thallus (Plate 6). The development of sporangia may be either intercalary (Plate 7) or terminal (Plate 8). Sporangia were oval or sometimes balloon shaped. Within the sporangia about 8 to 9 circular bodies were visible (Plate 9) and took lactophenol stain. Extensively branched and segmented rhizomycelia were seen.

4.4 ISOLATION OF ANAEROBIC FUNGI FROM CATTLE AND BUFFALO

A total of 17 isolates of rumen anaerobic fungi were obtained during the course of this investigation from cattle and buffalo. Isolation was done in 0.2% cellobiose containing 2% agar. The selection of the colonies from roll tubes was at random. Based on their growth under strict anaerobic conditions, the isolates were classified as obligatory anaerobic. Out of these isolates, seven from cattle and ten from buffalo were selected for further studies. The different isolates were again purified through roll tube technique till a single type of colony for each isolate was obtained. These isolates were maintained in both 1 per cent wheat straw containing media and 0.2 per cent cellobiose soft agar. Subculturing was done at seven days interval.

4.5 IDENTIFICATION OF RUMEN ANAEROBIC FUNGUS

Isolates from cattle were C1, C2, C3, C4, C5, Sm1 and Sm2. Isolates from buffalo were B1, B2, B3, B4, 1, 3, 4, 6, 8 and 11.

4.5.1 ISOLATE C1

Isolated from cattle (T1). Around 8 to 15 sporangia developed from single thallus. Thus, it was polycentric in nature (Plate 10). Sporangia were round and possessed stalk at the base. It had profusely branched unsegmented rhizomycelia. The

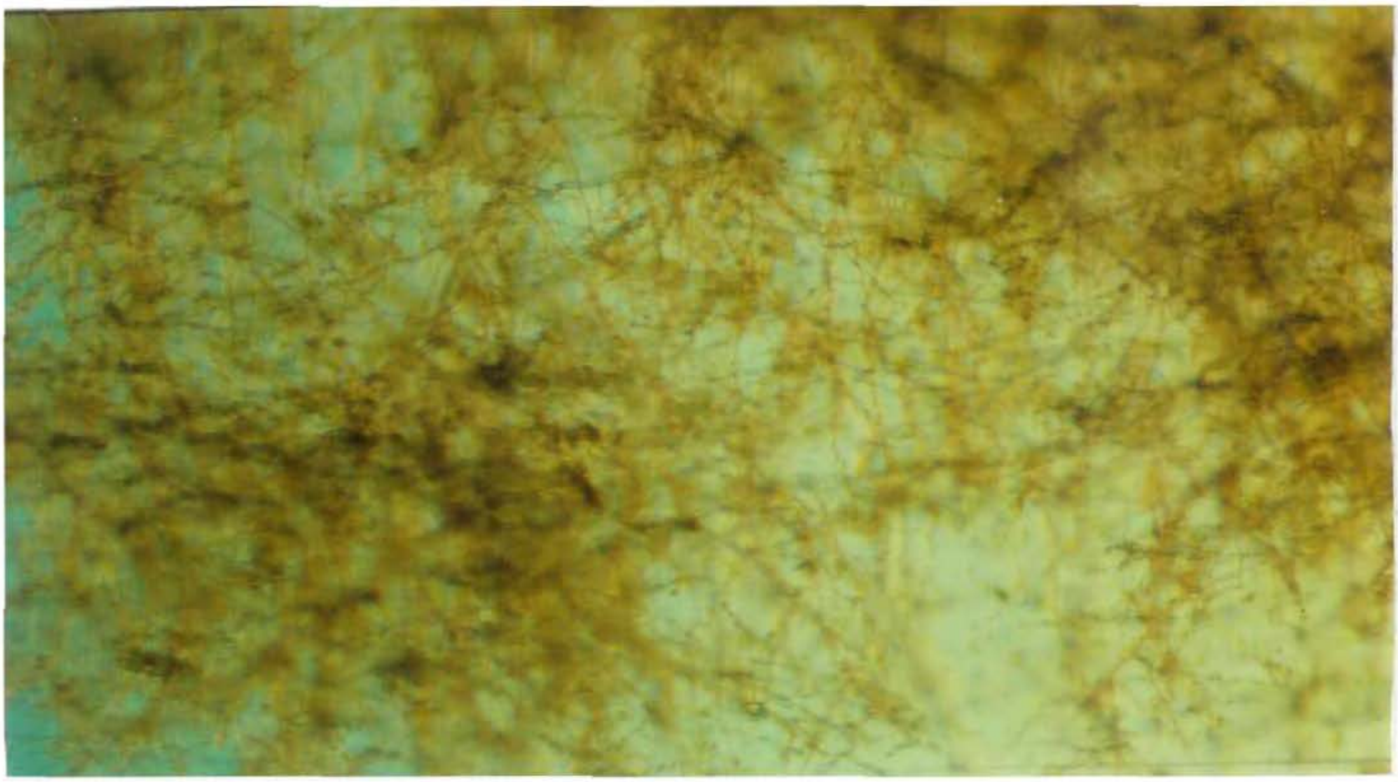


Plate 5: Extensively branched mycelial network of rumen fungus

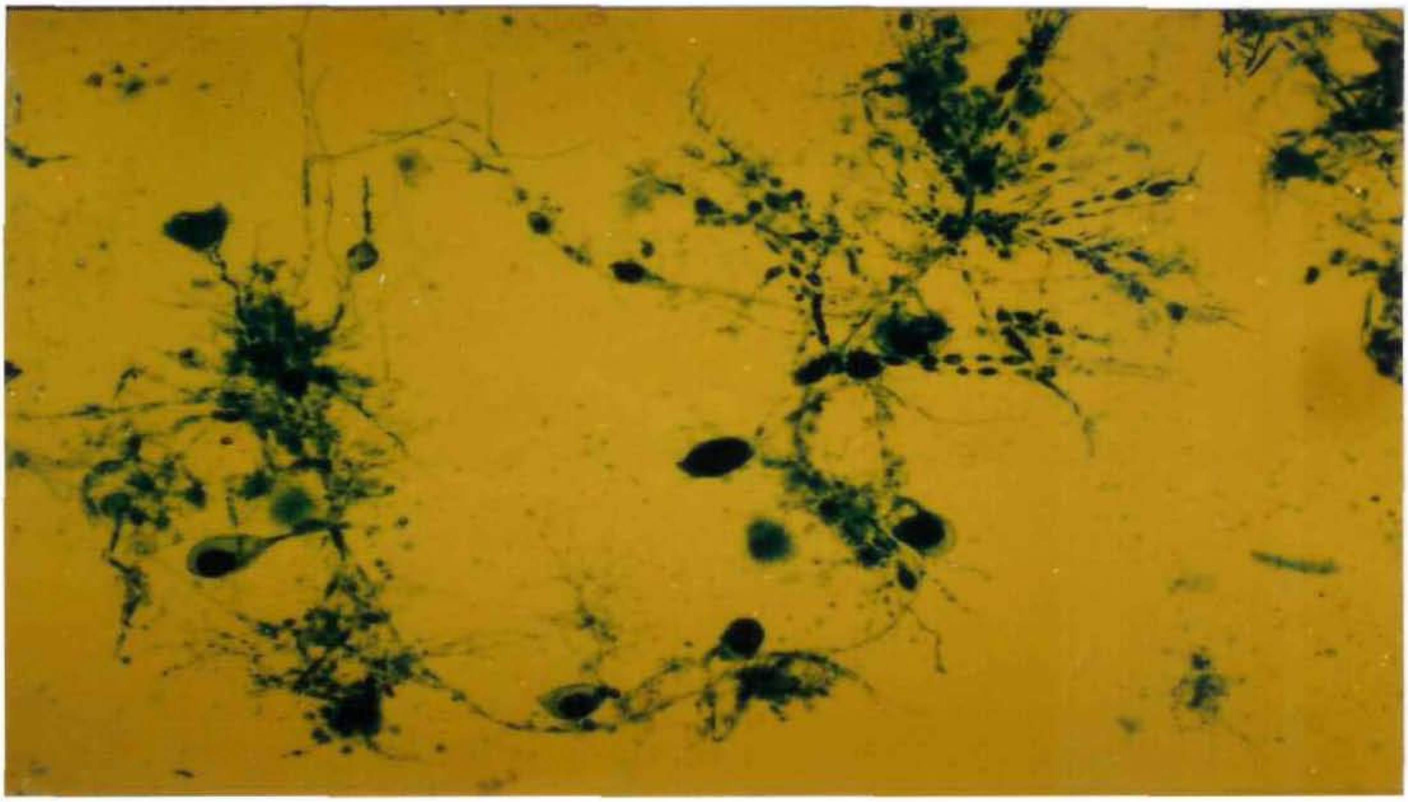


Plate 6: Polycentric fungus. Several sporangia developed from the thallus

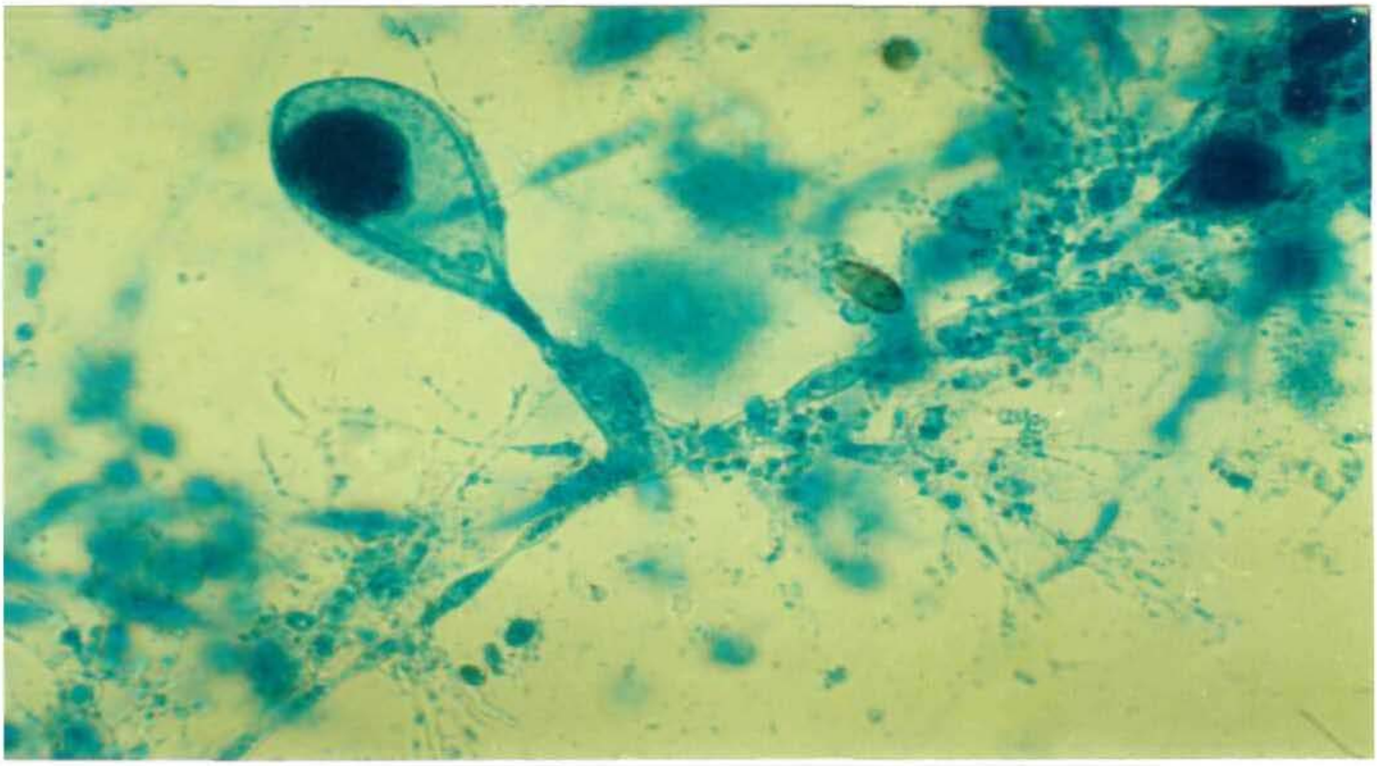


Plate 7: Intercalary development of sporangia

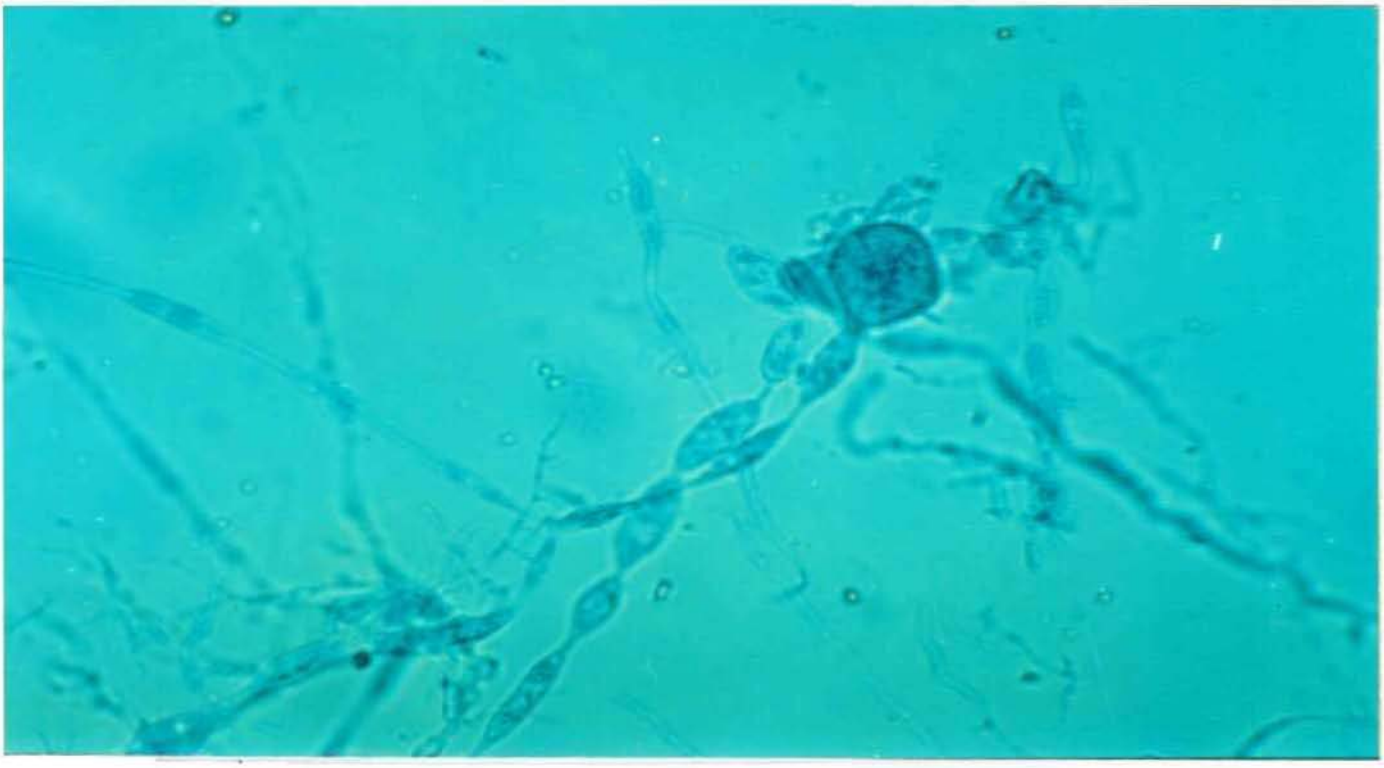


Plate 8: Terminal development of sporangia

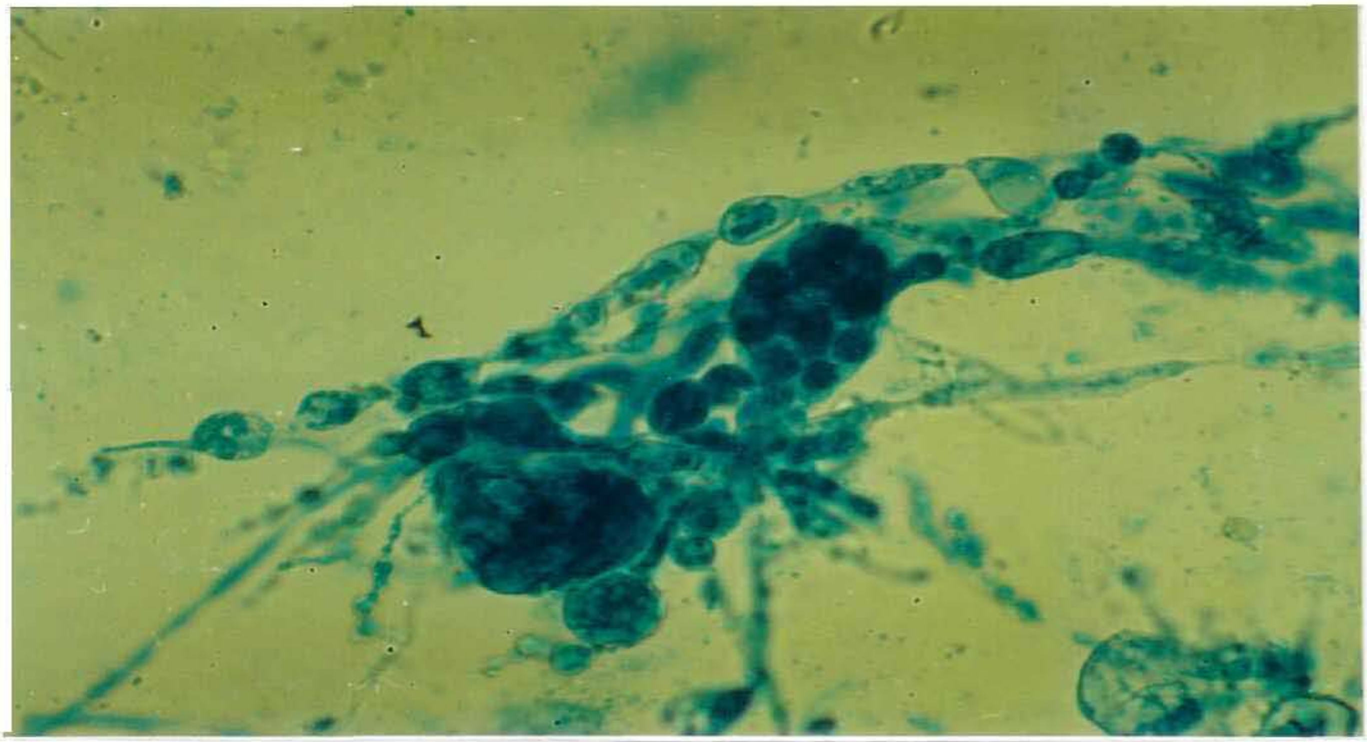


Plate 9: Several circular bodies present inside the sporangia of polycentric fungus

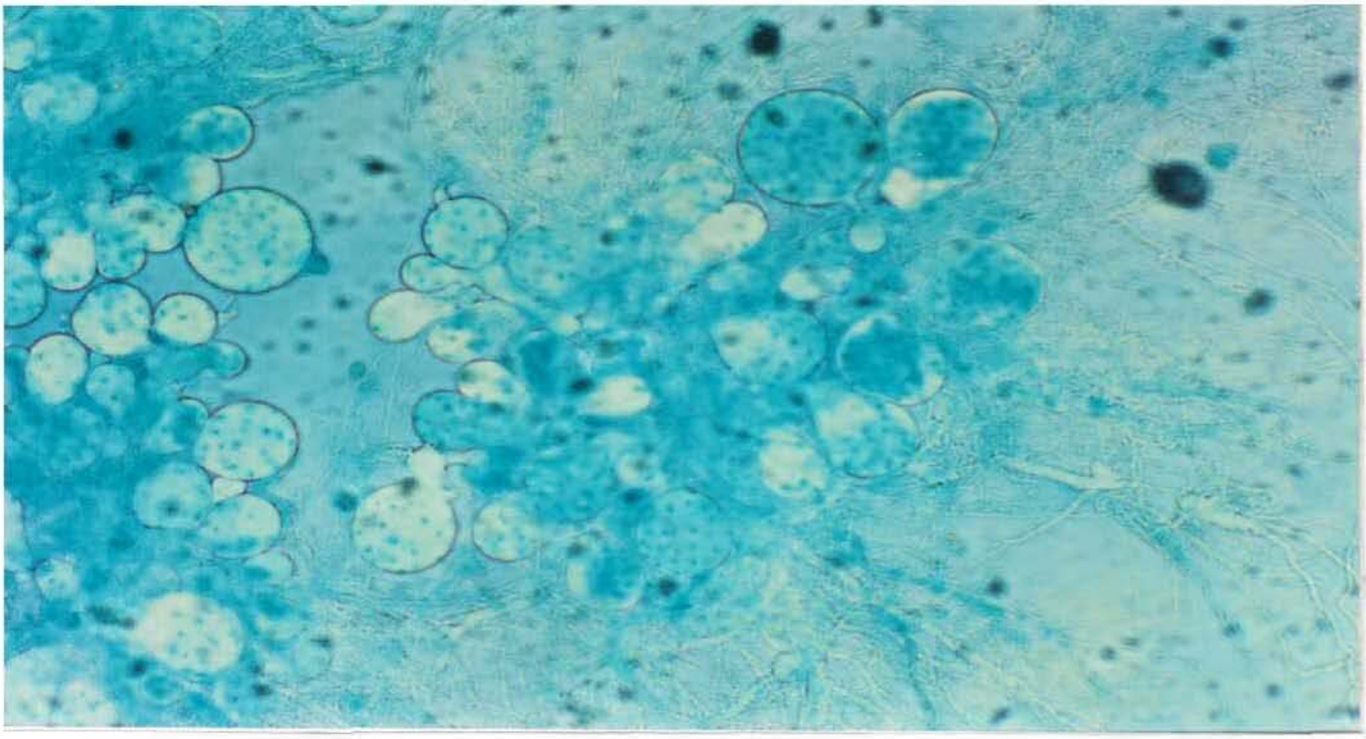


Plate 10: Microscopic view of isolate C1. Several round shaped sporangia with extensively branched rhizomycelia. Polycentric. Identified as Anaeromyces sp.

fungus was able to grow on straw and possessed uniflagellated zoospore. The isolate seemed to be *Anaeromyces* sp.

4.5.2 ISOLATE C2

It was isolated from the cattle receiving the diet T1. Very few sporangia (2 to 3) developed from single thallus (plate 11) and thus it was polycentric. Sporangia were completely circular and possessed segmented, extensively branched rhizomycelia. No stalk was present at the base of the sporangia. Some unusual structure was coming out of sporangia. It was able to grow over straw. Zoospores were uniflagellated and the isolate seemed to be *Anaeromyces* sp.

4.5.3 ISOLATE C3

Isolated from the cattle (T2). Single sporangium developed from the thallus. Thus it was a monocentric isolate. Sporangia were completely circular and multiple. Layers of sporangial wall were clearly visible. Profusely branched, unsegmented rhizomycelia were seen (Plate 12). Zoospores were polyflagellated. About five flagella were visible under microscope (Plate 13). The isolate seemed to be *Neocallimastix* sp.

4.5.4 ISOLATE C4

Isolated from cattle (T3). A number of sporangia developed from single thallus (Plate 14). Sporangia were small and round in shape. Unsegmented, extensively branched rhizomycelia were seen. No stalk at the base of sporangia. Zoospores were polyflagellated and were not able to grow on straw. Isolate was polycentric one and seemed to be *Anaeromyces* sp.

4.5.5 ISOLATE C5

This was isolated from cattle (T3). It was monocentric in nature as single sporangium developed from the thallus (Plate 15). Sporangium was circular in shape and no stalk was found at the base. Thick and unsegmented long rhizoid was seen and there was no immediate branching. The isolate was not able to grow on straw. Monoflagellated zoospore. Seemed to be *Piromyces* sp.

Plate 11: Microscopic view of isolate C2. Polycentric fungus with extensively branched and segmented rhizomycelia. Identified as Anaeromyces sp.

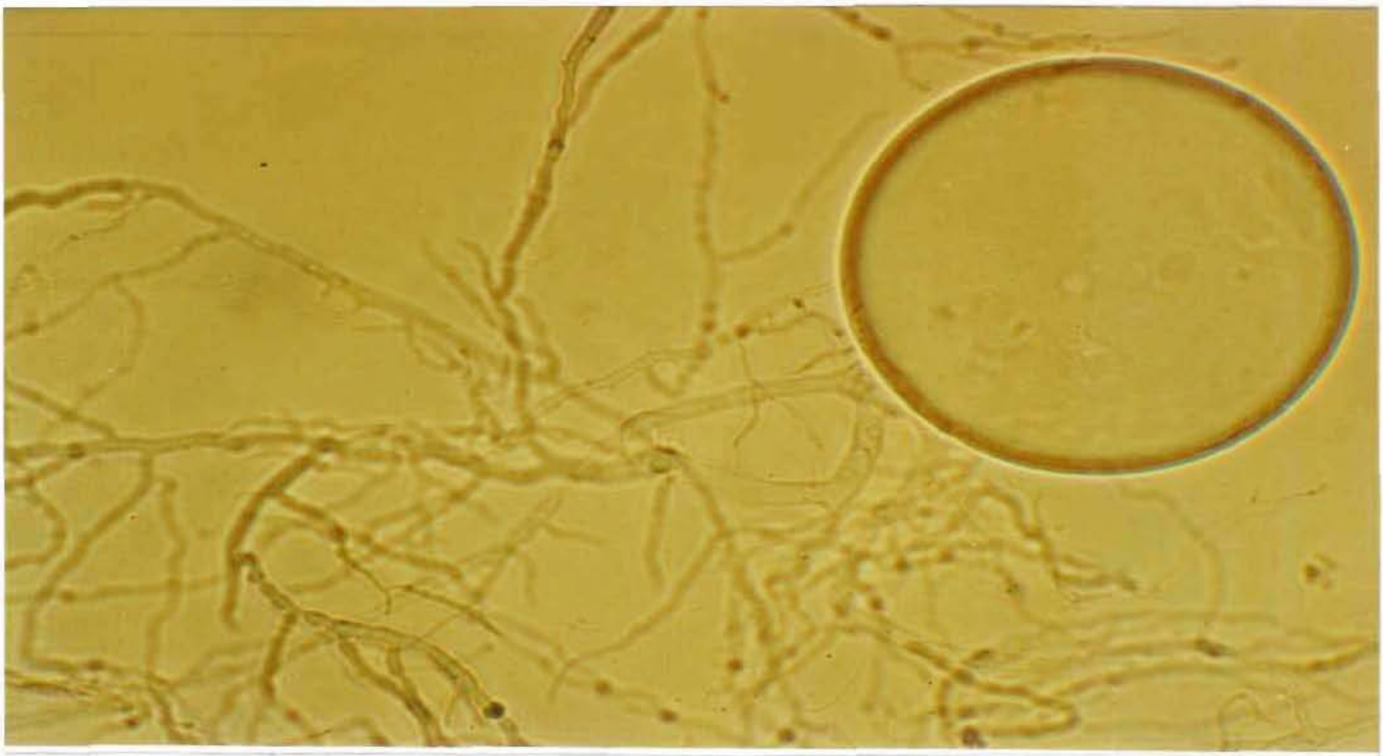


Plate 12: Microscopic view of isolate C3 (monocentric). Completely circular sporangia with extensively branched rhizomycelia. Identified as Neocallimastix sp.

Plate 13: Microscopic structure of polyflagellated zoospore (C3 isolate)

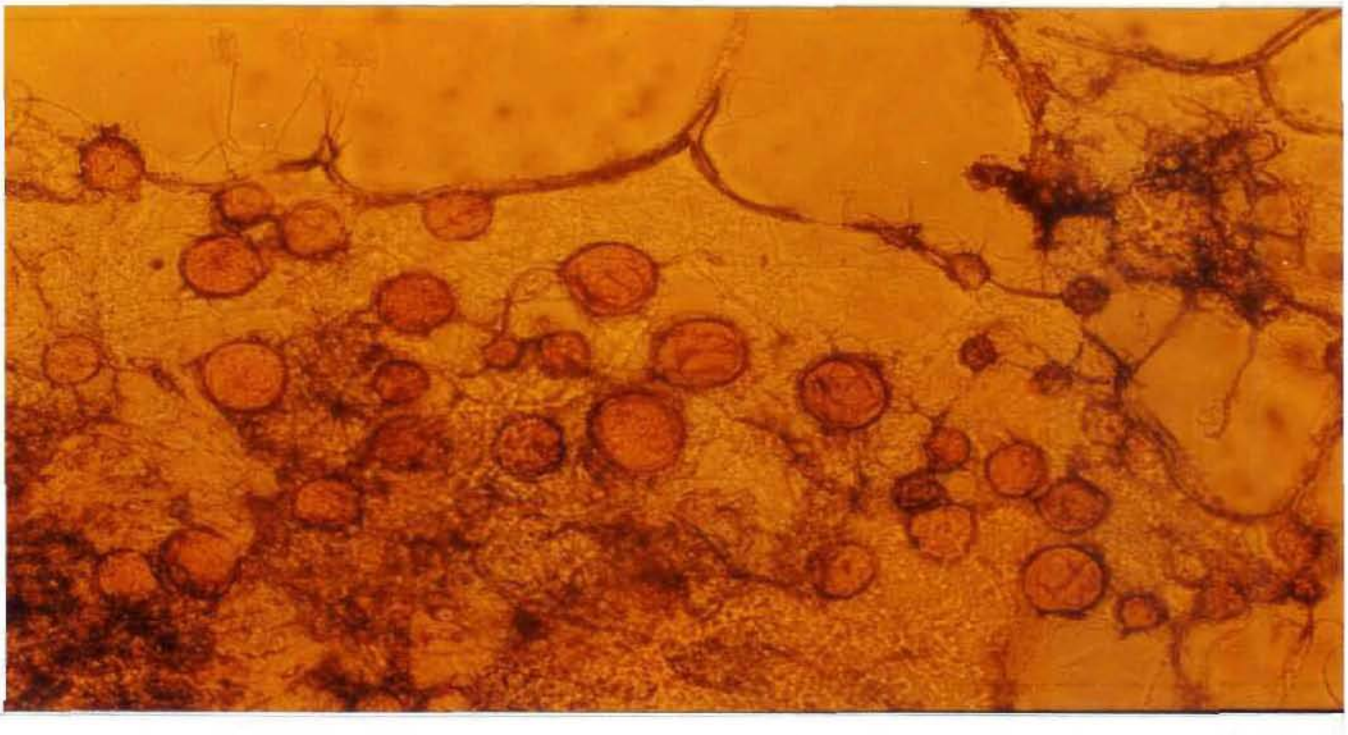


Plate 14: Microscopic view of C4 isolate. Several sporangia with finely branched, unsegmented rhizomycelia. Polycentric. Identified as Anaeromyces sp.

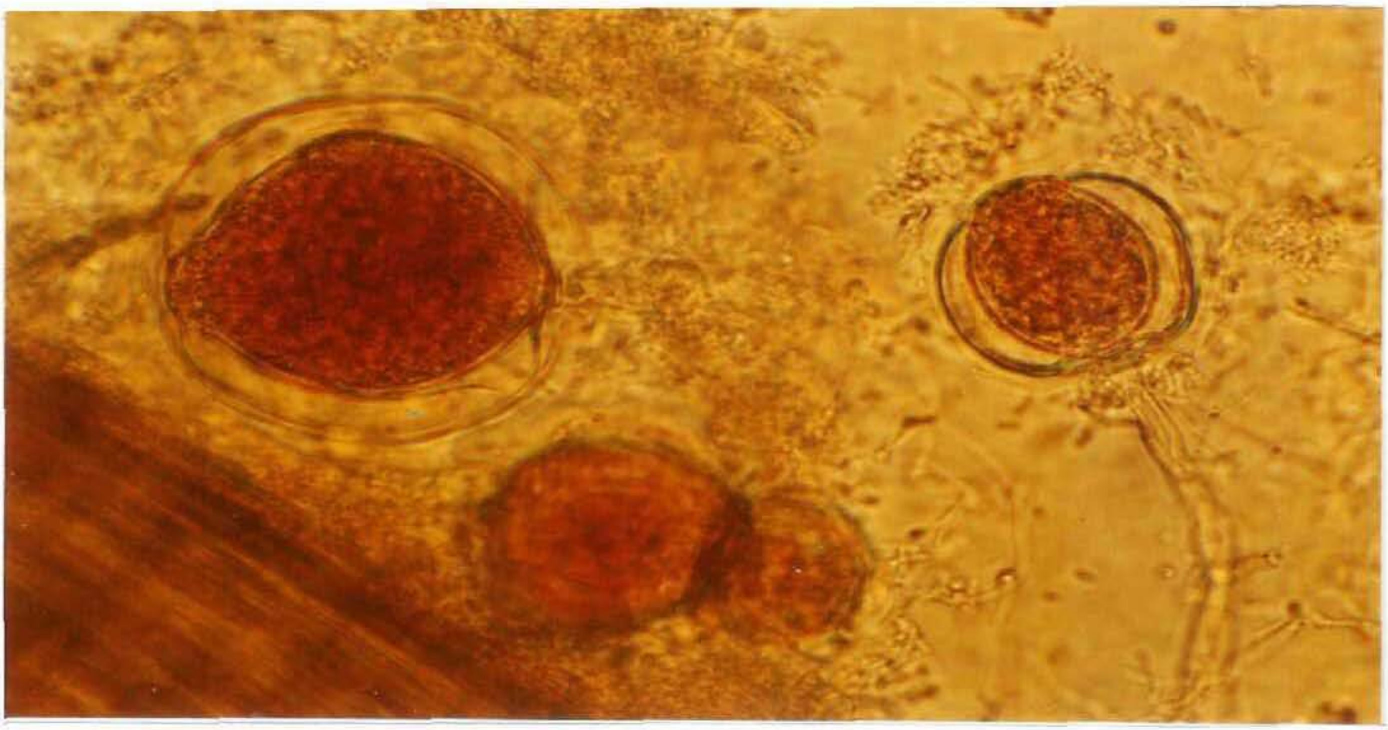


Plate 15: Microscopic view of C5 isolate. Monocentric fungus with long and thick rhizoid. No immediate branching of rhizoid. Identified as Piromyces sp.

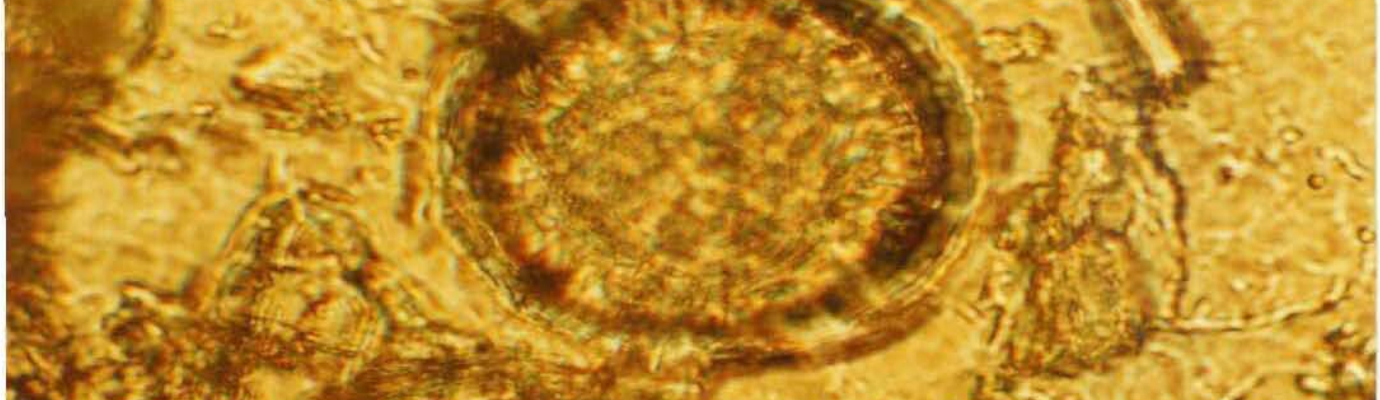


Plate 16: Microscopic view of Sm1 isolate. Monocentric fungus with extensively branched, unsegmented rhizomycelia. Identified as Piromyces sp.

4.5.6 ISOLATE Sm1

Isolated from cattle (T3). It was monocentric as single sporangium developed from the thallus (Plate 16). No stalk at the base of sporangia. Multiple layers of sporangial wall were clearly visible. Extensively unsegmented branched rhizomycelia. Monoflagellated zoospore. Able to grow over straw. Seemed to be *Piromyces* sp.

4.5.7 ISOLATE Sm2

Isolated from cattle (T3). Small, circular, single sporangium developed from thallus (Plate 17). Thus, it was monocentric. Presence of stalk at the base of sporangia. Rhizomycelia were unsegmented and extensively branched. Zoospores were monoflagellated. Able to grow over straw. Seemed to belong to *Piromyces* sp.

4.5.8 ISOLATE B1

This was isolated from the buffalo receiving the diet T1. Single sporangium developed from the thallus (Plate 18). It had round and medium sized sporangia. No stalk was observed at the base of sporangium. Thick, segmented and long rhizoid which branched terminally was seen. Not able to grow over straw. The zoospores were monoflagellated. It appeared to belong *Piromyces* sp.

4.5.9 ISOLATE B2

This was isolated from buffalo (T2). Single sporangium developed from the thallus (Plate 19), suggested the monocentric nature of fungus. Small oval shaped sporangia was seen, with a stalk at the base. Finely branched and unsegmented rhizomycelia were observed. The zoospores were monoflagellated. The isolate was able to grow over straw. Appeared to belong *Piromyces* sp.

4.5.10 ISOLATE B3

Isolated from buffalo (T3). Single sporangium developed from the thallus. Thus, it was a monocentric fungi. It had round and large sporangia (Plate 20). No stalk at the base of sporangia. Multiple layers of sporangial wall were visible. Extensively branched

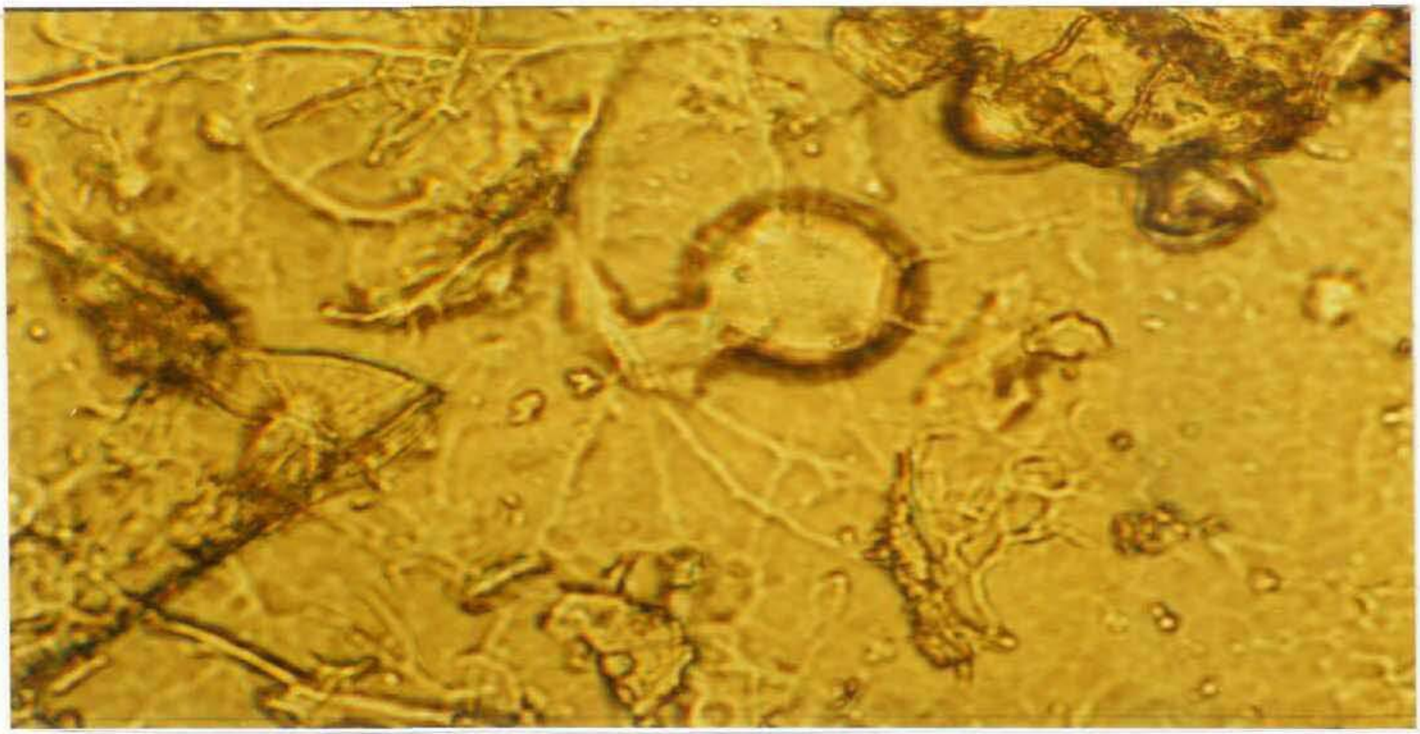


Plate 17: Microscopic view isolate Sm2 showing small and circular sporangia. Stalk at the base of sporangia. Monocentric. Branched rhizomycelia. Identified as Piromyces sp.

Plate 18: Microscopic view of isolate B1. Monocentric fungus with long rhizoid. No stalk at the base of sporangia. Identified as Piromyces sp.

Plate 19: Microscopic structure of B2 isolate. Monocentric fungus with oval shaped sporangia. Stalk at the base of sporangia. Extensively branched rhizomycelia. Identified as Piromyces sp.

Plate 20: Microscopic structure of B3 isolate. Monocentric fungus with extensively branched mycelia. Completely circular sporangia. Identified as Piromyces sp.

and thick rhizomycelia were observed at the beginning and finely branched at the end. Zoospores were monoflagellated (Plate 21) and able to grow over straw. It appeared to be *Piromyces* sp.

4.5.11 ISOLATE B4

Isolated from buffalo (T3). It was a monocentric fungi as single sporangium developed from the thallus. Small and circular sporangia were seen (Plate 22). No stalk at the base was noticed. Extensively branched, unsegmented rhizomycelia. Zoospore was monoflagellated and not able to grow over straw. It appeared to belong *Piromyces* sp.

4.5.12 ISOLATE 1

It was isolated from buffalo (T3). About 4 to 6 sporangia developed from single thallus (Plate 23). Polycentric in nature. Sporangia were small and oval shaped. Presence of stalk at the base of sporangia. Unsegmented, extensively branched rhizomycelia. Zoospores were polyflagellated. Able to grow over straw. Thus it appeared to be *Orpinomyces* sp.

4.5.13 ISOLATE 3

Isolated from buffalo (T3). Several sporangia developed from the thallus (Plate 24). Small, circular sporangia. Polycentric in nature. No stalk at the base of sporangia. Extensively branched, unsegmented, filamentous rhizomycelia. Not able to grow over straw. Polyflagellated zoospore. Appeared to belong *Orpinomyces* sp.

4.5.14 ISOLATE 4

Isolated from buffalo (T1). Several sporangia developed from the thallus. Thus, it was polycentric one. Large, circular sporangia (Plate 25). No stalk at the base of sporangia. Unsegmented, extensively branched rhizomycelia. Able to grow over straw. Uniflagellated zoospore. Appeared to belong *Anaeromyces* sp.

Plate 21: Microscopic structure of monoflagellated zoospore (B3 isolate)

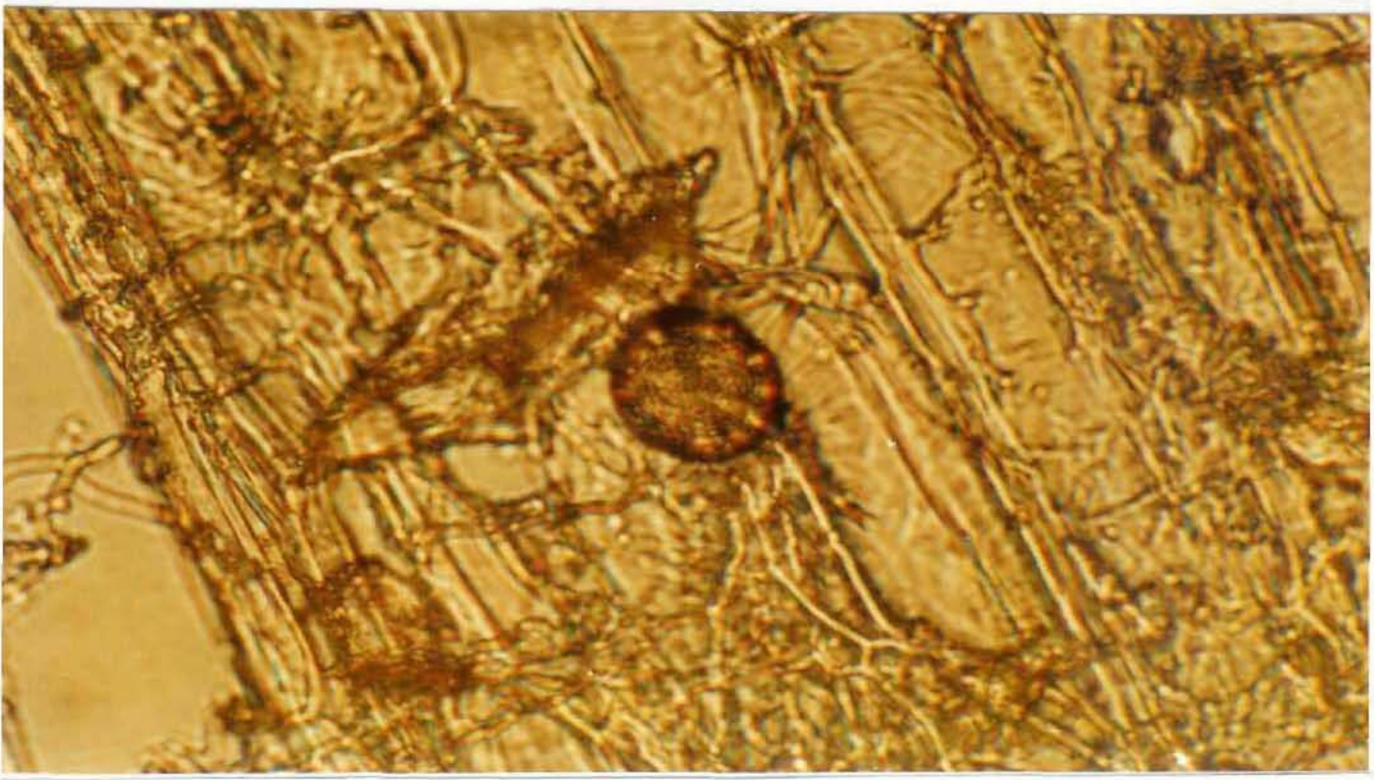


Plate 22: Microscopic view of isolate B4. Monocentric fungus with small, circular sporangia. No stalk at the base of sporangia. Identified as Piromyces sp.

Plate 23: Microscopic view of isolate 1. Polycentric fungus with oval shaped sporangia. Stalk at the base of sporangia. Branched rhizomycelia. Identified as Orpinomyces sp.

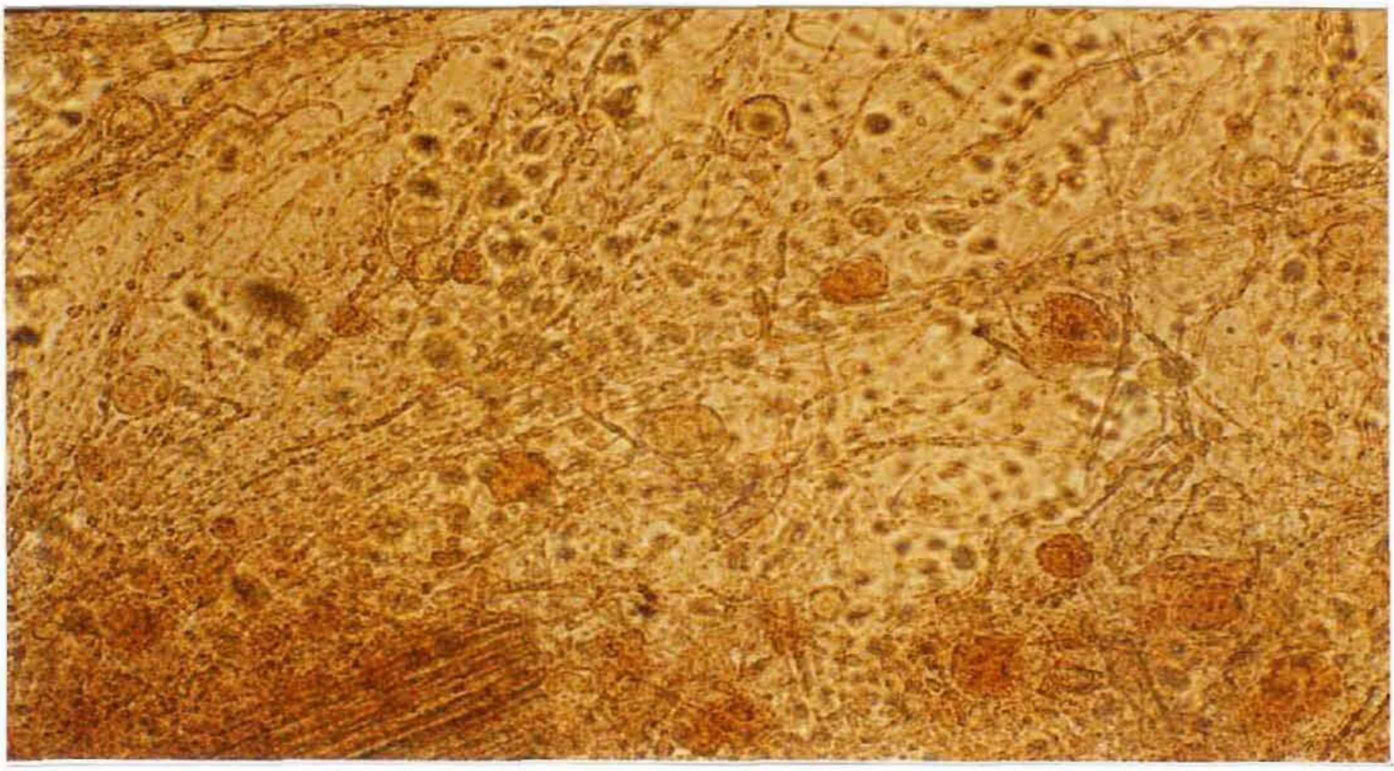


Plate 24: Microscopic structure of isolate 3. Polycentric fungus with several small sporangia. Extensively branched rhizomycelia. Identified as Orpinomyces sp.

Plate 25: *Microscopic view of isolate 4. Polycentric fungus with large sporangia. Unsegmented branched rhizomycelia. Identified as Anaeromyces sp.*

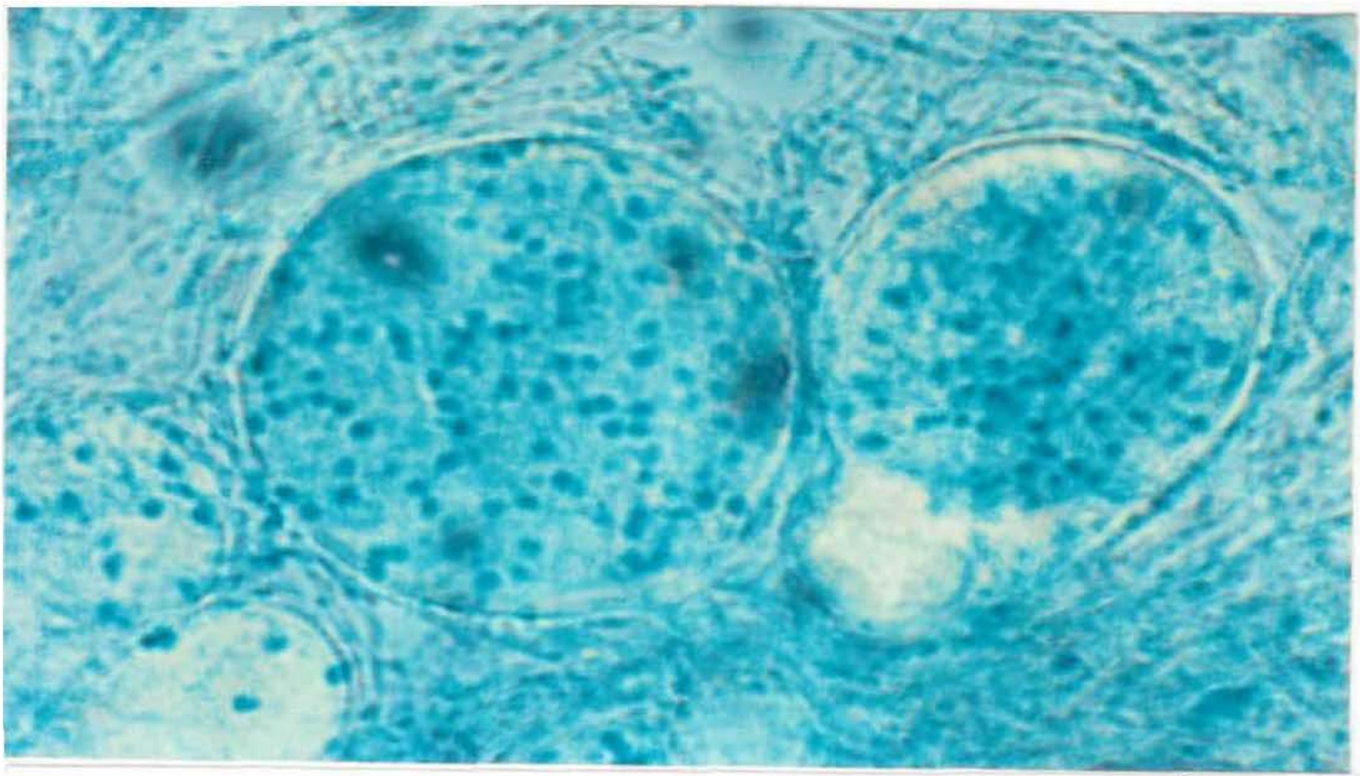


Plate 26: *Microscopic view of isolate 6. Polycentric fungus with large sporangia. Branched rhizomycelia. Identified as Anaeromyces sp.*

4.5.15 ISOLATE 6

Isolated from buffalo (T2). Several sporangia developed from single thallus (Plate 26). Circular and large sporangia. Poly centric in nature. Unsegmented, extensively branched rhizomycelia. No stalk at the base of sporangia. Matured sporangia were full of zoospores. Able to grow over straw. Uniflagellated zoospore. It appeared to belong *Anaeromyces* sp.

4.5.16 ISOLATE 8

Isolated from buffalo (T1). Several sporangia developed from the thallus (Plate 27). Polycentric in nature. Unsegmented, extensively branched rhizomycelia. Circular and large sporangia. No stalk at the base. Not able to grow over straw. Uniflagellated zoospore. Appeared to be *Anaeromyces* sp.

4.5.17 ISOLATE 11

Isolated from buffalo (T1). Several sporangia developed from single thallus (Plate 28). So it was polycentric. Small and circular sporangia. No stalk at the base of sporangia. Unsegmented rhizomycelia were finely branched. Polyflagellated zoospore. Able to grow over straw. Appeared to be *Orpinomyces* sp.

4.6 FECAL ISOLATE (BF3)

This was isolated from the feces of buffalo receiving the T3 diet. It was a monocentric as single sporangium developed from the thallus (Plate 29). Circular sporangia. Multiple layers of sporangial wall were visible. No stalk at the base of sporangia . Extensively branched and unsegmented rhizomycelia. Zoospores were polyflagellated. Able to grow over straw. Appeared to belong *Neocallimastix* sp.

4.7 MOTILITY OF ZOOSPORE

The motility of zoospore was tested with the C3 isolate. The zoospore of the isolate was polyflagellated. During motility, multiple flagella remained in the form of flagellar tuff and formed a single locomotory organ (Plate 30). After the formation of

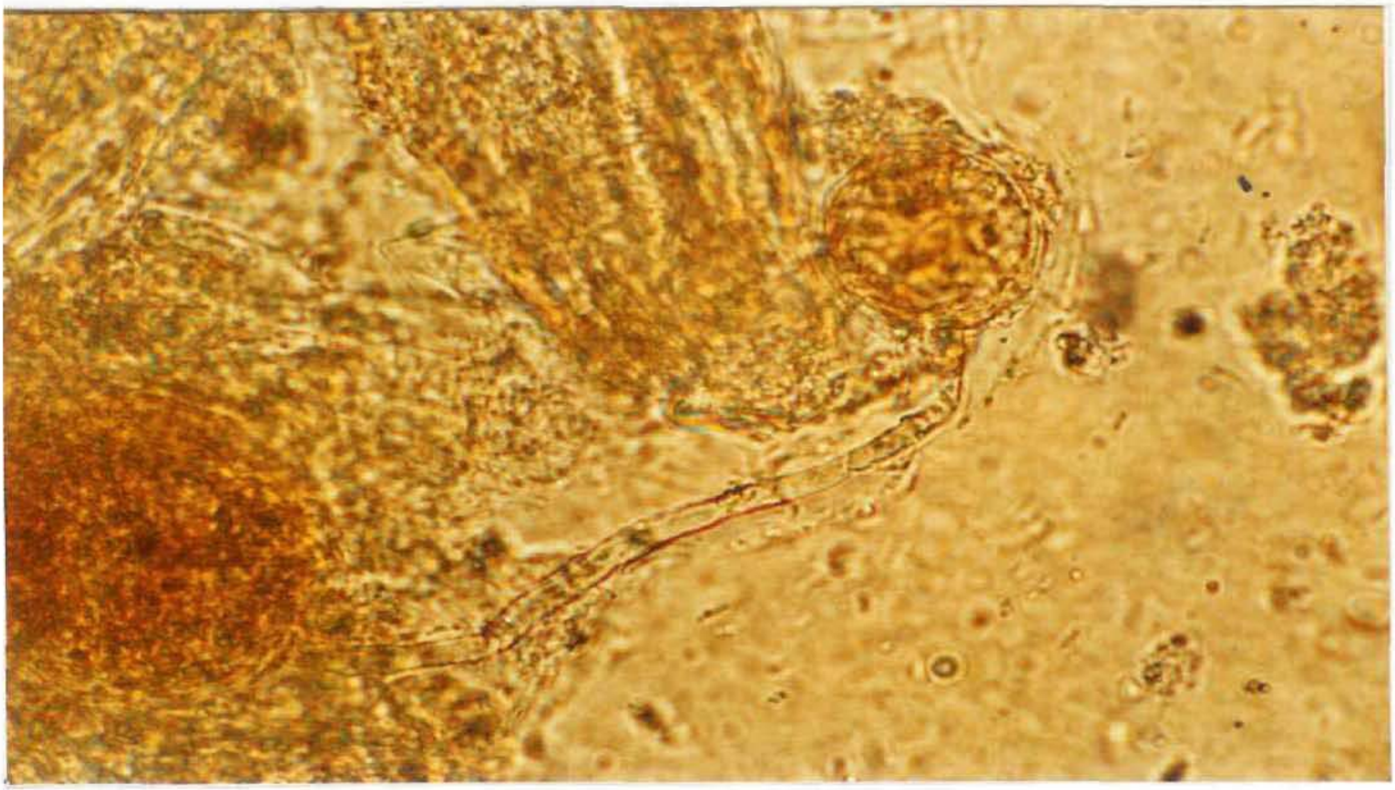


Plate 27: Microscopic structure of isolate 8. Polycentric fungus with circular sporangia. Unsegmented branched rhizomycelia. Identified as Anaeromyces sp.

Plate 28: Microscopic structure of isolate 11. Polycentric fungus with small, circular sporangia. Branched rhizomycelia. Identified as Orpinomyces sp.

Plate 29: Microscopic structure of BF3 isolate. Monocentric fungus with large sporangia. Branched rhizomycelia. Identified as Neocallimastix sp.

Plate 30: Formation of single locomotory organ by polyflagellated zoospore of C3 isolate.

single locomotory organ, flagellar tuff first bent forward and moved the zoospore in upward direction. Again flagellar tuff curved and forced the cell to move. Actually these were done through continuous and rapid beating of flagella.

4.8 GROWTH CHARACTERISTICS OF ISOLATES

Efforts were first made to grow all the isolates on cellobiose broth media. The isolates did not grow. Then the isolates were grown in 2% agar and subsequently agar concentration was reduced up to 0.3% (soft agar). With the last one, the growth was optimum. Actually the isolates were unable to grow without solid support. Isolate B3 was able to grow on xylan and cellulose also.

4.9 GAS PRODUCTION OF ISOLATES

Different isolates were grown on cellobiose soft agar and the gas production was judged visually after 4 days of incubation. Detail of the gas production has been presented in Table 4.3. From the table it could be seen that isolate number C1, C3, Sm1, Sm2, B3 and B4 produced profused gas (+ + + +); C2, C4, 6 and 8 produced moderate amount of gas (+ + +); B1, B2, 1, 3 produced small quantity of gas (+ +), while C5, 4 and 11 were negligible producer of gas (+).

4.10 VOLATILE FATTY ACIDS PRODUCTION

Out of the three major volatile fatty acids, the isolates produced only acetate. The acetate production by different isolates on cellobiose soft agar after 4 days of incubation is presented in Table 4.4. Maximum production was observed in case of B3 and C3 isolate and minimum observed in case of isolate 6.

4.11 DETAILED MORPHOLOGICAL FEATURES OF B3 AND C3 ISOLATE

4.11.1 ISOLATE B3

It was a monocentric isolate. Zoospores were monoflagellated. Size of the zoospore was 0.01 to 0.02 μm with circular shape. Sporangia too were circular in shape. Size of the sporangia was 1.5 x 1.5 μm. It possessed highly thick rhizoid (Plate 31). Thickness was upto 0.15 μm. Length of the flagella was 0.84 μm.

Table 4.3 Visual appraisal of gas producing ability of different anaerobic fungal isolate

Isolate	*Degree of gas production*
Buffalo	
B1	++
B2	++
B3	++++
B4	++++
1	++
3	++
4	+
6	+++
8	+++
11	+
Cattle	
C1	++++
C2	+++
C3	++++
C4	+++
C5	+
Sm1	++++
Sm2	++++

+	Negligible gas production
++	Lower quantity of gas production
+++	Moderate gas production
++++	Profused gas production

Table 4.4 Acetate production from different fungal isolates following 4 days of incubation

Isolate	*Acetate (nanomol/ml)*
Buffalo	
B1	0.4556
B2	3.0913
B3	14.2530
B4	9.1170
1	1.2200
3	1.0040
4	1.2700
6	0.4550
8	1.2210
11	1.3478
Cattle	
C1	6.0220
C2	5.9910
C3	12.9670
C4	6.1730
C5	2.9710
Sm1	7.5350
Sm2	7.4910

Plate 31: Microscopic view of extensively thick, branched and unsegmented rhizomycelia of B3 isolate.

Plate 32: Microscopic view of polyflagellated (five) zoospore (C3 isolate).

4.11.2 ISOLATE C3

The isolate was monocentric. Size of zoospore was 0.04 x 0.04 μm. Number of flagella may be up to five (Plate 32). Length of flagella was 0.14 μm. During early stage of development, sporangia were oval (Plate 33) and became circular (Plate 34) at the time of maturity. Size of the sporangia was 2.1 x 2.1 μm, with the rhizoid thickness between 0.05 to 0.2 μm.

4.12 NUMBER OF ZOOSPORES RELEASED PER SPORANGIUM

Number of zoospores were counted on the roll tube under inverted microscope. It was assumed that single colony developed from each zoospore or sporangium. After release of zoospore, these were not able to move further due to the presence of agar on cellobiose roll tube and further matured to become reproductive cell, i.e., sporangia. The number of zoospore released per sporangium was 15 to 18 (Plate 35).

4.13 ATTACHMENT OF STRAW WITH ANAEROBIC FUNGUS

Samples of rumen digesta were taken and seen under microscope. Oval shaped sporangia attached to fibre particle through long and thick rhizomycelia were observed (Plate 36). Under *in vitro* condition, straw particles were also heavily attacked by rhizomycelia (Plate 37). This attachment results in the weakening (breakdown) of the fibre particles.

4.14 AMINO ACID COMPOSITION OF B3 ISOLATE

The amino acid composition of dried anaerobic fungal cell (B3 isolate) is analysed in Water's HPLC. The chromatographic separation of standard amino acid mixture and *Piromyces* sp. B3 isolate are presented in Fig.4.1 and 4.2 respectively. The quantity of individual amino acid was calculated by comparing with the standard through computer programming. The concentration of each amino acid is presented in Table 4.5. The concentration of arginine and serine were at higher side. Moderate contents of amino acids were lysine (7.17 g/100 g total amino acids), isoleucine (6.08 g/100 g total amino acids), tyrosine (5.28 g/100 g total amino acids), proline (6.26 g/100 g total amino acids),

Fig.4.1 Chromatographic profile of standard amino acid mixture on HPLC

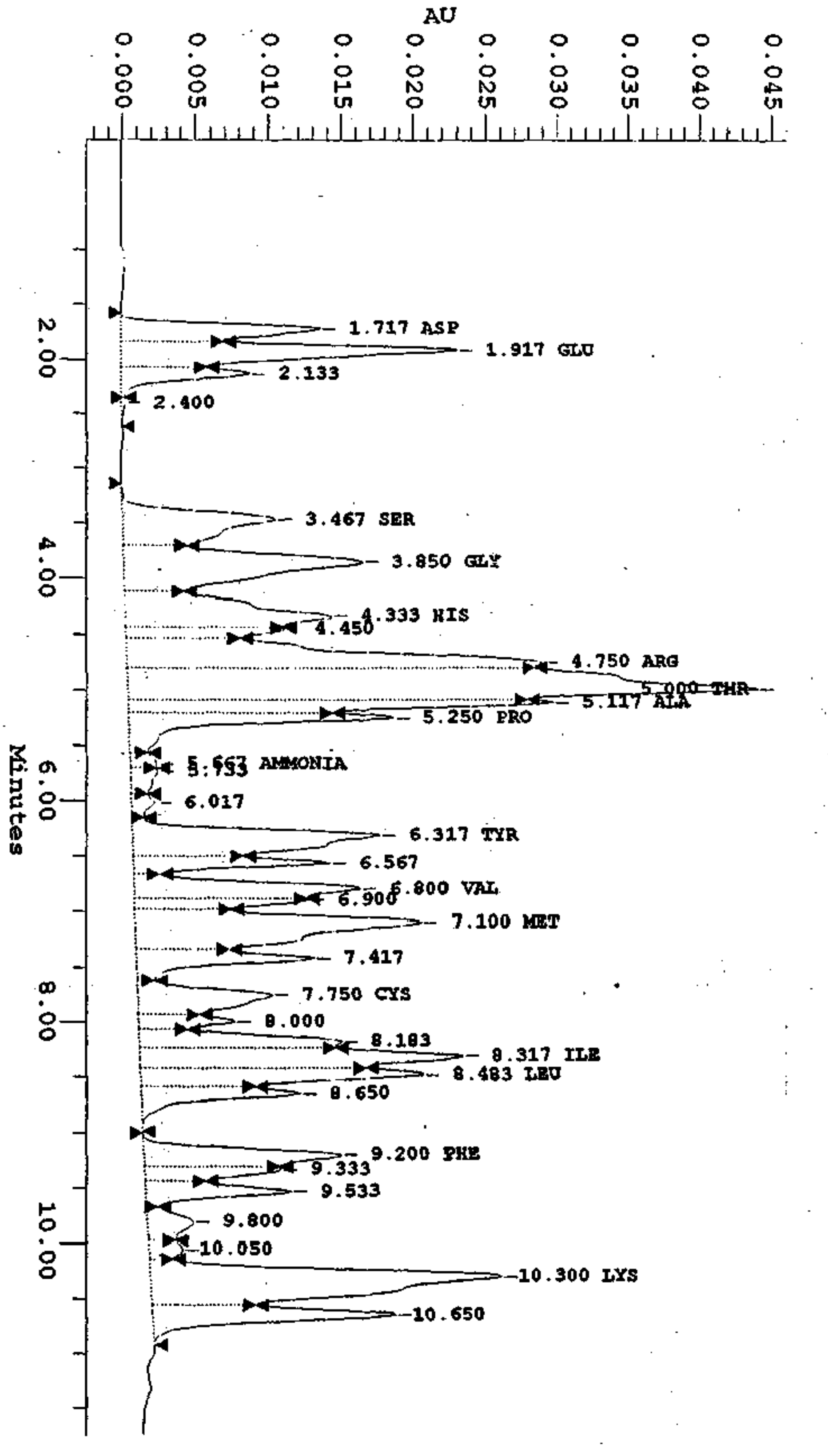
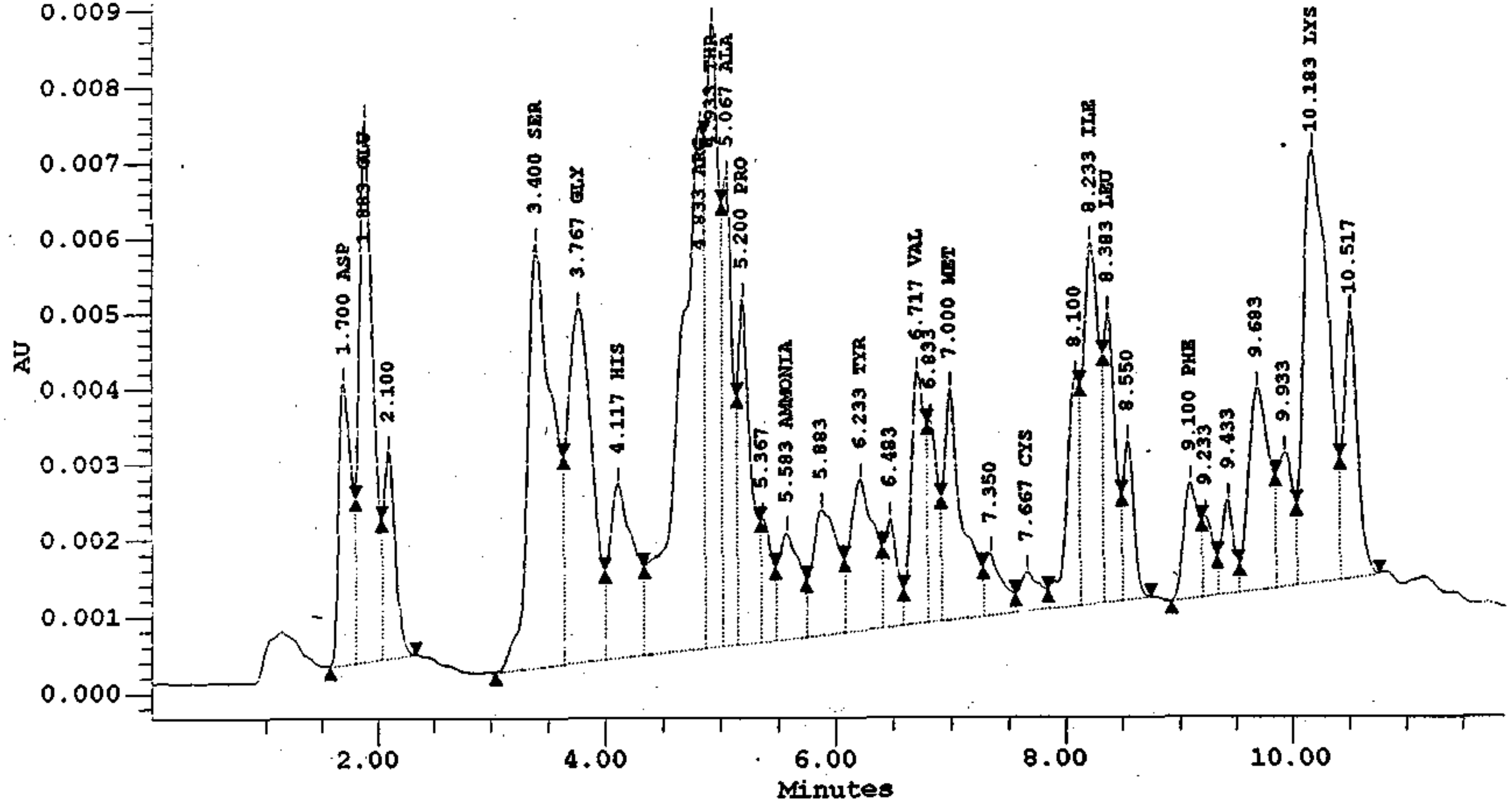

AU
0.000
0.005
0.010
0.015
0.020
0.025
0.030
0.035
0.040
0.045
Minutes
2.00
4.00
6.00
8.00
10.00
1.717 ASP
1.917 GLU
2.133
2.400
3.467 SER
3.850 GLY
4.333 HIS
4.450
4.750 ARG
5.000 THR
5.117 ALA
5.250 PRO
5.667 AMMONIA
5.733
6.017
6.317 TYR
6.567
6.800 VAL
6.900
7.100 MET
7.417
7.750 CYS
8.000
8.183
8.317 ILE
8.483 LEU
8.650
9.200 PHE
9.333
9.533
9.800
10.050
10.300 LYS
10.650

Fig.4.2 Chromatographic separation of amino acids from *Piromyces* sp. (B3 isolate) on HPLC



AU
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
Minutes
2.00
4.00
6.00
8.00
10.00
1.700 ASP
1.883 GLU
2.100
3.400 SER
3.767 GLY
4.117 HIS
4.833 ARG
4.933 THR
5.067 ALA
5.200 PRO
5.367
5.583 AMMONIA
5.883
6.233 TYR
6.483
6.717 VAL
6.833
7.000 MET
7.350
7.667 CYS
8.100
8.233 ILE
8.383 LEU
8.550
9.100 PHE
9.233
9.433
9.683
9.933
10.183 LYS
10.517

Table 4.5 Amino acid composition of hydrolysates prepared from rumen fungus (B3 isolate)

Amino acid	*Amount present (g/100 g total amino acids)*
Lysine	7.17
Isoleucine	6.08
Leucine	4.91
Phenylalanine	3.74
Alanine	4.44
Proline	6.26
Histidine	5.64
Arginine	11.90
Threonine	2.63
Glycine	4.38
Glutamic	9.18
Serine	11.87
Aspartic	7.16
Methionine	3.81
Cystine	0.65
Valine	4.90

Plate 33: Oval shaped, young sporangia of C3 isolate.

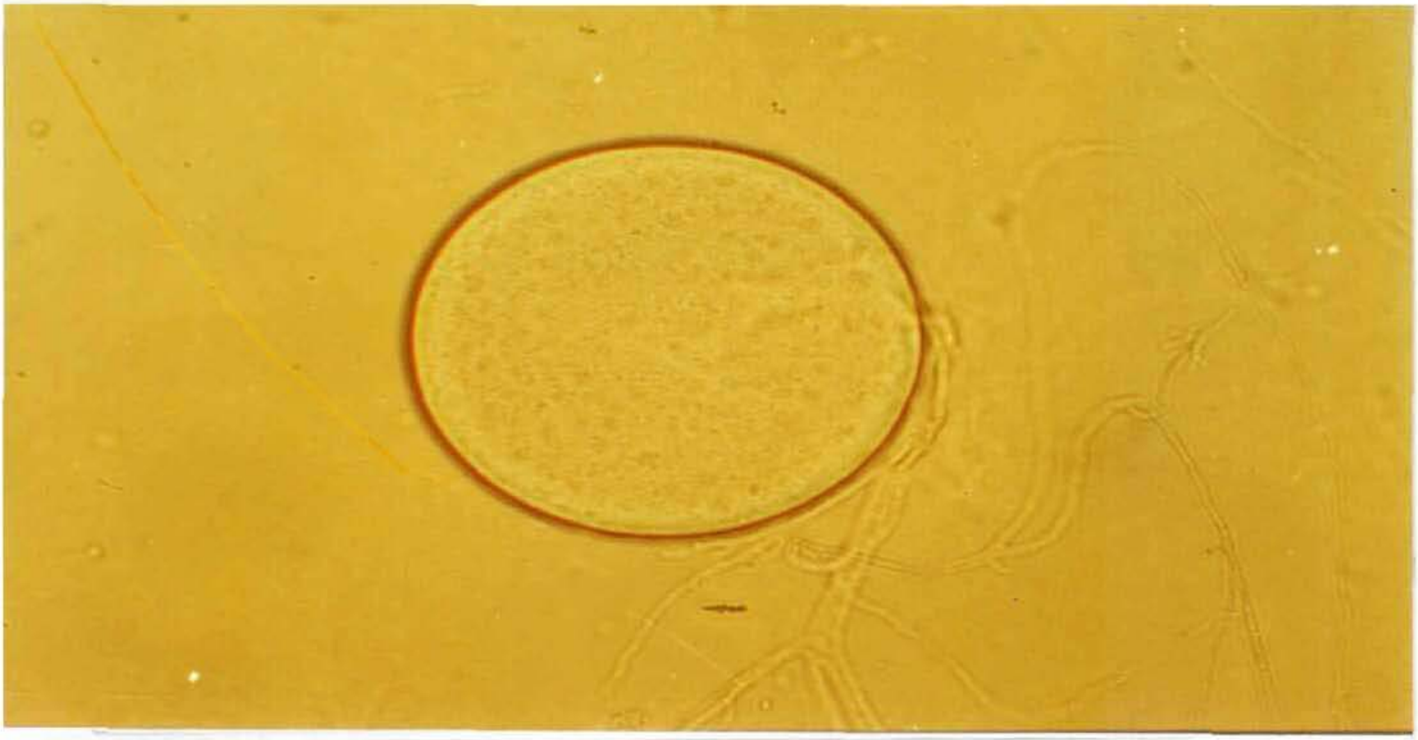


Plate 34: Matured, circular sporangium of C3 isolate.

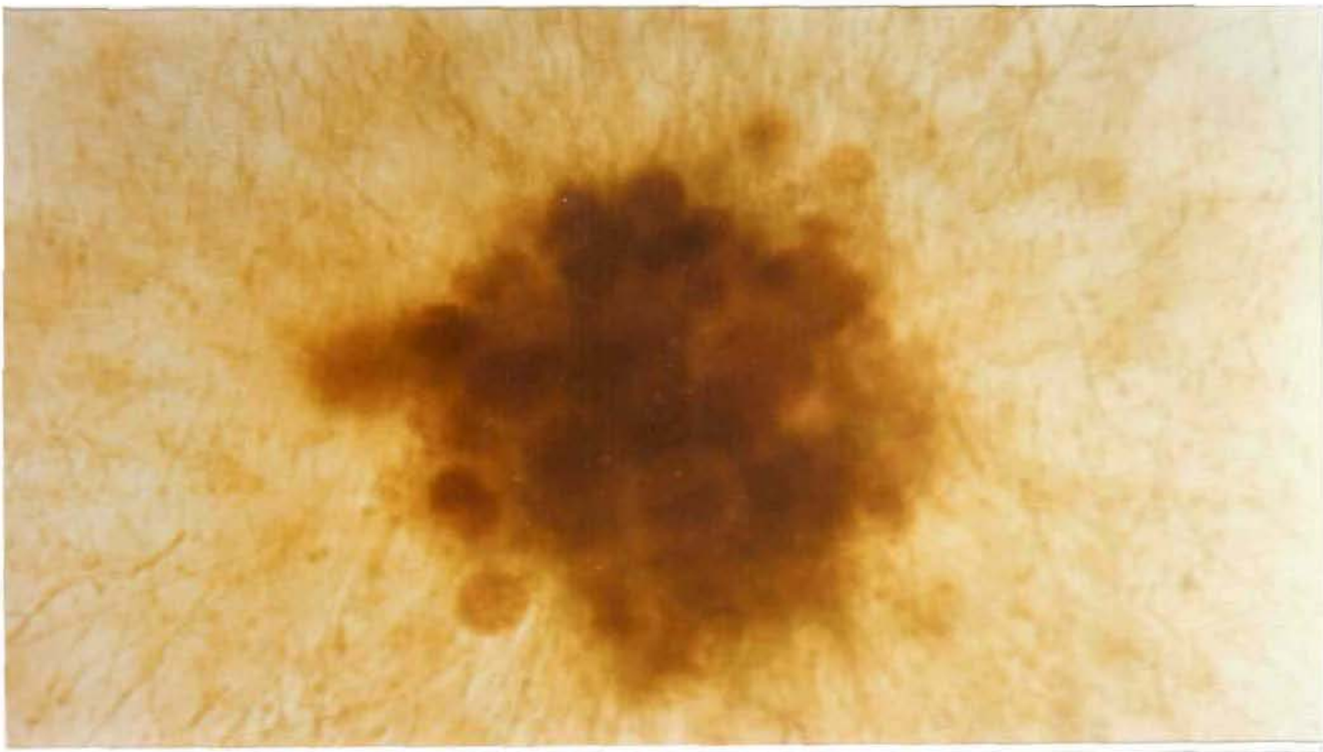


Plate 35: Aggregation of several sporangia to form the colony (zoom microscope).

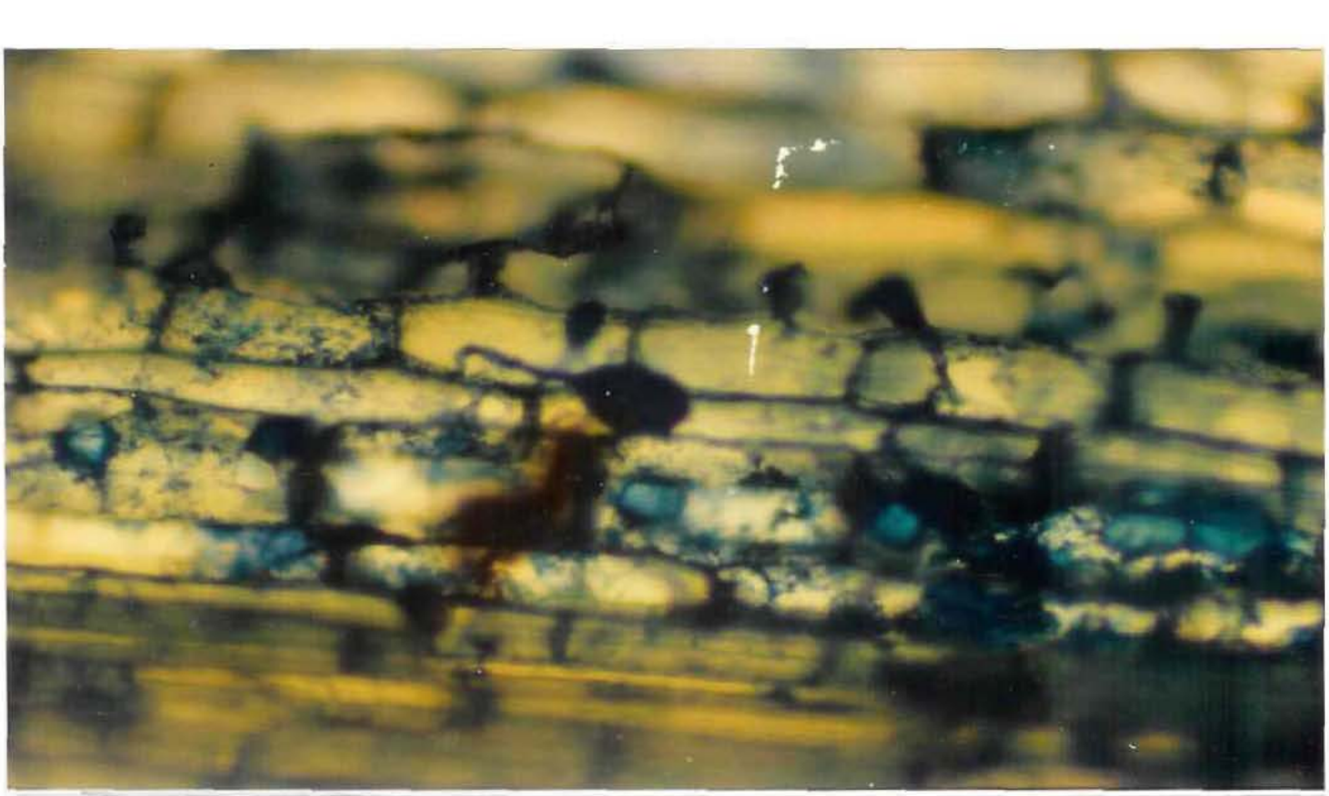


Plate 36: Samples of rumen digesta showing fungal (oval shaped) attachment with fibre.

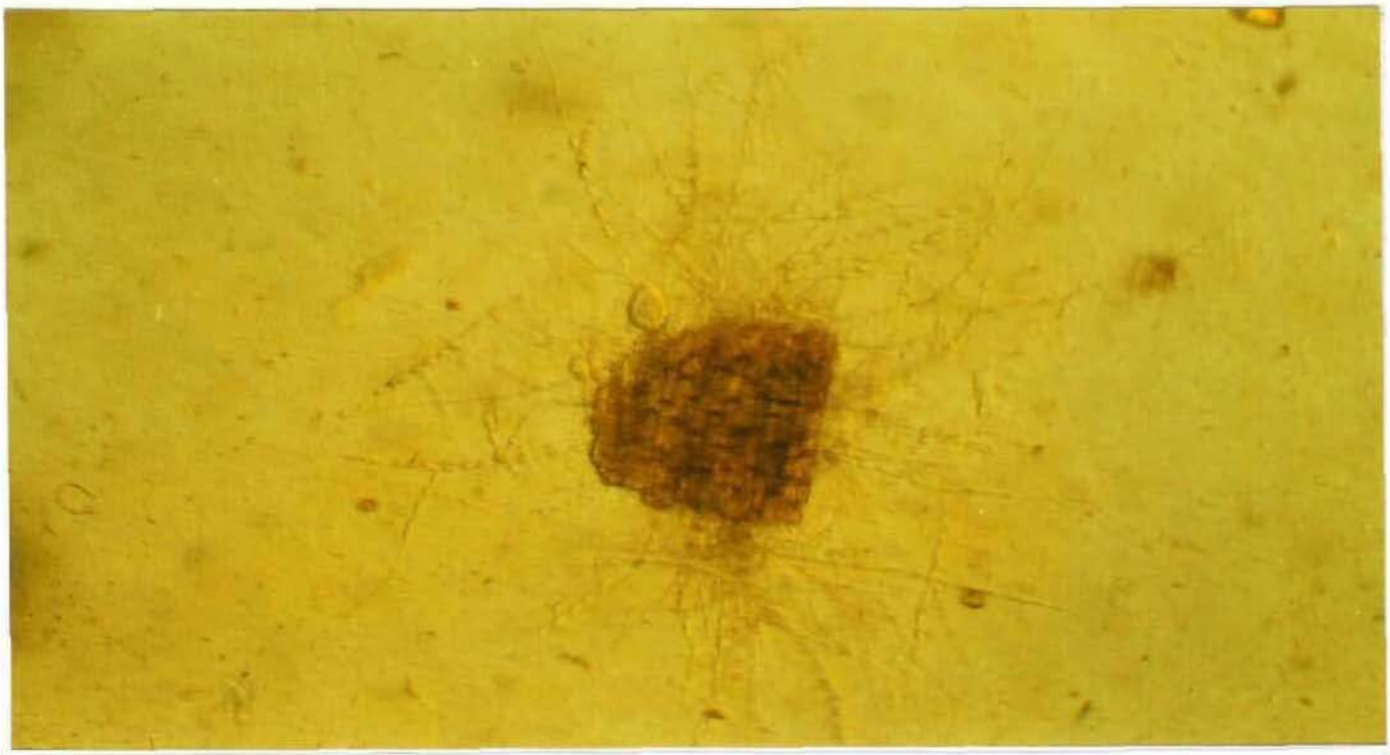


Plate 37: Extensive development of rhizomycelia over fibre particle (in vitro).

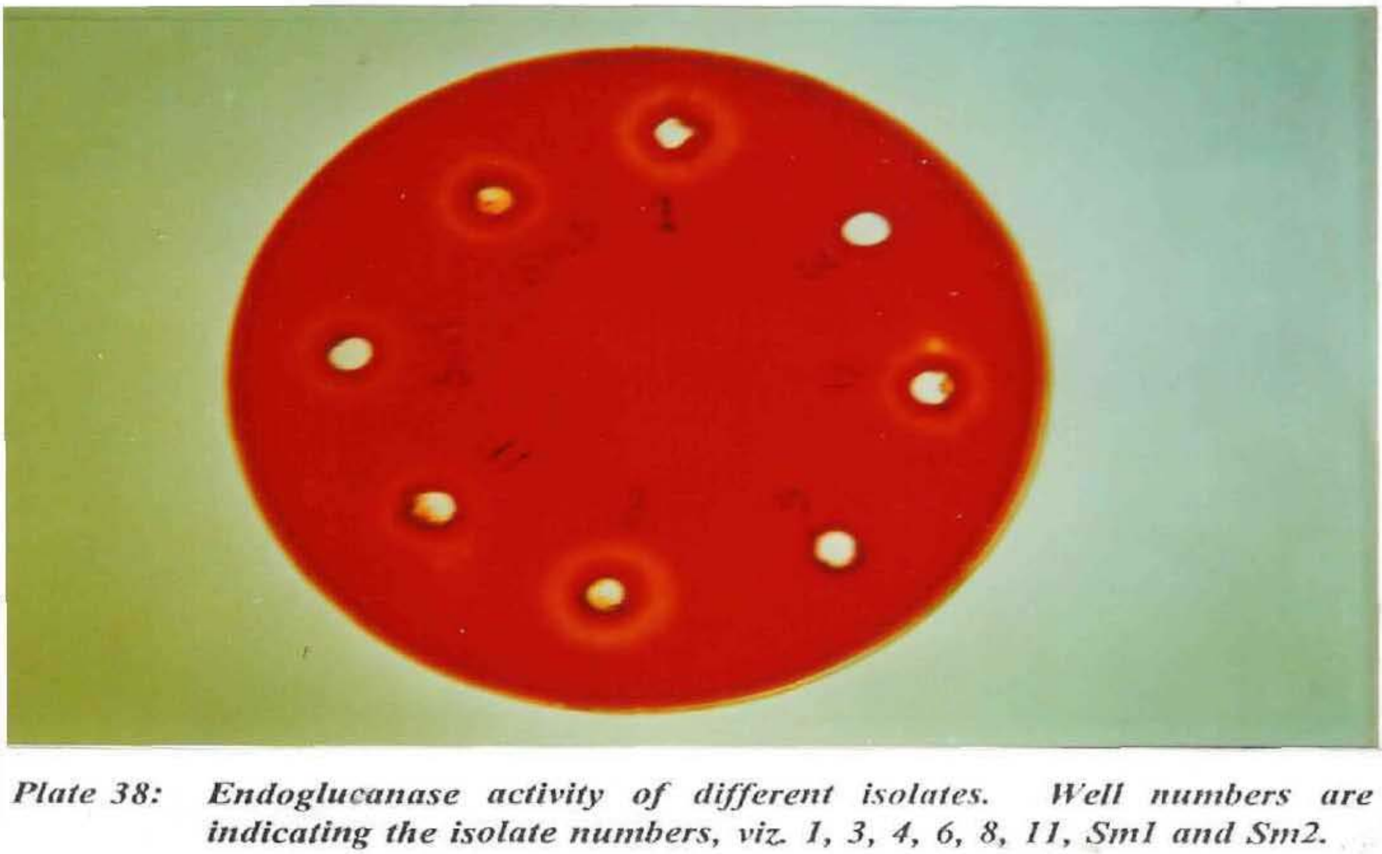


Plate 38: Endoglucanase activity of different isolates. Well numbers are indicating the isolate numbers, viz. 1, 3, 4, 6, 8, 11, Sm1 and Sm2.

histidine (5.64 g/100 g total amino acids), glutamic (9.18 g/100 g total amino acids), aspartic (7.16 g/100 g total amino acids). Other amino acids were leucine (4.91 g/100 g total amino acids), phenylalanine (3.74 g/100 g total amino acids), alanine (4.44 g/100 g total amino acids), threonine (2.63 g/100 g total amino acids), glycine (4.38 g/100 g amino acids), methionine (3.81 g/100 g total amino acids), cystine (0.65 g/100 g total amino acids) and valine (4.90 g/100 g total amino acids).

4.15 SCREENING OF FUNGAL ISOLATES FOR THE PRODUCTION OF ENDOGLUCANASE

The fungal isolates after 4 days of growth were screened for endoglucanase secretion through qualitative (congo red assay) as well as quantitative assay.

4.15.1 CONGO RED ASSAY

The yellow zone of clearance in CMC plate is the indicative of endoglucanase activity. From plate 38 and 39, it could be seen that isolate number 1, 8, C1, C3 and B3 produced bigger zone of clearance suggesting that they had better endoglucanase activity. Isolate number 4, 11, Sm1, Sm2, C2 and B2 produced smaller zone of clearance, i.e., these were moderate endoglucanase producer. Isolate number 3, 6, C4, C5, B1 and B4 were not able to produce any yellow zone of clearance, i.e., these isolates were not able to produce endoglucanase enzyme.

4.15.2 QUANTITATIVE ASSAY

The endoglucanase activity was measured by estimating the reducing sugars from carboxymethyl cellulose (CMC) after specific time of incubation at 50°C. The units of activity (IU) is defined as μmol of glucose equivalent released per minute by the culture supernatant. The endoglucanase activity of different isolates are presented in Table 4.6 The endoglucanase activity of isolate 1, 8, C1, C3 and B3 were towards higher side, ranging from 14.6990 to 15.9722 mIU/ml. In isolate number 4, 11, Sm1, Sm2, C2 and B2, the activities ranged from 3.9351 to 7.1759 mIU/ml. While the isolate number 3, 6, B1, B4, C4 and C5 did not possess any endoglucanase activity. Out of these seventeen isolates, B3 (*Piromyces* sp.) was selected for further enzyme studies.

Table 4.6 Endoglucanase activity of different isolates after 4 days of growth on cellobiose soft agar

Isolate	*Endoglucanase activity (mIU/ml)*
Buffalo	
B1	Nil
B2	3.9351
B3	15.9722
B4	Nil
1	15.0462
3	Nil
4	5.7870
6	Nil
8	15.5092
11	4.9768
Cattle	
C1	14.6990
C2	5.7870
C3	15.5092
C4	Nil
C5	Nil
Sm1	6.7129
Sm2	7.1759

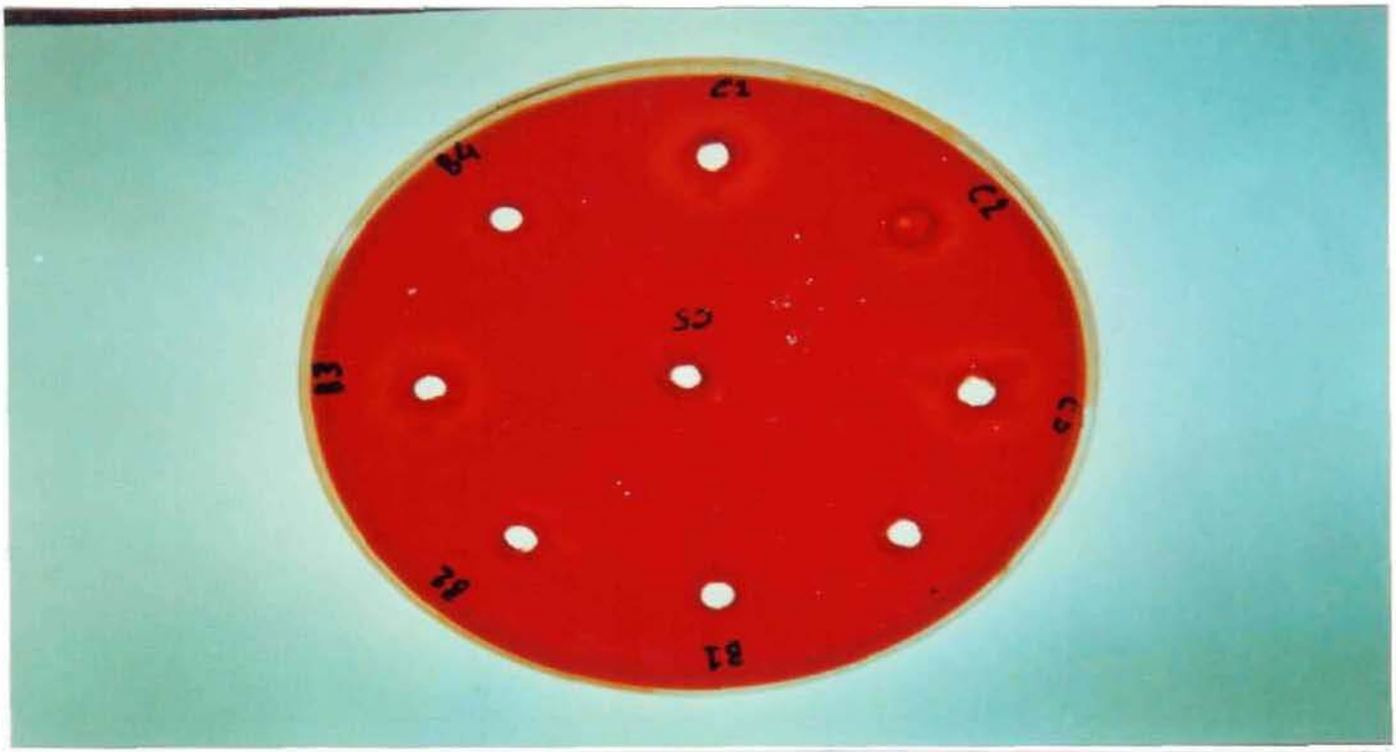


Plate 39: *Endoglucanase activity of diffeent isolates. Well numbers are indicating the isolate numbers, viz. B1, B2, B3, B4, C1, C2, C3, C4 and C5.*

Plate 40: *Endoglucanase activity of B3 isolate at different days after incubation. Well numbers are indicating the days after incubation.*

4.16 TIME OPTIMIZATION FOR HARVESTING ENZYME

The isolate B3 was grown in 500 ml flask containing 200 ml cellobiose soft agar media. Following withdrawal of culture supernatant regularly, endoglucanase activity was assayed quantitatively as well as qualitatively. The congo red assay of different days enzyme activity is given in Plate 40. The endoglucanase activity at different days after incubation is presented in Table 4.7. The maximum endoglucanase activity was found after 4 days of incubation (17.5925 mIU/ml).

4.17 EFFECT OF SONICATION ON THE RELEASE OF ENDOGLUCANASE FROM FUNGAL CELL

Fungal cells (sporangia and rhizomycelia) were subjected to sonication. The sonication of fungal cell did not release any endoglucanase enzyme. So it could be concluded that the enzyme production of this isolate is completely extracellular.

4.18 OTHER PLANT BIOMASS DEGRADING ENZYME

The avicelase, cellobiase and xylanase were also estimated in the culture supernatant of B3 isolate. The values were 0.4629 mIU/ml for avicelase, 19.3737 mIU/ml for cellobiase and 237.613 mIU/ml for xylanase. The specific activities were 0.0033 IU/mg protein for avicelase, 0.1409 IU/mg protein for cellobiase, 0.1186 IU/mg protein for endoglucanase and 1.7280 IU/mg protein for xylanase.

4.19 EFFECT OF pH ON DIFFERENT ENZYME ACTIVITY

4.19.1 ENDOGLUCANASE

The endoglucanase activity of culture supernatant was determined at 50°C in the pH range of 4.0 to 8.0. The studies depicted that the enzyme endoglucanase had a pH optima of 6.5 (Fig. 4.3).

4.19.2 CELLOBIASE

The cellobiase activity of the culture supernatant was determined at 50°C in the pH range of 4.0 to 8.0. A pH optima of 6.0 to 6.5 (Fig. 4.4) was found for the cellobiase enzyme.

Fig.4.3 Effect of pH on the extra-cellular endoglucanase activity of *Piromyces* sp. (B3 isolate)

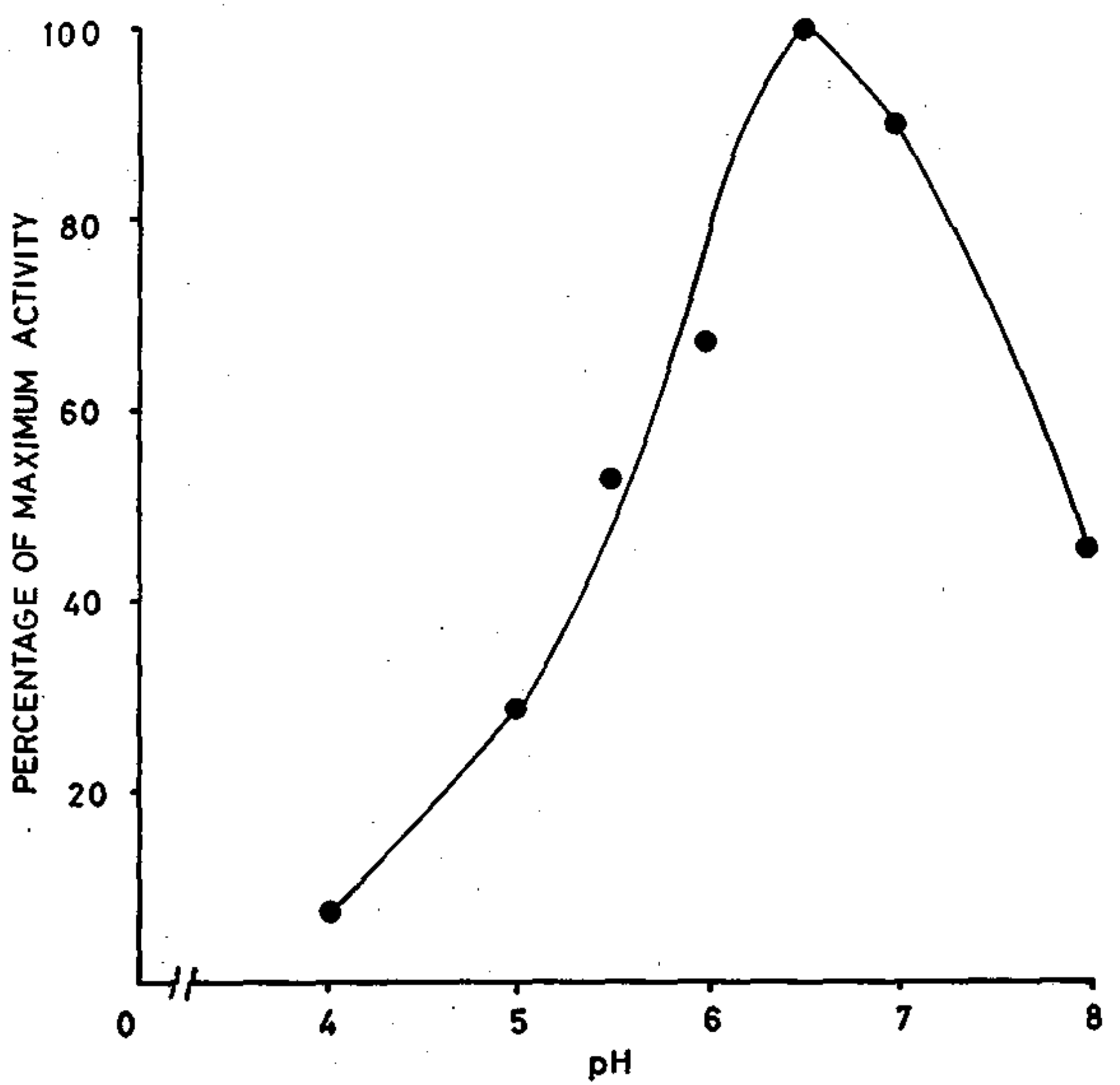

PERCENTAGE OF MAXIMUM ACTIVITY
100
80
60
40
20
0
4
5
6
7
8
pH

Fig.4.4 Effect of pH on the extracellular cellobiase activity of *Piromyces sp.* (B3 isolate)

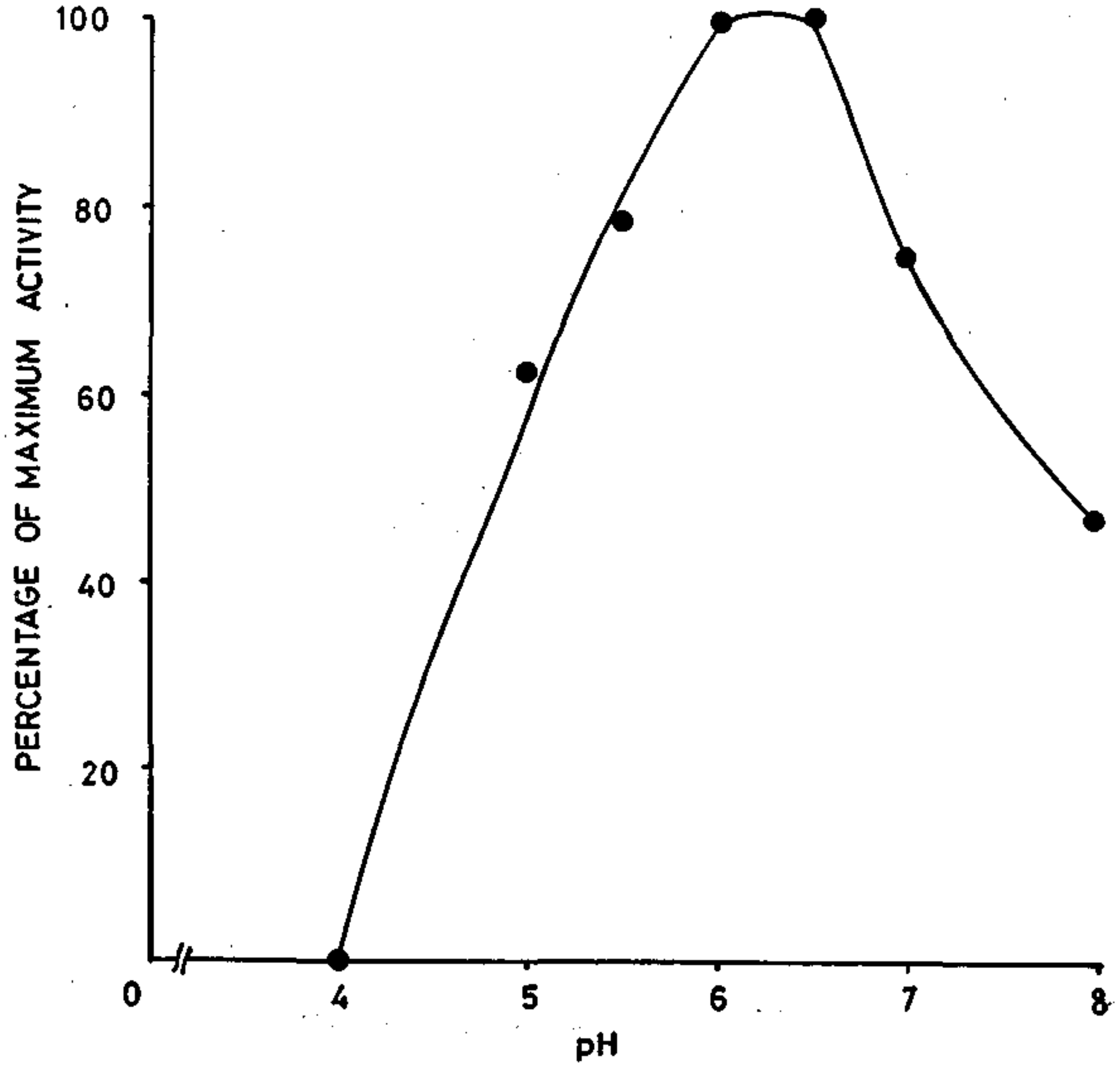

PERCENTAGE OF MAXIMUM ACTIVITY
100
80
60
40
20
0
4
5
6
7
8
pH

Table 4.7 Enzyme secretion by *Piromyces* sp. (B3 isolate) at different days after incubation

Days after incubation	*Endoglucanase activity (mIU/ml)*
1	1.1574
2	6.3657
3	11.1111
4	17.5925
5	16.4351
6	16.4351
7	10.4166
8	9.9537

4.19.3 XYLANASE

The xylanase activity of culture supernatant was determined at 50°C in the pH range of 4.0 to 8.0. The studies revealed a pH optima of 6.5 (Fig. 4.5) for xylanase enzyme.

4.20 PARTIAL PURIFICATION OF ENDOGLUCANASE AND XYLANASE

4.20.1 ENDOGLUCANASE

The culture supernatant having activity of 16.3194 mIU/ml was first precipitated with different levels, *viz.*, 40%, 60% and 80% of ammonium sulfate. It was seen that 60% ammonium sulfate was able to precipitate the endoglucanase enzyme completely. This level was subsequently used for precipitation of the enzyme.

4.20.1.1 Ammonium sulfate precipitation

Ammonium sulfate (60%) precipitated enzyme sample was dialysed overnight and endoglucanase activity was estimated. During this step, 42% of the endoglucanase activity was retained in the solution and 58% of activity was recovered in pellet. The specific activity of the dialysed fraction was 0.2473 IU/mg protein, indicating an increase of 2.08 fold after 60% ammonium sulfate precipitation followed by dialysis (Table 4.8).

4.20.1.2 DEAE Sepharose column chromatography

The next step of purification was attempted with DEAE Sepharose column chromatography. However, due to the unusual binding of enzyme protein with the anion exchanger, the enzyme failed to come out of the column and thus this step of purification was unsuccessful. Then fresh DEAE Sepharose anion exchanger was taken and 0.5 ml of enzyme was added to this. Subsequent washing of the column with different concentrations of buffer as described in materials and methods, gave several supernatants (S1, S2, S3, S4 and S5) and DEAE-SPC. The endoglucanase activity was detected only in DEAE-SPC fraction, not in S1, S2, S3, S4 and S5. Thus, it was

Fig.4.5 Effect of pH on the extracellular xylanase activity of *Piromyces* sp. (B3 isolate)

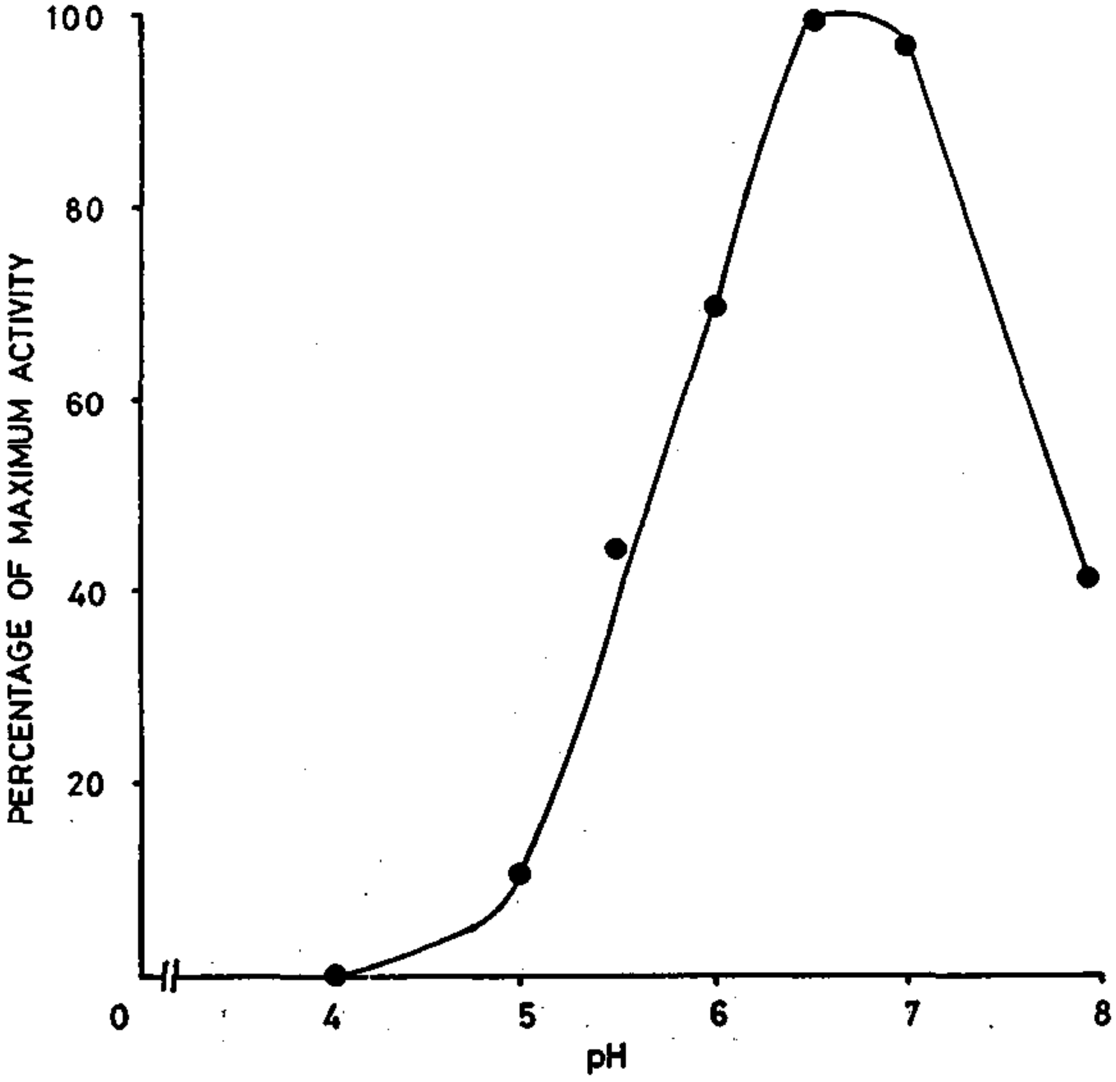

100
80
60
40
20
0
PERCENTAGE OF MAXIMUM ACTIVITY
4
5
6
7
8
pH

Table 4.8 Partial purification of endoglucanase from *Piromyces* sp. (B3 isolate) through hydroxylapatite column chromatography and Superose-12 gel filtration

Sl. No.	*Purification step*	*Total protein (mg)*	*Total activity (mIU)*	*Specific activity (IU/mg protein)*	*Yield (%)*	*Purification fold*
1.	Crude enzyme	13.750	1631.940	0.1186	100	1
2.	Ammonium sulfate precipitation	3.840	941.998	0.2473	58.21	2.08
3.	Hydroxylapatite	0.560	197.819	0.3532	12.12	2.97
4.	Superose 12 gel (FPLC)	0.2726	120.575	0.4420	7.30	3.72

confirmed that endoglucanase actually binds with the DEAE Sepharose anion exchanger.

4.20.1.3 Hydroxylapatite column chromatography

The dialysed sample was then applied in hydroxylapatite column. The elution of protein and distribution of activity is given in Fig. 4.6. In this case also, recovery of the activity was only 12.12 per cent (Table 4.8). The specific activity of endoglucanase was 0.3532 IU/mg protein indicating 2.97 fold of purification.

4.20.1.4 FPLC

Further purification of endoglucanase was attempted on Superose-12 gel filtration column fitted with FPLC. The elution of protein and distribution of endoglucanase activity is depicted in Fig. 4.7. The recovery of activity was only 7.3 per cent (Table 4.8), even lesser than hydroxylapatite column chromatography.

However, in mono-Q column (fitted with FPLC), the recovery of activity was around 43 to 50 per cent. The elution of protein and distribution of activity is presented in Fig. 4.8. Total of four peaks were obtained. Fold of purification and recovery of activity are presented in Table 4.9. First peak (C1) comprised of 7.8 per cent of the total activity and specific activity was 0.5761 IU/mg protein. The second peak (C2) comprised of 7.4 per cent of total activity and specific activity was 0.4261 IU/mg protein. The third peak (C3) comprised of 9.2 per cent of total activity and specific activity was 0.2264 IU/mg protein. Fourth peak (C4) comprised of 18.7 per cent of total activity and specific activity was 0.3583 IU/mg protein.

4.20.2 XYLANASE (PARTIAL PURIFICATION)

The culture supernatant having the xylanase activity of 237.613 mIU/ml was used for ammonium sulfate precipitation. By this step, 47 per cent of activity was

Fig.4.6 Chromatographic profile of ammonium sulfate precipitated endoglucanase on hydroxylapatite column

o-----o Protein, 280 nm
●——● Endoglucanase activity, mIU/ml

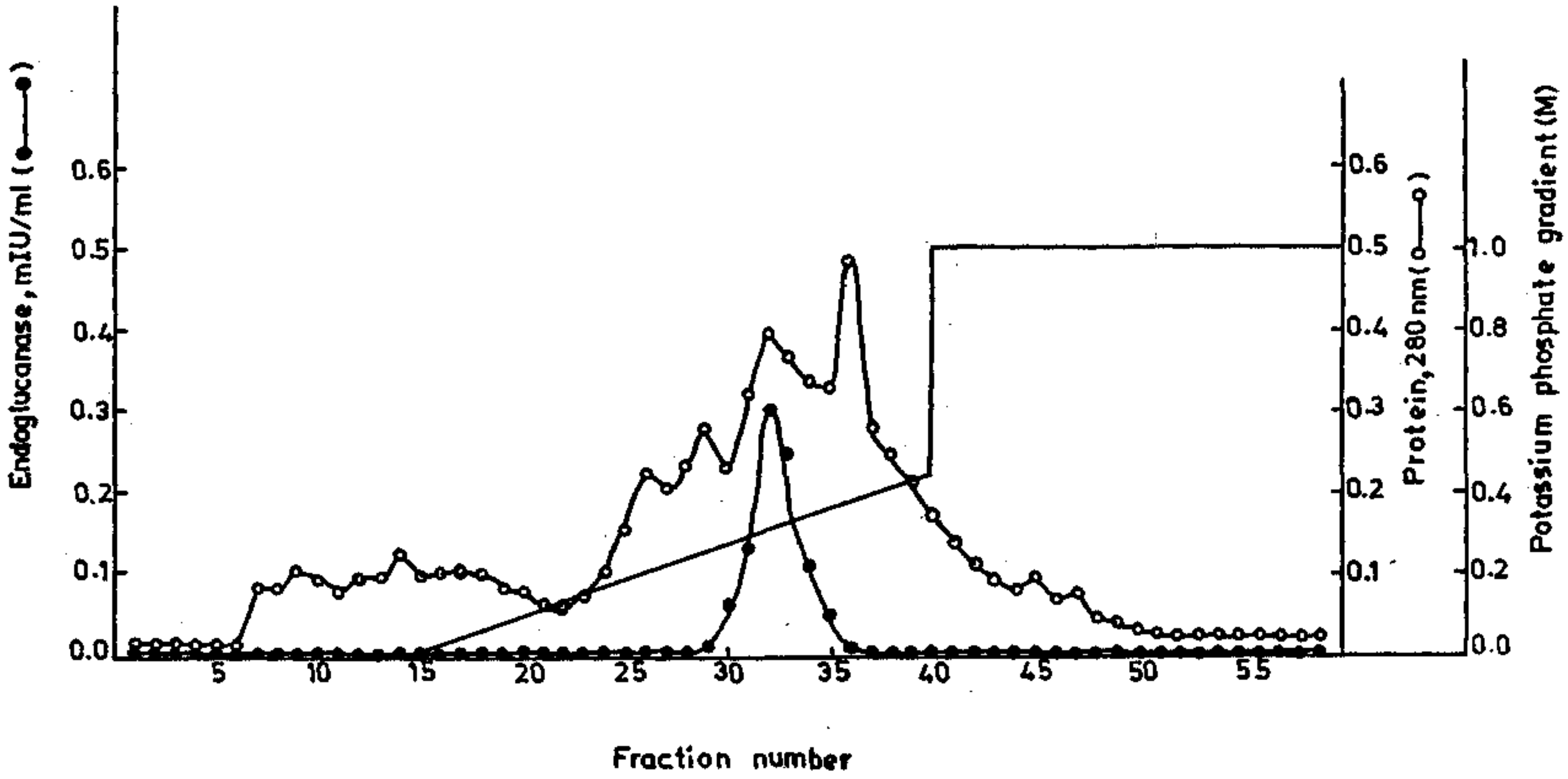
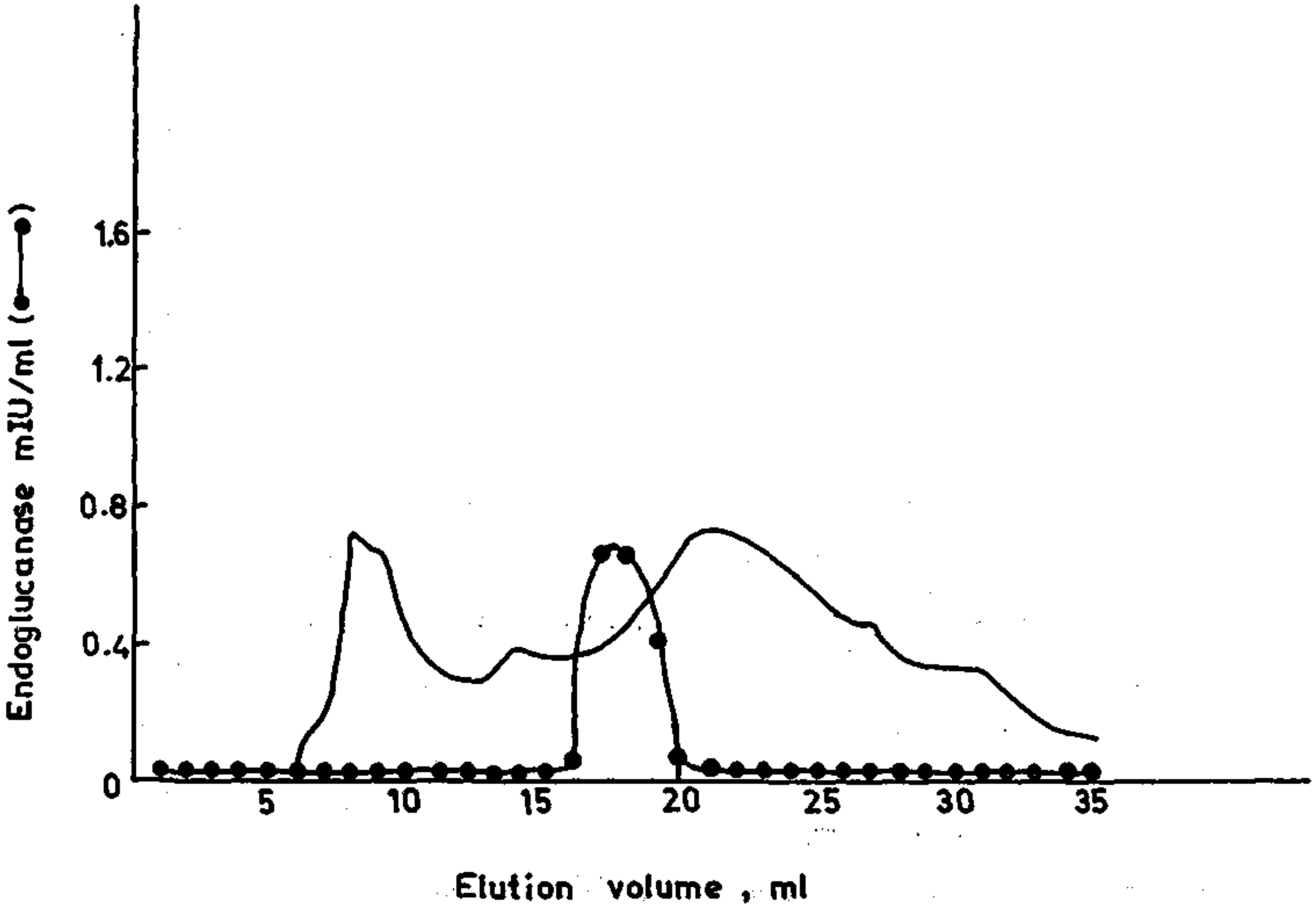

Endoglucanase, mIU/ml (●—●)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Protein, 280 nm (o—o)
0.1
0.2
0.3
0.4
0.5
0.6
Potassium phosphate gradient (M)
0.0
0.2
0.4
0.6
0.8
1.0
5
10
15
20
25
30
35
40
45
50
55
Fraction number

Fig.4.7 Chromatographic profile of ammonium sulfate precipitated endoglucanase on FPLC using Superose-12 gel filtration column

------- Protein, 280 nm

●——● Endoglucanase activity, mIU/ml



Endoglucanase mIU/ml (●—●)
1.6
1.2
0.8
0.4
0
5
10
15
20
25
30
35
Elution volume , ml

Fig.4.8 Chromatographic profile of ammonium sulfate precipitated endoglucanase and xylanase on FPLC using mono-Q column

------- Protein, 280 nm
o—o Endoglucanase activity, mIU/ml
●—● Xylanase activity, mIU/ml

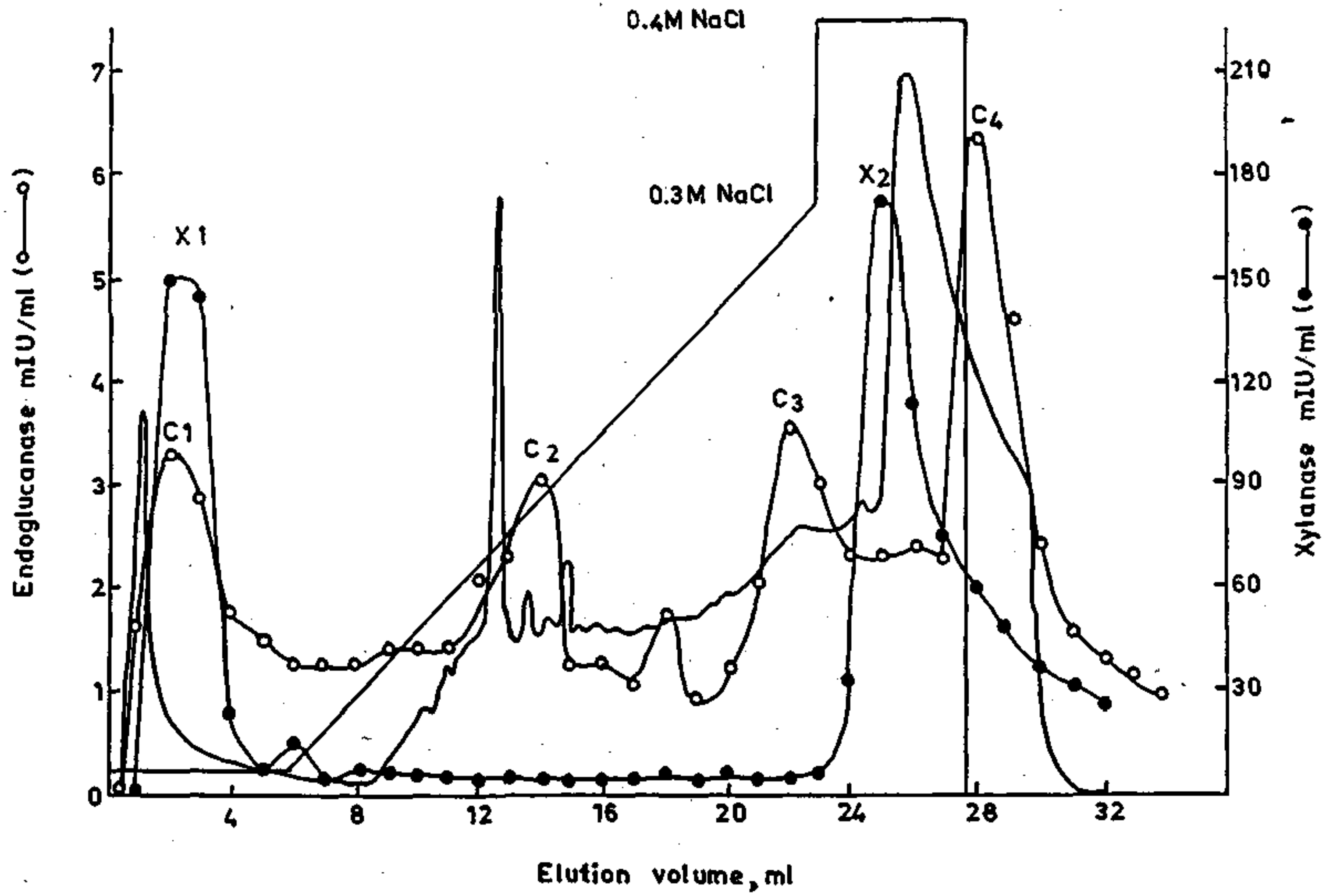

0.4M NaCl
0.3M NaCl
X 1
C1
C 2
C3
X2
C4
Endoglucanase mIU/ml (○——○)
Xylanase mIU/ml (●——●)
Elution volume, ml
0
1
2
3
4
5
6
7
4
8
12
16
20
24
28
32
30
60
90
120
150
180
210

Table 4.9 Partial purification of endoglucanase from *Piromyces* sp. (B3 isolate) through mono-Q column (FPLC)

Sl. No.	*Purification step*	*Total protein (mg)*	*Total activity (mIU)*	*Specific activity (IU/mg protein)*	*Yield (%)*	*Purification fold*
1.	Crude enzyme	13.750	1631.940	0.1186	100	1
2.	Ammonium sulfate precipitation	3.840	941.998	0.2473	58.21	2.08
3.	Mono-Q column (FPLC)					
	Peak I (C1)	0.223	128.484	0.5761	7.80	4.87
	Peak II (C2)	0.284	121.022	0.4261	7.40	3.59
	Peak III (C3)	0.667	151.020	0.2264	9.20	1.90
	Peak IV (C4)	0.854	306.033	0.3583	18.70	3.02

recovered and 53 per cent was lost in supernatant (Table 4.10). The specific activity of the dialysed sample was 2.9147 IU/mg protein, indicating an increase of 1.68 fold. This sample was then directly applied to mono-Q column connected with FPLC. Elution of protein and xylanase activity are depicted in Fig. 4.8. The recovery of activity was 23 per cent. The xylanase was eluted in two peaks (X2 and X2). X1 peak comprised of 8.2 per cent of total activity with a specific activity of 58.308 IU/mg protein. X2 peak comprised of 14.2 per cent of total activity with a specific activity of 7.7451 IU/mg protein (Table 4.10). The fold of purification were 128.8 and 17.1 for X1 and X2 peak, respectively.

4.21 SDS-PAGE

4.21.1 ENDOGLUCANASE

Dialysed enzyme along with the molecular weight marker were run on separating gel containing 0.1 per cent carboxymethyl cellulose. After staining, four activity bands (Plate 41) were detected. Rf values for each marker protein was calculated and plotted against the log MW of individual protein. Standard curve was drawn. From the migration of each activity band, Rf value was calculated. The MW was determined from the standard curve. MW of different isoenzymes of endoglucanase were 100 kDa, 66 kDa, 39.8 kDa and 33 kDa (Table 4.11).

4.21.2 XYLANASE

Dialysed enzyme along with the molecular weight marker were run on separating gel containing 0.1 per cent xylan. After staining, four xylanase activity bands were detected (Plate 42). A standard curve was drawn by plotting the Rf values against log MW of marker proteins. The Rf values for each activity band were calculated and MW was determined from the standard curve. MW of different isoforms of xylanase were 177 kDa, 100 kDa, 63 kDa and 50 kDa (Table 4.12).

Table 4.10 Partial purification of xylanase from *Piromyces* sp. (B3 isolate) through mono-Q column (FPLC)

Sl. No.	*Purification step*	*Total protein (mg)*	*Total activity (IU)*	*Specific activity (IU/mg protein)*	*Yield (%)*	*Purification fold*
1.	Crude enzyme	13.750	23.7618	1.7281	100	1
2.	Ammonium sulfate precipitation	3.840	11.1925	2.9147	47.1	1.68
3.	Mono-Q column (FPLC)					
	Peak I (X1)	0.0337	1.9650	58.3080	8.20	128.8
	Peak II (X2)	0.4449	3.4450	7.7451	14.50	17.1

Table 4.11 ***Relative mobility (Rf) of the molecular weight markers and different isoenzymes of endoglucanase on SDS-PAGE.***

Distance travelled by dye = 9.1 cm

Proteins	***MW***	***Log MW***	***Distance travelled by protein (cm)***	***Relative mobility (Rf)***
Markers				
Bovine serum albumin	66,000	4.8195	4.6	0.5054
ß-amylase	50,000	4.6989	5.7	0.6263
Alcohol dehydrogenase	38,000	4.5797	7.0	0.7692
Carbonic anhydrase	31,000	4.4913	8.0	0.8791
Cytochrome C	12,400	4.0934	8.5	0.9340
Endoglucanase				
1st band	100,000	5.00	3.0	0.3296
2nd band	66,069	4.82	4.6	0.5054
3rd band	39,810	4.60	6.5	0.7142
4th band	33,113	4.52	7.4	0.8131

Table 4.12 Relative mobility (Rf) of the molecular weight markers and different isoenzymes of xylanase on SDS-PAGE.

Distance travelled by dye ≈ 7.5 cm

Proteins	*MW*	*Log MW*	*Distance travelled by protein (cm)*	*Relative mobility (Rf)*
Markers				
Myosin	200,000	5.3010	0.35	0.0466
ß-galactosidase	116,250	5.0653	1.25	0.1666
Phosphorylase b	97,400	4.9890	1.45	0.1933
Serum albumin	66,200	4.8208	2.15	0.2866
Ovalbumin	45,000	4.6532	3.70	0.4933
Xylanase				
1st band	177,827	5.25	0.7	0.0933
2nd band	100,000	5.00	1.8	0.2400
3rd band	63,095	4.80	2.7	0.3600
4th band	50,000	4.70	3.6	0.4800

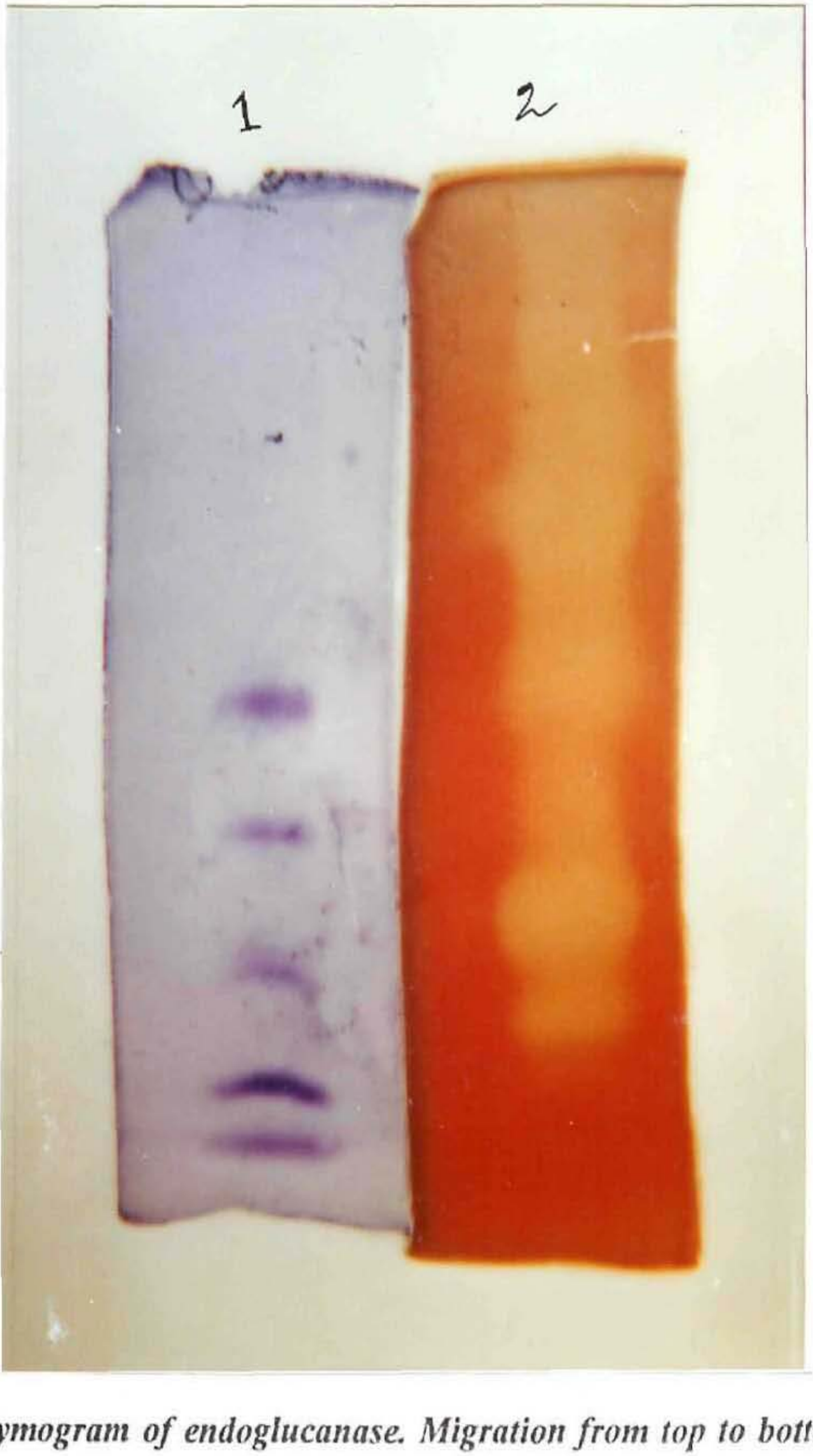


Plate 41: *Zymogram of endoglucanase. Migration from top to bottom. Lane 1, A set of molecular weight marker: BSA (Mr = 66,000); ß-amylase (Mr = 50,000); alcohol dehydrogenase (Mr = 38,000); carbonic anhydrase (Mr = 31,000) and cytochrome C (Mr = 12,400). Lane 2, Ammonium sulfate precipitated enzyme. Activity stained.*

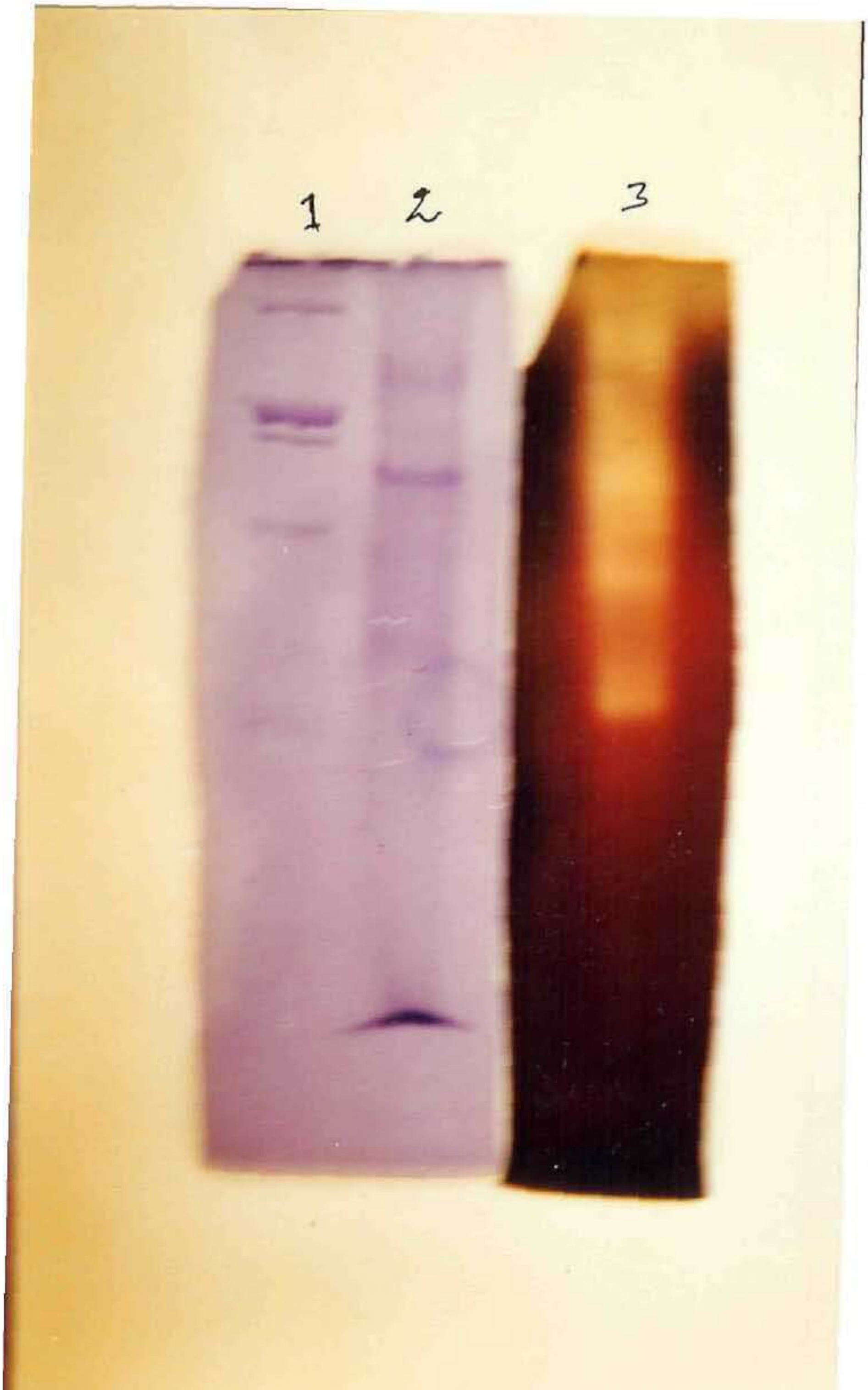


Plate 42: *Zymogram of xylanase. Migration from top to bottom.*
Lane 1, A set of molecular weight marker: Myosin (Mr = 200,000); β-galactosidase (Mr = 116,250); phosphorylase b (Mr = 97,400); serum albumin (Mr = 66,200) and ovalbumin (Mr = 45,000).
Lane 2, Ammonium sulfate precipitated enzyme. Protein stained.
Lane 3, Ammonium sulfate precipitated enzyme. Activity stained.

4.22 *IN VITRO* STUDIES

4.22.1 CHEMICAL COMPOSITION OF WHEAT STRAW

The milled (1 mm particle size) and dried wheat straw was taken for the *in vitro* studies. The chemical composition of the straw sample was as follows: organic matter 88.45% and total ash 11.55%.

4.22.2 *IN VITRO* GAS PRODUCTION

In vitro gas production at different hours of incubation are depicted in the Fig. 4.9 and 4.10 for buffalo and cattle, respectively. During the use of buffalo rumen liquor as a source of inoculum, total gas production (ml) were 93.33 ± 3.52 (T1), 57.00 ± 1.15 (T2), 73.30 ± 0.88 (T3) and 99.33 ± 2.90 (T4). Statistical analysis revealed that gas production by different groups of organisms differed significantly ($P < 0.01$). Gas production in T1 (bacteria and fungi) and T4 (bacteria, fungi and fungal isolate) was statistically at par (Table 4.13). But in the presence of either fungi alone (T2) or bacteria alone (T3), the gas production differed significantly between T2 and T3 and also with T1 and T4. When cattle was used as source of rumen inoculum, total gas production (ml) were 86.00 ± 1.52 (T1), 57.66 ± 0.66 (T2), 67.00 ± 1.15 (T3) and 92.00 ± 0.57 (T4). Statistical analysis revealed that gas production in T2 and T3 groups differed significantly from T1 and T4 ($P < 0.01$). The results further revealed that there was no additional effect of fungal isolate as probiotic (Table 4.14). Gas producing ability of only fungi (T2) and only bacteria (T3) differed significantly between each other, the values being higher for bacteria only compared to fungi only.

4.22.3 *IN VITRO* DRY MATTER DIGESTIBILITY (%)

IVDMD per cent for different treatments are presented in Table 4.13 and 4.14. The values (%) were 36.33 ± 0.78 (T1), 23.93 ± 0.52 (T2), 28.93 ± 0.69 (T3) and 38.56 ± 0.58 (T4) for buffalo. From the statistical analysis, it was found that T2 and T3 differed significantly from T1 and T4 ($P < 0.01$). The IVDMD per cent differed significantly between only fungi (T2) and only bacteria (T3). When source of rumen inoculum was cattle, IVDMD per cent were 30.60 ± 0.78 (T1), 18.73 ± 0.94 (T2),

Fig.4.9 *In vitro* gas production by different types of inoculum (buffalo) at different hours of incubation

T1 = Mixed inoculum (bacteria + fungi)
T2 = Fungi only
T3 = Bacteria only
T4 = Mixed inoculum + *Piromyces* sp. (B3 isolate)

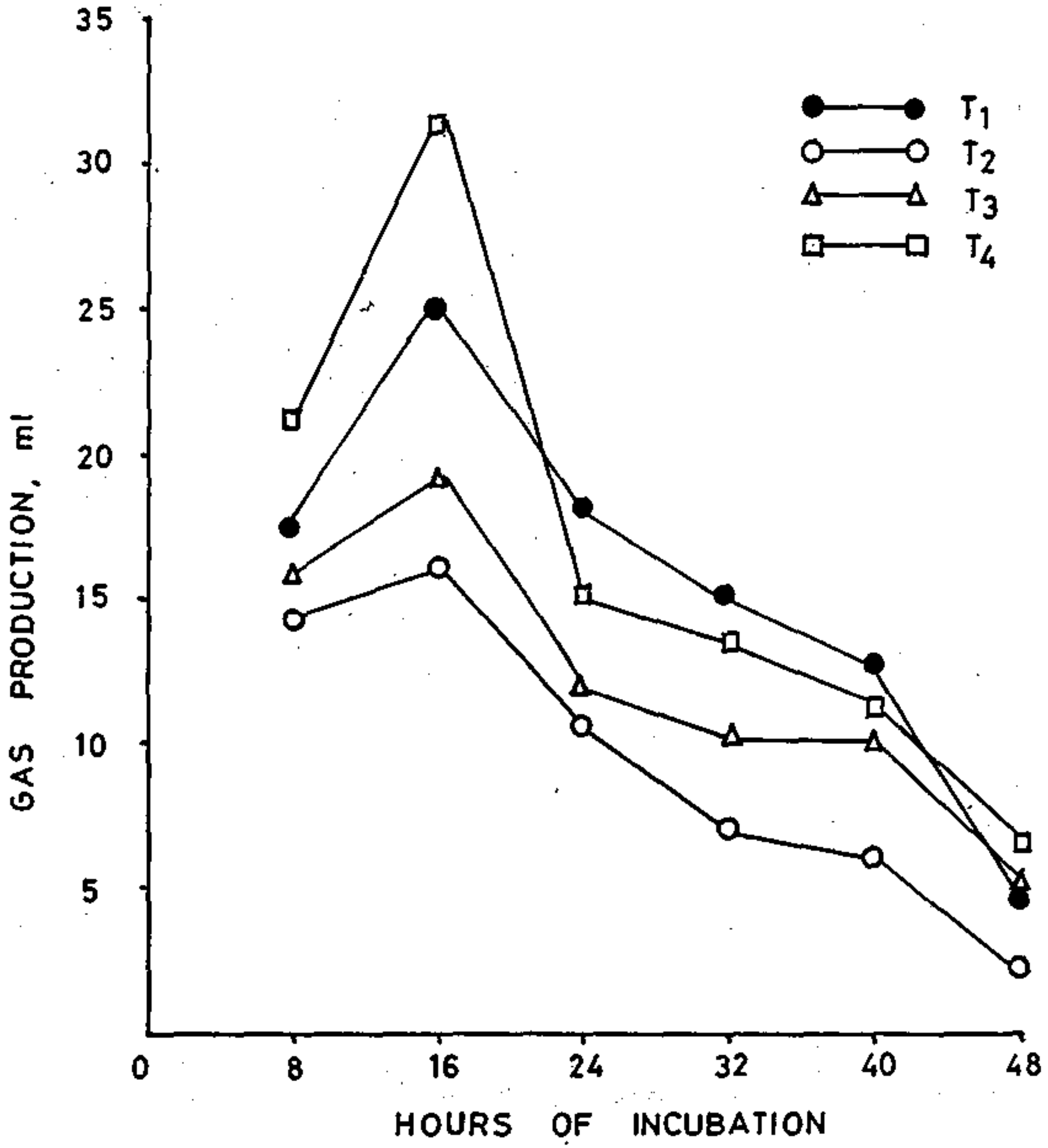
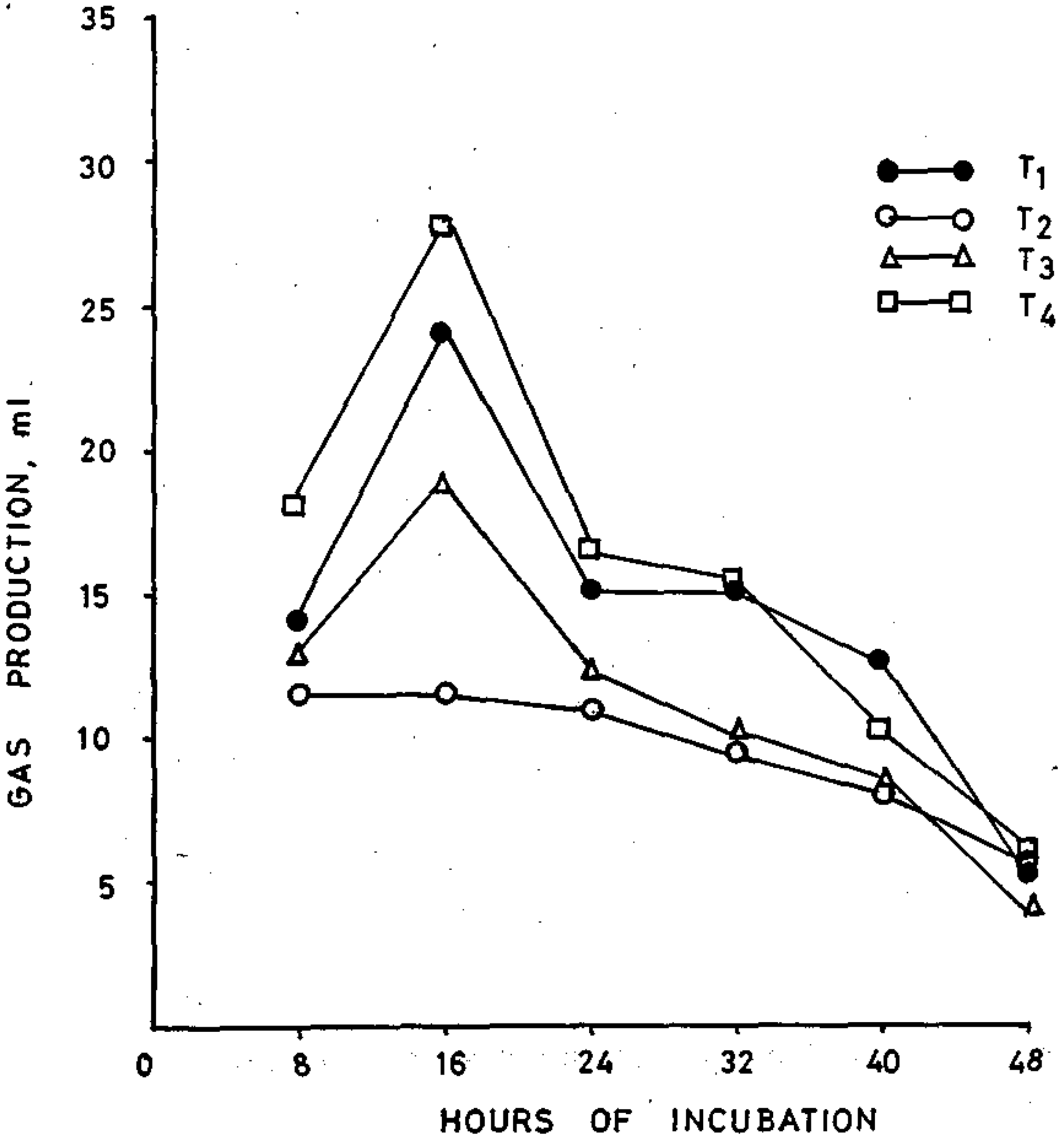

T$_1$
T$_2$
T$_3$
T$_4$
GAS PRODUCTION, ml
35
30
25
20
15
10
5
0
8
16
24
32
40
48
HOURS OF INCUBATION

Fig.4.10 *In vitro* gas production by different types of inoculum (cattle) at different hours of incubation

T1 = Mixed inoculum (bacteria + fungi)
T2 = Fungi only
T3 = Bacteria only
T4 = Mixed inoculum + *Piromyces* sp. (B3 isolate)



35
30
25
20
15
10
5
0
8
16
24
32
40
48
GAS PRODUCTION, ml
HOURS OF INCUBATION
T_1
T_2
T_3
T_4

Table 4.13 Role of different groups of organisms on rumen parameters under *in vitro* system in buffalo

Sl. No.	*Rumen parameters*	*Mixed rumen inoculum (T1)*	*Fungi only (T2)*	*Bacteria only (T3)*	*Mixed rumen inoculum + fungal isolate (T4)*	*CD*
1.	Total gas production** (ml)	93.33^{a} ± 3.52	57.00^{b} ± 1.15	73.30^{c} ± 0.88	99.33^{a} ± 2.90	16.2095
2.	IVDMD** (%)	36.33^{a} ± 0.78	23.93^{b} ± 0.52	28.93^{c} ± 0.69	38.56^{a} ± 0.58	4.3093
3.	IVOMD** (%)	53.96^{a} ± 0.12	41.73^{b} ± 0.84	46.86^{c} ± 0.14	53.76^{a} ± 0.67	4.1910
4.	TVFA concentration** (mM/100 ml SRL)	19.33^{a} ± 0.66	10.33^{b} ± 0.33	15.33^{c} ± 0.33	20.66^{a} ± 0.88	4.0369
5.	Molar proportion of acetate**(%)	64.03^{a} ± 1.24	80.96^{b} ± 0.82	61.50^{a} ± 1.05	60.13^{a} ± 1.21	7.7648
6.	Molar proportion of propionate** (%)	23.56^{a} ± 0.72	10.23^{b} ± 0.60	24.90^{a} ± 1.60	27.33^{a} ± 1.49	8.1948
7.	Molar proportion of butyrate(%)	12.40 ± 0.62	8.80 ± 0.34	13.60 ± 0.72	12.53 ± 0.98	NS

* $P < 0.05$; ** $P < 0.01$; NS = Non-significant; ± S.E.

Table 4.14 Role of different groups of organisms on rumen parameters under *in vitro* system in cattle

Sl. No.	*Rumen parameters*	*Mixed rumen inoculum (T1)*	*Fungi only (T2)*	*Bacteria only (T3)*	*Mixed rumen inoculum + fungal isolate (T4)*	*CD*
1.	Total gas production** (ml)	86.00^{a} ± 1.52	57.66^{b} ± 0.66	67.00^{c} ± 1.15	92.00^{a} ± 0.57	7.4369
2.	IVDMD** (%)	30.60^{a} ± 0.78	18.73^{b} ± 0.94	25.93^{c} ± 0.57	34.40^{a} ± 0.75	6.8374
3.	IVOMD** (%)	50.76^{a} ± 0.32	38.36^{b} ± 0.61	44.76^{c} ± 0.35	52.60^{a} ± 1.40	5.8572
4.	TVFA concentration** (mM/100 ml SRL)	18.66^{a} ± 0.66	10.66^{b} ± 0.66	15.00^{c} ± 0.57	20.00^{a} ± 0.57	3.9375
5.	Molar proportion of acetate** (%)	60.13^{a} ± 1.21	79.63^{b} ± 0.83	62.03^{a} ± 1.41	63.36^{a} ± 1.63	8.40
6.	Molar proportion of propionate** (%)	26.30^{a} ± 0.43	9.86^{b} ± 0.33	26.63^{a} ± 1.01	24.20^{a} ± 0.75	4.9482
7.	Molar proportion of butyrate (%)	11.36 ± 0.40	9.83 ± 1.13	11.33 ± 0.55	12.43 ± 1.03	NS

* $P < 0.05$; ** $P < 0.01$; NS = Non-significant; ± S.E.

25.93 ± 0.57 (T3) and 34.40 ± 0.75 (T4). Statistical analysis indicated that treatments differed significantly ($P < 0.01$). While T1 and T4 did not differ significantly, T2 and T3 differed significantly. Hence, dry matter degradation was different for bacteria and fungi (Table 4.14), with higher IVDMD per cent recorded for bacteria only than fungi only.

4.22.4 IVOMD (%)

IVOMD (%) for different treatments are presented in Table 4.13. The values in buffalo were 53.96 ± 0.12, 41.73 ± 0.84, 46.86 ± 0.14 and 53.76 ± 0.67 per cent for T1, T2, T3 and T4, respectively. From the statistical analysis, it was found that degradation of organic matter using four types of inoculum was significantly different ($P < 0.01$). But the T1 and T4 did not differ significantly indicating no beneficial effect from added fungal isolate. When source of rumen liquor was cattle, the values (%) were 50.76 ± 0.32, 38.36 ± 0.61, 44.76 ± 0.35 and 52.60 ± 1.40 for T1, T2, T3 and T4, respectively (Table 4.14). Statistical analysis revealed that degradation of straw organic matter using different types of inoculum differed significantly ($P < 0.01$). But there was no significant difference between T1 and T4 indicating no effect of added fungal isolate.

4.23 TOTAL VOLATILE FATTY ACIDS (mM/100 ml SRL)

TVFA for different treatments are presented in Table 4.13. Total volatile fatty acids production (mM/100 ml SRL) in buffalo were 19.33 ± 0.66, 10.33 ± 0.33, 15.33 ± 0.33 and 20.66 ± 0.88 for T1, T2, T3 and T4, respectively. From statistical analysis, it was found that TVFA production was significantly different among the types of inoculum ($P < 0.01$). But T1 and T4 did not differ significantly, indicating no effect of additional fungal inoculum. T2 and T3 also differed significantly. When the source of rumen inoculum was cattle, the values (mM/100 ml SRL) were 18.66 ± 0.66, 10.66 ± 0.66, 15.00 ± 0.57 and 20.00 ± 0.57 for T1, T2, T3 and T4, respectively (Table 4.14). Statistical analysis revealed that the various types of inoculum differed significantly ($P < 0.01$) regarding the production of VFA. But T1 and T4 did not differ significantly.

4.24 MOLAR PROPORTION OF INDIVIDUAL VOLATILE FATTY ACIDS

In case of buffalo rumen liquor, the proportion of individual volatile fatty acids in T1, T2, T3 and T4, respectively were acetic acid, 64.03 ± 1.24, 80.96 ± 0.82, 61.50 ± 1.05 and 60.13 ± 1.21; propionic acid, 23.56 ± 0.72, 10.23 ± 0.60, 24.90 ± 1.60 and 27.33 ± 1.49; butyric acid, 12.40 ± 0.62, 8.80 ± 0.34; 13.60 ± 0.72 and 12.53 ± 0.98 (Table 4.13). From statistical analysis, it was seen that T2 differed significantly ($P < 0.01$) from T1, T3 and T4 with respect to molar proportion of acetate and propionate.

In case of cattle rumen inoculum, the proportions of individual volatile fatty acids in T1, T2, T3 and T4, respectively were acetic acid, 60.13 ± 1.21, 79.63 ± 0.83, 62.03 ± 1.41 and 63.36 ± 1.63; propionic acid, 26.30 ± 0.43, 9.86 ± 0.33, 26.63 ± 1.01 and 24.20 ± 0.75; butyric acid, 11.36 ± 0.40, 9.83 ± 1.13 and 11.33 ± 0.55 and 12.43 ± 1.03 (Table 4.14). Statistical analysis revealed that the inoculum T2 differed significantly ($P < 0.01$) from other three inoculum, i.e., T1, T3 and T4 with respect to molar proportion of acetate and propionate. But molar proportion of butyrate using the four inoculum did not differ significantly.

Chapter 5

Discussion

5. DISCUSSION

While studying the diurnal changes in the concentrations of microorganisms in the rumen of sheep fed limited diets once daily, Warner (1966) showed a significant increase in the population density of the flagellate *Callimastix frontalis* 1 h after the sheep were fed. In 1975, Orpin first established that these flagellates are of fungal origin and challenged the hypothesis of non-existence of anaerobic fungi in nature, as postulated by Foster (1949). But till mid seventies of this century, there does not exist even a single report regarding the rumen anaerobic fungi, though extensive studies about rumen organisms had been initiated from 1950 onwards. According to Bauchop (1983), the failure to recognize the anaerobic fungi could be attributed not only to mycological dogma, but also to the practice among rumen microbiologists, of working with strained rumen fluid and discarding the solid digesta, with which the vegetative stage of the fungus is associated. Once Orpin in 1975 brought to the light the existence of anaerobic fungi in rumen, it was readily accepted by the scientists, especially rumen microbiologists and it is a reality now. Today, it is known that rumen anaerobic fungi possess a minority status in the rumen eco-club. But the role it plays in fibre degradation in rumen is significant. The presence of fungi in rumen and the role it plays has generated lot of interest among the scientists (animal nutritionists, rumen microbiologists, biochemists and biotechnologists). A considerable work is going on in many laboratories in the world on the different aspects of rumen anaerobic fungi. In the present study, the interest was mainly focussed on anaerobic fungi from buffalo rumen, its characteristics, biochemistry and the role it plays in fibre degradation. The on-going text discusses the results obtained from the present study.

5.1 ENUMERATION OF FUNGAL POPULATION

Fungal population was enumerated by counting the colonies which appeared in roll tube following incubation at 39°C. Actually, on the agar surface, zoospores can not migrate, however, they mature to become vegetative sporangia. After bursting of sporangia, several zoospores are released which mature again and form visible colonies. These colonies are counted.

In case of buffalo, fungal population in rumen was found to be more in T3 animal (roughage:concentrate::100:0) all time, compared to either T1 animal (wheat straw:concentrate::50:50) or T2 animal (oat fodder:concentrate::50:50). From Table 4.1, it could be realised that fungal populations were more at 1 h after feeding, 2.42 x 10^3/ml, 2.56 x 10^3/ml and 5.41 x 10^3/ml in T1, T2 and T3 animal, respectively, and gradually declined to reach a level of 1.21 x 10^3/ml, 1.36 x 10^3/ml and 2.51 x 10^3/ml for T1, T2 and T3 animal, respectively at 4 h after feeding. Similarly, in cattle rumen also, the fungal population was always more in T3 animal (roughage:concentrate::100:0) as compared to either T1 animal (wheat straw:concentrate::50:50) or T2 animal (oat fodder:concentrate::50:50). From the Table 4.2, it could be seen that the fungal population were more at 1 h after feeding, 2.57 x 10^3/ml, 2.67 x 10^3/ml and 5.14 x 10^3/ml in T1, T2 and T3 animal, respectively and gradually declined to reach a level of 1.39 x 10^3/ml, 1.34 x 10^3/ml and 2.43 x 10^3/ml for T1, T2 and T3 animal, respectively at 4 h after feeding. The population density of rumen anaerobic fungi is usually in the range of 4 x 10^2 to 10^5/ml, fluctuates greatly and rapidly over the feeding period of the host animal (Orpin, 1975; Akin, 1987; Ushida *et al.,* 1989). Theodorou *et al.* (1990a) enumerated fungal population from steers as cell wall degrading thallus forming unit (TFU). Population ranged from 2.20 x 10^4 to 28.6 x 10^4 TFU/g of rumen dry matter. The number of *Neocallimastix* sp. zoospores in the rumen of cows during first 3 h after feeding with hay-silage-concentrate diets varied from 7 x 10^3 to 5.4 x 10^5/ml; the number of uniflagellated zoospores varied from 10^4 to 10^5/ml (Kostyukovsky *et al.*, 1991). But in the experimental animals of present study, fungal population was comparatively lower. This may either be due to the lower population of fungi within rumen itself or due to less release of zoospore or sporangia from fibre particles during stirring of rumen liquor. Fungi are supposed to be attached with fibre particles through extensive rhizomycelia (Bauchop, 1979). In case of animals receiving either T1 or T2 diet, fungal population were lower due to the type of diet on which they were maintained. Higher population in animal receiving T3 diet was due to 100 per cent roughage, that too 50 per cent from wheat straw and 50 per cent from oat fodder. Large number of fungal population were detected in animal maintained on high fibrous diet compared to low fibrous or pelleted diet (Bauchop, 1979). This is due to the fact that fungi need solid support for their growth and thus invade the fibrous materials. High concentrate diet or easily digestible nutrients can not provide that sort of solid support for a longer time for growth and maturity of fungus. Consequently, the high concentrate diet can not support higher fungal population. The maximum fungal population was observed at 15-30 minutes after feeding (Orpin, 1974). In the present study, the highest fungal population was observed after 1 h of feeding. The maximum number of fungi found after feeding is the result of stimulus received from soluble nutrients of the ingested materials. The soluble chemicals released from the fibrous feeds induce the sporangia to burst out, releasing spores which then move towards the fibre particles (Orpin, 1975).

5.2 ENUMERATION OF FUNGAL POPULATION IN FECES

Anaerobic fungi identical in appearance to the rumen fungi have been reported to be present in the feces of Ethiopian Zebu cattle, gaur, bacterian camel, antelope, Arabian oryx, Ethiopian fat tailed sheep, bongo, vicuna, mara, Asian elephant, Zebra (Milne *et al.*, 1989), blue duiker (Dehority and Varga, 1991), llama (Marvin-Sikkema *et al.*, 1992), Przewalski's horse, nilgai, axis deer, wapiti onager (Lawrence, 1993), yak (Sijtsma and Tan, 1993).

During the present study , an effort was also made to count and isolate the anaerobic fungus from the fecal samples of cattle and riverine buffalo. In reverine buffalo, anaerobic fungal population ranged from 6.5×10^{3} to 6.8×10^{3} per gram of fecal dry matter, while in the case of cattle, the values ranged from 5.3×10^{3} to 5.6×10^{3} per gram of fecal dry matter. Reports by other workers (Theodorou *et al.*, 1990a; Davies *et al.*, 1993) suggest that the population size of anaerobic fungi in feces can be equivalent to that of rumen digesta (at 10^{4} to 10^{5} thallus forming unit per gram fecal dry matter) and tend to decline slowly after drying. Present finding is the first report on the presence of anaerobic fungus in the feces of riverine buffalo (Samanta *et al.*, 1996). In the caecum, fibrous materials (escaping rumen degradation) are supposed to be digested by bacteria only. Our studies established that in riverine buffalo, anaerobic fungi also contribute in fibre digestion at caecum level in addition to bacteria.

5.3 TYPES OF COLONY

Based on the appearance of colony on cellobiose agar surface, three types of colonies were observed, viz., type A, type B and type C. In case of type A colony, three zones were visible; the first was due to the aggregation of sporangia, the second was due to the growth of thick rhizomycelia and third one resulted from the finer branching of mycelia. Fungus of type A was monocentric nature. Type B colonies were completely transparent due to either having extensively spread or less developed rhizomycelia. Fungus of these colonies were polycentric in nature. Type C colonies were of monocentric fungus. Akin and Rigsby (1987) classified the colonies on cellobiose agar into three categories. In their case, type A colony was due to prominent rhizomycelia without sporangia. At 96 h and later, these colonies had developed both oval and small globose sporangia in the rhizomycelia. Type B colonies revealed sporangia with a thick wall, packets with multiple flagella and rhizoidal structures devoid of cytoplasm, indicating a fungus of different nature of growth rate from type A. Type D differed from type A and B by having a central cluster of round bodies, radiating rhizoids and much reduced rhizomycelia.

5.4 CHARACTERISTICS OF FUNGAL ISOLATES

The different isolates were identified based on zoospore morphology, development of sporangia (one or more), nature of rhizomycelia (Theodorou *et al.*, 1996). On perusal of Table 5.1, it can be seen that both polycentric and monocentric fungi are present in the rumen ecosystem of crossbred cattle and reverine buffalo. Out of 17 anaerobic fungal isolates, nine were classified as polycentric and eight were monocentric, based on the number of sporangia developed from thallus. Trinci *et al.* (1994) also broadly classified the anaerobic fungus into two categories (monocentric and polycentric) based on the number of sporangia developed from the thallus. Similarly, based on number of flagella present in zoospore, isolate C5, Sm1, Sm2, B1, B2, B3 and B4 were identified as *Piromyces* sp. , as all these isolates possessed single flagella. The sporangia of these isolates were circular and possessed unsegmented rhizomycelia. The isolates from sheep, horse, elephant, deer and goat are classified as *Piromyces* genus, based on monocentric nature of growth, uniflagellated zoospores,

Table 5.1 Characteristics of different fungal isolates

Isolate	*Source*	*Nature of growth*	*Shape of sporangia*	*Rhizomycelia*	*Presence of stalk at the base of sporangia*	*Zoospore flagellation*	*Gas production*	*Endoglucanase activity*	*Name of the species*
C1	Cattle	Polycentric	Circular	Unsegmented	Present	Monoflagellated	++++	High	*Anaeromyces*
C2	Cattle	Polycentric	Circular	Segmented	Absent	Monoflagellated	+++	Low	*Anaeromyces*
C3	Cattle	Monocentric	Circular	Unsegmented	Absent	Polyflagellated	++++	High	*Neocallimastix*
C4	Cattle	Polycentric	Circular	Unsegmented	Absent	Polyflagellated	+++	Nil	*Anaeromyces*
C5	Cattle	Monocentric	Circular	Unsegmented	Absent	Monoflagellated	+	Nil	*Piromyces*
Sm1	Cattle	Monocentric	Circular	Unsegmented	Absent	Monoflagellated	++++	Low	*Piromyces*
Sm2	Cattle	Monocentric	Circular	Unsegmented	Present	Monoflagellated	++++	Low	*Piromyces*
B1	Buffalo	Monocentric	Circular	Segmented	Absent	Monoflagellated	++	Nil	*Piromyces*
B2	Buffalo	Monocentric	Oval	Unsegmented	Present	Monoflagellated	++	Low	*Piromyces*
B3	Buffalo	Monocentric	Circular	Unsegmented	Absent	Monoflagellated	++++	High	*Piromyces*
B4	Buffalo	Monocentric	Circular	Unsegmented	Absent	Monoflagellated	++++	Nil	*Piromyces*
1	Buffalo	Polycentric	Oval	Unsegmented	Present	Polyflagellated	++	High	*Orpinomyces*
3	Buffalo	Polycentric	Circular	Unsegmented	Absent	Polyflagellated	++	Nil	*Orpinomyces*
4	Buffalo	Polycentric	Circular	Unsegmented	Absent	Monoflagellated	+	Low	*Anaeromyces*
6	Buffalo	Polycentric	Circular	Unsegmented	Absent	Monoflagellated	+++	Nil	*Anaeromyces*
8	Buffalo	Polycentric	Circular	Unsegmented	Absent	Monoflagellated	+++	High	*Anaeromyces*
11	Buffalo	Polycentric	Circular	Unsegmented	Absent	Polyflagellated	+	Low	*Orpinomyces*
BF3	Buffalo	Monocentric	Circular	Unsegmented	Absent	Polyflagellated	Not detected	Not detected	*Neocallimastix*

+, negligible; ++, little; +++, moderate; ++++, profused extensive and filamentous rhizomycelia (Orpin, 1977b; Gold *et al.*, 1988; Li *et al.*, 1990; Ho *et al.*, 1993a).

The isolate C3 (from cattle) was *Neocallimastix* sp. Its monocentric nature of growth and presence of numerous flagella in zoospore determined its classification under the genus *Neocallimastix*. Based on zoospore morphology, monocentric nature and extensive rhizomycelia several workers also classified their isolate as *Neocallimastix* (Heath *et al.*, 1983; Orpin and Munn, 1986; Ho *et al.*, 1993b). The fecal isolate (BF3) from buffalo also belonged to *Neocallimastix* sp. due to its monocentric nature, polyflagellation of zoospore and extensively branched rhizomycelia. Marvin-Sikkema (1992) also classified their one of the fecal isolates as *Neocallimastix* L2 based on morphological features.

Isolate C1, C2, C4, 4, 6 and 8 were classified as *Anaeromyces* sp. due to polycentric growth, monoflagellated zoospore. Breton *et al.* (1990) and Ho *et al.* (1993d) used the monoflagellation of zoospore and polycentric nature for classifying their isolate as *Anaeromyces* sp. Isolate 1, 3 and 11 belonged to *Orpinomyces* sp. as their zoospores were polyflagellated and sporangia possessed branched rhizomycelia. Beoton *et al.* (1989) used polyflagellation of zoospore for classification of their isolate.

5.5 METABOLIC PRODUCTS OF ANAEROBIC FUNGUS

The gas production by different isolates was visually judged (Table 4.3) and the acetate production was determined in GLC (Table 4.4). There was no increase in peak height of propionate as compared to control, where media was not inoculated with anaerobic fungus. This indicated that anaerobic fungi did not produce propionate as well as butyrate. The fermentation end products of anaerobic fungus can be influenced by a variety of cellular and environmental factors, including the presence of other microorganisms. In place of mitochondria, anaerobic fungi possess hydrogenosomes which are capable of coupling the metabolism of hexose to cellular energy formation with the concomitant generation of hydrogen (Yarlett *et al.*, 1986b). Thus, they convert hexose to formate, acetate, lactate, ethanol, carbon dioxide and hydrogen (Bauchop and Mountfort, 1981; Lowe *et al.*, 1987b; Phillips and Gordon, 1988; Borneman *et al.*, 1989). When cultured in the presence of methanogenic bacteria, the fermentation profile is shifted away from electron sink products, such as ethanol and lactate, towards the more reduced products, such as acetate and formate (Bauchop and Mountfort, 1981).

5.6 NUMBER OF ZOOSPORES RELEASED PER SPORANGIUM

It was observed that about 15 to 18 zoospores were released from single sporangium. *Neocallimastix* sporangium can release 2-38 zoopspores under *in vitro* conditions (Orpin, 1975).

5.7 ATTACHMENT OF STRAW WITH ANAEROBIC FUNGI

Both, under *in vitro* and *in vivo* conditions anaerobic fungi were observed to attach and penetrate wheat straw. Orpin (1977a) provided the first account of plant cell wall degradation by anaerobic fungi, by demonstrating their close association with plant biomass with the help of ^{14}C uptake from barley awns. Anaerobic fungi penetrate and preferentially colonize tissues traditionally regarded as resistant to degradation, such as recalcitrant sclerenchyma and vascular tissues (Akin *et al.*, 1989; Borneman and Akin, 1990). They can even colonize highly recalcitrant plant materials, such as palm press fiber and wood (Ho *et al.*, 1991; Joblin and Naylor, 1993). Wheat straw, rice straw, maize stalk, soyabean hulls and grasses are also reported to be colonized by anaerobic fungi (Akin *et al.*, 1983,1989; Lowe *et al.*, 1987c; Grenet and Barry, 1988; Theodorou *et al.*, 1989; Ho *et al.*, 1991; Roger *et al.*, 1992).

5.8 AMINO ACID COMPOSITION OF B3 ISOLATE

On perusal of Table 4.5, it is crystal clear that fungal isolate is rich in arginine (11.90 g/100 g total amino acids) and serine (11.87 g/100 g total amino acids). If we consider methionine and cystine, quantity was 4.46 g/100 g total amino acids. Lysine, isoleucine, tyrosine, proline, histidine, glutamic and apartic were also present at moderate level. The amino acid composition of the isolate can be compared with an important cellulolytic bacteria, i.e., *Ruminococcus albus* (Purser and Buechler, 1966), for which the values (g/100 g total amino acids) are 4.4, 6.3, 1.3, 7.5, 7.1, 5.2, 10.9, 2.5, 9.6, 3.6, 10.7, 3.6, 8.0, 5.9, 1.7, 3.8, 0.6 and 5.6 for threonine, valine, methionine, isoleucine, leucine, phenylalaline, lysine, histidine, aspartic, serine, glutamine, proline, glycine, alanine, cystine, tyrosine, diaminopimelic and arginine, respectively. The values (g/100 g total amino acids) for another important cellulolytic bacteria *Bacteroides succinogenes* are 6.4, 4.7, 2.9, 5.7, 7.9, 5.0, 9.7, 2.3, 11.1, 3.6, 11.6, 5.0, 5.5, 6.1, 1.4, 4.5 and 6.3 for threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, aspartic, serine, glutamic, proline, glycine, alanine, cystine, tyrosine and arginine, respectively (Purser and Buechler, 1966). In the case of methionine, the quantity is more than *R. albus* and *B. succinogenes* (3.81 vs. 1.3 and 2.9). Regarding the content of cystine, it is lower in B3 isolate than in either *R. albus* or *B. succinogenes* (0.65 vs. 1.7 or 1.4, respectively). Methionine and lysine are reported to be most limiting amino acids for growing steers (Richardson and Hatfield, 1978). Thus, anaerobic fungi are playing a vital role by providing these two limiting amino acids to meet their tissue requirements. Purser and Buecheler (1966) did a comparison between the amino acid composition of rumen bacteria (mixed) and protozoa (mixed), the content (g/100 g total amino acid) of three amino acids were 9.3 and 10.1 for lysine, 2.6 and 2.2 for methionine, 1.0 and 1.0 for cystine, respectively. Thus, in the isolate (B3), the content of methionine (3.81 g 1/00 g total amino acids) was higher as compared to either rumen bacteria and protozoa.

The principal source of amino acids of ingesta, leaving the rumen and entering the alimentary tract, are from bacterial, protozoal and undigested food proteins. The relative proportions, together with the amino acid composition of each of these fractions, are the principal factors influencing the distribution of amino acids at tissue level (Purser and Buchler, 1966). But the present studies point out that anaerobic rumen fungi also may govern the tissue availability and distribution of major amino acids in the host animal when the microbial protein production is limited or when amino acid requirements are high, especially in high yielding animals where ruminally produced microbial protein may not met the amino acid need of the host animal (Merchen and Titgemeyer, 1992). Under these circumstances, if the ration is formulated judiciously which is capable of maintaining a fungal population towards higher side, the adverse situation arising from the lower level limiting amino acids can be easily overcome.

5.9 ENDOGLUCANASE ACTIVITY OF DIFFERENT ISOLATES

Based on congo red assay and quantitative assay (Table 4.6), the isolate C1, C3, B3, 1 and 8 were found to have higher endoglucanase activity, whereas the isolate C2, Sm1, Sm2, B2, 4 and 11 had lower endoglucanase activity. However, the isolate C4, C5, B1, B4, 3 and 6 did not show any endoglucanase activity at all. Relatively few microorganisms produce enzymes capable of degrading the crystalline, hydrogen bonded, highly ordered polymer of cellulose. Among them important are *Trichoderma* sp., *Fusarium solani, Clostridium thermocellum, anerobic fungi* (Wood and Garcia-Campayo, 1990; Wood, 1991; Wilson and Wood, 1992). In the present study, only 11 out of the total of 17 isolates had endoglucanase activity, suggesting thereby that not all the strains of the rumen fungi are able to produce the endoglucanase enzyme for the degradation of dietary cellulose.

5.10 OPTIMUM TIME FOR HARVESTING ENZYME

It was observed that maximum endoglucanase secretion by isolate B3 happened at 96 hours after inoculation (Table 4.7). Barichievich and Calza (1990) and Gomez De Segura and Fevre (1993) found the maximum cellulase and xylanase activity by the rumen fungi after 3 to 4 days of inoculation. In the present studies, similar trend was observed, and the enzyme activity was found to decline afterwards.

5.11 EFFECT OF SONICATION ON THE RELEASE OF ENDOGLUCANASE FROM FUNGAL CELL

Location of the enzymes appears to be strain dependent (Sijtsma and Tan, 1993). In the present studies, sonication of fungal biomass, viz. sporangia and rhizomycelia does not release any endoglucanase and xylanase enzyme, indicating that the isolate B3 produces these enzymes extracellularly. Mountfort and Asher (1985) observed that sonication of fungal tissue (*N. frontalis*) in the absence or presence of 1 to 10 per cent (vol/vol) Tween 80 or TET, also failed to release endoglucanase from the cells. When *N. frontalis* is grown on xylan, xylanase was found to be present mainly in the culture fluid, and only 10 per cent of the activity was found to be associated with fungal rhizoid (Mountfort and Asher, 1989). In contrast, Lowe *et al.* (1987a) found that no cellulase and only 15 per cent of the xylanase activity, produced by *Neocallimastix* R1 were extracellular, implying thereby that the enzyme was mainly located intracellularly.

5.12 DIFFERENT HYDROLYTIC ENZYMES OF B3 ISOLATE

In the present studies, it was observed that B3 isolate had the following enzyme activities: endoglucanase (16.319 mIU/ml), cellobiase (19.3737 mIU/ml), very high level of xylanase (237.613 mIU/ml) and very low level of avicelase (0.4629 mIU/ml). Sijtsma and Tan (1993) reported that *Piromyces* CS1 isolate secreted the four enzymes viz. ß-1,4-glucosidase (21 mIU/ml), ß-xylosidase (58 mIU/ml), xylanase (840 mIU/ml) and endoglucanase (116 mIU/ml). Compared to the enzyme activities obtained by the above workers from *N. frontalis* with regard to the different enzymes, the enzyme activities obtained from isolate B3, in the present case were much lower.

5.13 EFFECT OF pH ON DIFFERENT ENZYME ACTIVITIES

The optimum pH for different enzymes were 6.5 for endoglucanase (Fig. 4.3), 6.0 to 6.5 for cellobiase (Fig. 4.4) and 6.5 for xylanase (Fig. 4.5). These values were close to physiological pH range of rumen and allowed to accrue maximum benefit of these hydrolytic enzymes for the degradation of plant biomass. Mountfort and Asher (1989) reported that pH optima for endoglucanase is 6.0 and for xyalanase it is 5.5.

5.14 PARTIAL PURIFICATION OF ENDOGLUCANASE

Purification and fractionation of enzymes are cumbersome due to aggregation of several hydrolytic enzymes. The presence of extracellular complexes of cellulolytic and other hydrolytic enzymes in the culture fluid of anaerobic fungus has been reported for *Neocallimastix* as well as for *Piromyces* sp. (Wilson and Wood, 1992; Teunissen *et al.*, 1993b). In the present study, the ammonium sulfate (60%) precipitation yielded only 58.21 per cent of endoglucanase activity with the improvement of specific activity by 2.08 fold only.

Attempt to partially purify and fractionate through DEAE sepharose column chromatography, hydroxylapatite column chromatography and superose 12 gel filtration (connected with FPLC) was not much encouraging due to unusual binding of the enzyme protein with DEAE sepharose gel and the low yield through latter two steps. Gomez De Segura and Fevre (1993) also observed a significant loss in enzyme activity during anion exchange chromatography. However, Li and Calza (1991a) observed 71.2 per cent recovery of endoglucanase, 76 per cent of avicelase and 85 per cent of ß-1, 4-glucosidase through the hydroxylapatite column chromatography.

Most of the microorganisms produce isoenzymes. Differences in enzyme activities found after growth on the different substrates could be the result of variations in isoenzymes synthesized. Several forms of isoenzymes for endoglucanase from anaerobic fungi were separated by gel filtration chromatography, native polyacrylamide gel electrophoresis and isoelectric focussing (Wood *et al.*, 1986; Barichevich and Calza, 1990). The multiple forms of endoglucanase might represent distinct enzymes or proteolytic degradative products or those resulted from postranslational modifications including glycosylation. By using hydroxylapatite column chromatography, Li and Calza (1991a) separated seven well defined endoglucanase peaks, having specific activities ranging from 0.77 to 15.66 IU/mg protein. Through the use of mono-Q column (FPLC), it was possible in the present study, to separate four well defined peaks, having specific activities ranging from 0.2264 to 0.5761 IU/mg protein indicating 1.9 to 4.87 fold of purification (Table 4.9).

On SDS-PAGE zymogram, four yellow bands were visualized for endoglucanase enzyme after congo red assay. The molecular weight of these bands were 100 kDa, 66 kDa, 39.8 kDa and 33 kDa. The endoglucanase isoenzymes fractionated by Li and Calza (1991a) had a molecular weight of 29 kDa, 95 kDa, 125 kDa, 20 kDa, 50 kDa and 120 kDa for C1, C2, C3, C4, C5, C6 and C7 peak, respectively.

5.15 PARTIAL PURIFICATION OF XYLANASE

The culture supernatant having xylanase activity 237.618 mIU/ml and specific activity 1.728 IU/mg protein was used for partial purification. Ammonium sulfate precipitation yielded only 47.1 per cent of xylanase activity with an increase in specific activity by 1.68 fold. This indicated that a substantial amount of activity was lost in the supernatant during ammonium sulfate precipitation. Gomez De Segura and Fevre (1993) obtained 78.5 per cent yield of xylanase activity and 1.2 fold purification through ammonium sulfate precipitation.

- Attempt to fractionate xylanase enzyme by mono-Q column (FPLC) permitted only 23 per cent recovery through two well defined peaks, viz., X1 and X2 (Fig. 3.8). The specific activity of X1 peak was 58.308 IU/mg protein indicating a whopping increase in purification by 128.8 fold. While in case of X2 peak (7.7451 IU/mg protein), the purification increased by 17 fold only. Gomez De Segura and Fevre (1993) were able to increase the purification by 13 fold for xylanase II and by 1.5 fold for xylanase I. The molecular weight of these fractions were 45 kDa and 70 kDa for xylanase I and II, respectively. But in the present investigation, four yellow bands were detected on SDS-PAGE zymogram after congo red assay. The molecular weight of these activity bands were 177 kDa, 100 kDa, 63 kDa and 50 kDa. Therefore, on SDS-PAGE zymogram, it was found that the two proteins (100 kDa and 63 kDa) exhibited both endoglucanase as well as xylanase activity. This might be due to the presence of common catalytic domain, capable of triggering both endoglucanase and xylanase activity.

5.16 *IN VITRO* STUDIES

5.16.1 *IN VITRO* GAS PRODUCTION FROM STRAW

In vitro gas production using rumen liquor containing mixed rumen organisms (T1), rumen liquor containing fungi alone (T2) and rumen liquor containing bacteria alone (T3) were significantly different in both buffalo and cattle. No difference was noticed between T1 and T4 (whole rumen liquor containing mixed organism plus fungal isolate). This indicated that there was no additional significant effect on gas production due to addition of the fungal culture (B3 isolate). The *in vitro* gas production from straw (0.5 g) can vary from 28.25 to 44.75 ml within 24 hours of fermentation (Sirohi, 1993). The gases are produced during the fermentation of substrates. The total volume of gas production depends upon the nature of the substrate, *in vitro* conditions, source of inoculum etc. If any one of these factors is changed, the type of fermentation is altered leading to differences in gas production. In the present investigation, higher values for total gas production were obtained in T1 and T4 in both the species, i.e., cattle and buffalo.

Though the gas production in rumen is directly related to fermentation of the substrates, it is more related to fibre degradation rather than the fermentation of soluble sugars. However, the lower gas production in T2 (fungi only) and T3 (bacteria only) reveals that these organisms, when used in isolation, and not as mixed culture of all the members of the rumen ecosystem, fail to degrade the plant fibre substantially.

5.16.2 IN VITRO DRY MATTER DIGESTIBILITY (IVDMD %) OF STRAW

On perusal of Table 4.13, it was found that IVDMD per cent was slightly higher with buffalo rumen liquor as compared to cattle. Digestibility of dry matter and other nutrients can vary between buffalo and cattle (Bhatia *et al.*, 1979; Parthasarthy, 1980; Sangwan *et al.*, 1987). Ichhponani and Sidhu (1966) found higher DM digestibility in buffalo while Walli and Mudgal (1978) did not find any significant difference in the DM digestibility of the two species.

The addition of antibiotics against either bacteria (T2) or fungi (T3) caused significant reduction of DM digestibility. The reduction was more prominent while using the antibiotics against bacteria. This suggests that the presence of bacteria is more essential for the overall dry matter digestibility of the feed compared to fungi. While using antibiotics either against bacteria or fungi, the dry matter degradation of coastal bermuda grass and alfalfa was lower for fungi, but the bacteria were most active (Windham and Akin, 1984) as far as the DM degradation was concerned.

Using the antibiotics against fungi and bacteria in an *in vitro* rumen system, Malakar and Walli (1995) observed that rumen fungi alone were less active in degrading plant biomass in both cattle as well as buffalo, as compared to bacteria alone. In the present study, the addition of fungal isolate B3 to the mixed rumen culture did not produce any additional benefit in terms of degrading DM. It is quite intriguing that although the rumen anaerobic fungi has a well developed enzyme system to degrade fibre, the results obtained from the *in vitro* studies do not reflect that trend.

5.16.3 IN VITRO ORGANIC MATTER DIGESTIBILITY OF STRAW

The average IVOMD values (%) for buffalo and cattle were 53.96 ± 0.12 and 50.76 ± 0.32 for mixed rumen culture (bacteria plus fungi). There was no significant difference in organic matter digestibility between buffalo and cattle (Walli and Mudgal, 1982). In present investigation, the treatment groups were found to differ significantly with regard to the degradation of organic matter, both in cattle and buffalo. The value was highest in the system which had mixed rumen inoculum (T1) and lowest in the system containing fungus only (T2). Furthermore, the degradability of organic matter between the treatments containing fungi alone and the bacteria alone, differed significantly ($P < 0.01$). Bacterial population was however, more efficient in degrading organic matter than that of fungi alone in the rumen *in vitro* system (Malakar, 1991). Thus, the presence of bacterial population appears to be more essential in digesting the feed organic matter in the rumen than the presence of fungi alone. The addition of fungal isolate (T4) did not improve the degradation of straw organic matter over the control (T1). However, twice daily dosing of calves, kept in isolation, with a *Neocallimastix* culture, prior to weaning, caused 25 per cent increase in feed intake and 8 per cent increase in weight gain in 6 to 12 weeks (Theodorou *et al.*, 1990b). In this investigation, added fungal isolate (B3) perhaps did not get sufficient time to get re-established in the rumen ecosystem, provided under *in vitro* conditions, and thus failed to enhance the DM/OM digestibility of straw.

5.16.4 TOTAL VOLATILE FATTY ACID CONCENTRATION

From Table 4.13 and 4.14, it could be observed that volatile fatty acid concentration did not differ much between buffalo and cattle (19.33 ± 0.66 vs. 18.66 ± 0.66). The variation between the antibiotic treatments was significant ($P < 0.01$). In case of buffalo, inhibiting the bacterial activity caused a significant reduction in total volatile fatty acids (mM/100 ml SRL) from 19.33 ± 0.66 to 10.33 ± 0.33. However, by suppressing the fungal activity using cyclohexamide, the value reduced 19.33 ±

0.66 to 15.33 ± 0.33 showing that the absence of fungi caused much less reduction than the absence of bacteria. The addition of fungal isolate did not produce any significant enhancement in TVFA production. Malakar and Walli (1995) also observed similar kind of trend while suppressing the activities of bacteria or fungi through selective antibiotics.

Pant and Roy (1970) did not observe any difference in volatile fatty acid production in rumen between buffalo and cattle corroborating with the results obtained in the present investigation. Volatile fatty acids are the end product of substrate degradation in rumen. The general pattern of plant biomass degradation in rumen at particular time is governed by dynamic equilibrium existing between different groups of complex microbial population apart from the other factors like, the type of feed, frequency of feeding, quantity of feed, dietary roughage concentrate ratio, flow rate of digesta and the sampling time. However, in the *in vitro* system, it is mainly the type of substrate and the type of microbial culture which govern the concentration of TVFA. The results of the present investigation are also a pointer in the same direction as the treatment differences were mainly due to the cultural differences.

5.16.5 MOLAR PROPORTION OF INDIVIDUAL VOLATILE FATTY ACIDS

From the Table 4.13 and 4.14, it was quite clear that the acetate proportion was much higher in T2 group (fungi only) from T1, T3 and T4. The trend was similar in both buffalo and cattle. In T2 group, penicillin and streptomycin were added to suppress the activity of bacteria and allowed only fungi to ferment wheat straw. This resulted the higher molar proportion of acetate, being 80.96 ± 0.82 for buffalo and 79.63 ± 0.83 for cattle in contrast to 64.03 ± 1.24 and 60.13 ± 1.21 for buffalo and cattle, respectively in mixed culture (T1). Several reports suggest that fermentation by anaerobic fungi results in conversion of monosaccharides into acetate, lactate, formate, ethanol, carbon dioxide and hydrogen (Bauchop and Mountfort, 1981; Lowe *et al.*, 1987b; Phillips and Gordon, 1988; Borneman *et al.*, 1989). The proportion of acetate in T1, T3 and T4 groups did not differ significantly. Propionate proportion was significantly low in T2 group as compared to T1, T3 and T4 groups. The results of the present *in vitro* studies clearly establish that when the rumen liquor contains only fungi in the incubation system, i.e., when the bacterial population is suppressed through the use of antibiotics, the type of fermentation favours acetate production and inhibits propionate production. The molar proportion of propionate in T1 (presence of bacteria and fungi), T3 (presence of bacteria) and T4 (presence of bacteria, fungi and isolate), did not differ significantly among each other. This also indicated that there was no effect on the molar proportion of propionate after the addition of the fungal isolate. The butyrate proportion remained unaffected by different groups of organisms in the *in vitro* incubation studies.

Looking at the results on gas production, DMD and OMD, TVFA concentrations obtained from the *in vitro* rumen studies, in which one of the treatments, i.e., T4 was as good as a single culture (isolate) of rumen anaerobic fungi used as a probiotic supplement to see whether it enhances the fermentation rate more so the degradation of plant fibre (i.e., wheat straw); it can be concluded that the supplementation of probiotic fungi to the fibre diet did not prove beneficial the way it was contemplated. One reason could be the limitations of the *in vitro* experiments, which are just short term studies. It is just possible that once the fungi is separated from rumen digesta and subjected to various processes during its isolation, including the different kind of growth media used on which it is grown, it may need more than 48 hours (as used in the *in vitro* studies) to get re-established in the rumen ecosystem. That is perhaps the reason that after using the rumen fungi as a probiotic on long term basis, Theodorou *et al.* (1990b) were able to get increased weight gain and feed intake in calves. Thus, long term studies are needed using probiotic fungal isolates from rumen scaled up for growth studies of calves, to re-establish the results obtained by Theodorou *et al.* (1990b).

Chapter 6

Summary and Conclusion

6. SUMMARY AND CONCLUSION

Rumen anaerobic fungi have attracted a considerable attention globally since its discovery in mid-seventies, not only because of their uniqueness among fungal species existing in nature but also because of their ability to attack, penetrate and utilize all categories of plant biomass including highly lignified fibre. Reports about the crucial role played by the anaerobic fungi in the digestion of herbivorous animals provided the source of inspiration to plan and conduct the present investigation, entitled "Studies on anaerobic fungi from cow and buffalo rumen with respect to cellulolytic activity and fibre degradability". Since most of the work reported on anaerobic fungi was deemed fit to extend the studies to buffalo rumen also and simultaneously compare it with cattle rumen.

The whole study was divided into 3 parts. The first part consisted of the *in vivo* studies, in which three fistulated cattle and three fistulated buffaloes were fed 3 dietary combinations, viz. 50 per cent through roughage and 50 per cent through concentrate (T1 and T2) and 100 per cent roughage comprising of green fodder and straw (T3).

The main purpose of this study was to see the effect of diet and time of sampling on anaerobic fungal population in rumen. The studies also included a comparison of cattle and buffalo rumen with respect to anaerobic fungal population.

The second part of the study was the main part of the present investigation involving the isolation of rumen fungi having cellulolytic and xylanolytic activity from the rumen digesta of the fistulated cattle and buffalo used in the first part of the study. Subsequently, the studies included the selection of the promising isolate having potential plant biomass degrading enzyme activity and partial purification of endoglucanase and xylanase.

The third part of the studies concerned the use of the promising rumen fungal isolate as probiotic supplement in the *in vitro* rumen system to see its effect on the enhancement of fibre degradation and VFA production.

The salient achievements of the investigation are summarized as follows:

6.1 FUNGAL POPULATION

6.1.1 POPULATION IN BUFFALO RUMEN

The fungal population in buffalo varied with the type of diet and the time after feeding. The maximum fungal population was found on 100 per cent roughage diet, at 1 h after feeding. The number was 5.41 x 10^3/ml (T3). But when the diet comprised of 50 per cent concentrate and rest 50 per cent either from wheat straw or green fodder, the maximum population was 2.42 x 10^3/ml (T1) or 2.56 x 10^3/ml (T2), respectively. Irrespective of sampling time, the average fungal population for three dietary combinations upto 4 h after feeding were 1.47 x 10^3/ml for T1, 1.60 x 10^3/ml for T2 and 3.17 x 10^3/ml for T3, suggesting the highest average fungal population on 100 per cent roughage diet.

6.1.2 POPULATION IN CATTLE RUMEN

The fungal population in cattle also varied with the diet as well as with the time after feeding. The maximum fungal population in cattle also was found to be on 100 per cent roughage based diet. The value at 1 h after feeding was 5.14 x 10^3/ml on this diet. The maximum population observed at 1 h after feeding in T1 and T2 groups were 2.57 x 10^3/ml and 2.67 x 10^3/ml, respectively. The average fungal population upto 4 h after feeding were 1.67 x 10^3/ml, 1.69 x 10^3/ml and 3.03 x 10^3/ml in T1, T2 and T3, respectively. Thus, the trend in the ruminal fungal population with regard to dietary combination and time of sample was similar in cattle and buffaloes.

6.1.3 POPULATION IN FECAL SAMPLES

The average fungal population in buffalo fecal sample was 6.5 x 10^3 to 6.8 x 10^3/g fecal dry matter, while in the case of cattle, value was 5.3 x 10^3 - 5.6 x 10^3/g fecal dry matter. Thus, the population of anaerobic fungi was higher in fecal matter of cattle and buffalo than its population in the rumen digesta.

6.2 TYPES OF ANAEROBIC FUNGI IN THE RUMEN

Both polycentric and monocentric fungi were observed in the rumen of Indian buffalo and cattle. Out of 17 isolates selected arbitrarily, 9 were polycentric in nature and 8 were monocentric in nature. In polycentric group, several sporangia are found to develop from the same thallus, while in the case of monocentric type, single sporangium develops from the thallus.

Based on the number of flagella in zoospore of the selected fungal isolates, these were classified and accordingly the isolate C3 was *Neocallimastix* sp.; C5, Sm1, Sm2, B1, B2, B3 and B4 were *Piromyces* sp.; C1, C2, C4, 4, 6 and 8 were *Anaeromyces* sp. while isolate number 1, 3 and 11 were *Orpinomyces* sp.

6.3 TYPE OF FUNGUS IN FECAL SAMPLES OF CATTLE AND BUFFALO

The fecal fungal isolate (BF3) of buffalo was classified as *Neocallimastix* sp. The zoospores were monoflagellated and the sporangia were equipped with highly branched rhizomycelia.

6.4 NUMBER OF ZOOSPORES RELEASED PER SPORANGIUM

The number of zoospores released range from 15 to 18 in monocentric fungus.

6.5 INDIVIDUAL VOLATILE FATTY ACIDS PRODUCTION BY THE ISOLATES

All the isolates produced acetate in higher proportion during their growth on cellobiose soft agar which, in fact, it appeared to be the main acid produced by the rumen fungi. The isolates B3 and C3 produced maximum of this acid compared to other isolates.

6.6 AMINO ACID COMPOSITION OF THE SELECTED ISOLATE B3

The selected isolate, i.e., B3 (*Piromyces* sp.) had a moderate level of lysine (7.17 g/100 g total amino acids). Cystine plus methionine level was also at moderate (4.46 g/100 g total amino acids). Thus, the fungal isolate selected, appears to be a good source of limiting amino acids.

6.7 ENDOGLUCANASE ACTIVITY

Out of 17 isolates, five isolates (C1, C3, B3, 1 and 8) had higher endoglucanase activity, six isolates (C2, Sm1, Sm2, B2, 4 and 11) had moderate endoglucanase activity and rest of the six isolates (C4, B1, B4, 3 and 6) did not possess any endoglucanase activity. Thus, the isolate having highest enzyme activity, i.e., B3, was selected for further biochemical and nutritional studies.

6.8 DIFFERENT ENZYME ACTIVITY OF B3 ISOLATE

The culture supernatant of B3 isolate possessed several hydrolytic enzyme activities, viz., endoglucanase, 17.5925 mIU/ml, avicelase, 0.4629 mIU/ml, cellobiase, 19.3737 mIU/ml and xylanase, 237.613 mIU/ml. Sonication of fungal cells failed to release any hydrolytic enzyme, indicating the extracellular nature of enzyme production by the fungal isolate.

6.9 OPTIMUM pH OF DIFFERENT ENZYMES

The pH optima determined for the different hydrolytic enzymes were: 6.5 for endoglucanase, 6.0 to 6.5 for cellobiase and 6.5 for xylanase.

6.10 PARTIAL PURIFICATION OF ENDOGLUCANASE

Sixty per cent ammonium sulfate saturation of enzyme supernatant increased the specific activity from 0.1188 IU/mg protein to 0.3473 IU/mg protein indicating 2.08 fold purification of the enzyme.

DEAE Sepharose unusually caused binding of the enzyme and failed to get any recovery of the enzyme, leaving aside its separation.

Hydroxylapatite column chromatography allowed only 12.12 per cent recovery of activity. The specific activity of endoglucanase was 0.3532 IU/mg protein, indicating 2.97 fold purification of the enzyme.

Purification through Superose-12 gel filtration (FPLC) also failed to give much recovery of enzyme.

Purification through mono-Q column (FPLC) resulted in the recovery of around 50 per cent of total activity of the enzyme. Total of four well defined peaks were obtained. The specific activities were 0.5761 IU/mg protein for C1 peak, 0.4261 IU/mg protein for C2 peak, 0.2264 IU/mg protein for C3 peak and 0.3583 IU/mg protein for C4 peak. The fold of purification ranged from 1.90 to 4.87.

6.11 PARTIAL PURIFICATION OF XYLANASE

Sixty per cent ammonium sulfate saturation improved the specific activity of xylanase from 1.7280 to 2.9147 IU/mg protein, indicating 1.68 fold purification.

On mono-Q column (FPLC), the recovery of activity was 23 per cent. Two well defined peaks were obtained. The specific activities were 58.308 IU/mg protein for X1 peak and 7.7451 IU/mg protein for X2 peak, indicating 128.8 and 17.1 fold purification for X1 and X2 peaks, respectively. Thus, higher fold of purification were obtained for xylanase than for endoglucanase through mono-Q column (FPLC).

6.12 MOLECULAR WEIGHT OF DIFFERENT ISOENZYMES OF ENDOGLUCANASE

Using the SDS-PAGE technique, it was found that endoglucanase existed in four different isozyme forms. The MW of different isozymes (endoglucanase) were 100 kDa, 66 kDa, 39.8 kDa and 33 kDa.

6.13 MOLECULAR WEIGHT OF DIFFERENT ISOENZYMES OF XYLANASE

From zymogram of xylanase, using the same technique as for endoglucanase, it was found that xylanase also existed in four isoforms, having MWs of 177 kDa, 100 kDa, 63 kDa and 50 kDa.

6.14 *IN VITRO* RUMEN STUDIES ON ENHANCEMENT OF DEGRADABILITY BY THE ISOLATE

6.14.1 GAS PRODUCTION

In presence of both bacteria and fungi, total gas production during 48 h incubation *in vitro* was 93.33 ± 3.52 ml and 86.00 ± 1.52 in buffalo and cattle, respectively. Addition of antibiotics (either against bacteria or fungi) significantly reduced the gas production, the reduction being more when bacterial growth was suppressed than when fungal growth was suppressed. No significant effect on gas production was found following addition of B3 isolate in mixed culture (bacteria plus fungi), though apparently the increase was there.

6.14.2 *IN VITRO* DRY MATTER AND ORGANIC MATTER DIGESTIBILITY

In the *in vitro* rumen system, using wheat straw as the substrate, the addition of antibiotics against bacteria or fungi to suppress their activities/growth, resulted in reduction in the dry matter and organic matter digestibility in comparison to the mixed rumen culture (without any antibiotics).

In IVDMD, the reduction was from 36.33 to 23.93 per cent in buffalo and 30.60 to 18.73 per cent in cattle with respect to bacterial suppression. During suppression of fungal activity the IVDMD were 28.93 per cent and 25.93 per cent in buffalo and cattle respectively.

IVOMD also reduced from 53.96 to 41.73 and 46.86 per cent for bacterial and fugal activity suppression respectively in buffalo. In cattle, the values reduced from 50.76 to 38.56 and 44.76 for suppression of bacteria and fungus, respectively.

Addition of the selected fungal isolate B3 to the mixed rumen culture (without antibiotics) did not cause any significant increase in the *in vitro* DM and OM digestibilities of rumen.

6.14.3 TOTAL VOLATILE FATTY ACID CONCENTRATION

Addition of antibiotics against bacteria or fungi resulted in the reduction of TVFA concentration in the *in vitro* rumen system, compared to when mixed rumen culture without any antibiotics was used. The reduction was from 19.33 to 10.33 mM/100 ml SRL in buffaloes and from 18.66 to 10.66 mM/100 ml SRL in cattle with respect to bacterial suppression.

Reduction in TVFA concentration was also observed when antibiotics against fungi was used. The values were 15.33 mM/100 ml SRL for buffalo and 15.00 mM/100 ml SRL for cattle.

Addition of the selected fungal isolate B3 to the mixed rumen culture did not cause any significant increase in the TVFA concentration of the *in vitro* system, using straw as the substrate, though apparently the isolate addition did cause some increase in TVFA concentration.

These trends in TVFA concentrations were similar in cattle and buffaloes and the species difference was non-significant.

6.14.4 MOLAR PROPORTION OF INDIVIDUAL FATTY ACIDS

The molar proportion of acetate, propionate and butyrate were similar when mixed rumen inoculum (bacteria plus fungi), bacteria only and mixed inoculum plus B3 isolate. The trend is similar in both buffalo and cattle. Acetate proportion is significantly higher (around 80%) when wheat straw is fermented only by fungi.

Acetate production was significantly higher (80.96% for buffalo and 79.63% for cattle), when wheat straw was fermented by fungi alone (antibiotics used against bacteria) than when the straw was fermented by bacteria alone (antibiotics used against fungi), the values being 61.50 per cent for buffalo and 62.03 per cent for cattle.

No significant difference was observed in the proportion of acetate, propionate and butyrate when the inoculum used was either mixed rumen culture or mixed rumen culture plus the fungal isolate B3.

The trend in the VFA proportions using different inoculum was similar in cattle and buffalo rumen.

As the fibrolytic saprophytes of graminaceous plants, anaerobic fungi have evolved over millions of years to occupy a unique niche in the gastrointestinal tract of herbivorous animals, where they act as primary colonizer of plant cell walls. Discovery and descriptions of their presence in the digestive tract of ruminants have challenged the traditional belief that fungi require oxygen to grow, and have thus, brought about a reappraisal of accepted theory regarding the digestion of plant biomass by herbivorous mammals. In addition to plant biomass digestion through enzymatic and mechanical means, fungi also serve as better source of amino acids, specially lysine, cystine and methionine as compared to either anaerobic bacteria or protozoa.

Besides their saprophytic role in animals, anaerobic fungi have certain important characteristics which are applicable to industry and agriculture, particularly in relation to the utilization of crops, crop residues and their by-products. The fibrolytic activity of anaerobic fungi is comparable with that of aerobic organism, currently used as a source of cellulase and hemicellulase. The anaerobic fungi are widely distributed throughout the world among the wild and domesticated herbivores. Some of these organisms may have higher fibrolytic activity than the species known today. The enhanced cellulolysis in *Trichoderma reesei* (strain C30) is the consequence of an intensive programme of mutagenesis and selection for hypercellulolytic activity. Similar way, if it is possible to apply selection pressure to anaerobic fungi either through conventional mutation or molecular means, it is quite likely that mutant forms (anaerobic fungi) could be produced with an even greater activity towards cellulosic substrates than the already available wild species. Another important thing is that anaerobic fungi exist in digestive tract in a highly competitive ecosystem alongside other microorganisms which grow at a faster rate. It is suggested that these mini creatures survive in digestive tract, because of their invasive growth habit and consortium of plant biomass degrading enzymes.

The present investigations depict that anaerobic fungi do exist in the rumen of reverine buffalo and the type of fungi and their cellulolytic and xylanolytic enzyme activities are similar with respect to cattle and buffaloes.

Using the anaerobic culture techniques, a promising fungal B3 was isolated from the buffalo rumen and subsequently used for the production of endoglucanase and xylanase enzymes. The enzymes were partially purified through biochemical techniques to achieve the purification of 1.90 to 4.87 fold for endoglucanase and 17.1 and 128.8 fold for two peaks of xylanase enzyme.

When the promising fungal isolate B3 was used as a probiotic in the mixed rumen culture in the *in vitro* system, the increase in DM and OM digestibility and the increase in TVFA and the proportions of individual VFA was non-significant.

It may be concluded that inspite of the fact that a promising rumen fungal isolate exhibiting higher hydrolytic enzyme activities, i.e., endoglucanase and xylanase (the latter being very much higher compared to former), its presence as a probiotic in the *in vitro* system did not produce the desired results of enhancing DM and OM digestion. It is just possible that the fungus could not re-establish itself in the new rumen environment in such a short span of 48 h. Perhaps it needed more time to get re-adapted in the rumen ecosystem. May be long term feeding studies on the growing ruminants (cattle, buffalo, goats) using the probiotic fungi could provide an explanation to this intriguing question.

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