

**IN VITRO BIODEGRADATION OF SUGARCANE
BAGASSE BASED RATIONS USING ZOOSPORES OF
DIFFERENT FUNGI**



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

**MASTER OF VETERINARY SCIENCE
IN
DAIRYING
(ANIMAL NUTRITION)**

**BY
SHELKE SACHIN KARBHARI
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**DIVISION OF DAIRY CATTLE NUTRITION
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R)**

KARNAL - 132001 (HARYANA), INDIA

2007

Regn. No. 2080506

*Dedicated to
My Family
And
My Guide*

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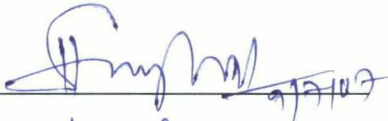
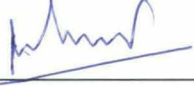
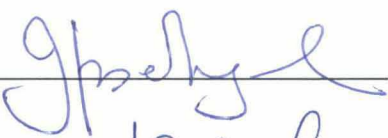

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This is to certify that the thesis entitled, "**IN VITRO BIODEGRADATION OF SUGARCANE BAGASSE BASED RATIONS USING ZOOSPORES OF DIFFERENT FUNGI**" submitted by **SHELKE SACHIN KARBHARI** towards the partial fulfilment of the award of the degree of **MASTER OF VETERINARY SCIENCE** in **DAIRYING (ANIMAL NUTRITION)** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: June 13, 2007


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ACKNOWLEDGEMENTS

First and foremost, I bow my head to thank the Almighty for having bestowed me with all what I needed if not what I wanted and showing me the right path at life's cross roads.

I am extremely grateful and profoundly obliged to my major advisor, **Dr. (Mrs.) Aruna Chhabra**, Principal Scientist, Dairy Cattle Nutrition Division, National Dairy Research Institute, Karnal, for her sagacious guidance, invaluable suggestions, constant encouragement and constructive criticism during the entire course of this study, which enabled me to bring the problem in hand to a successful end. My indebtedness is reserved for her goodwill and patience during the entire period of my research work.

I would like to express my heartfelt thanks to Dr. A.K. Puniya, Senior Scientist, DM Division and Dr. Kishan Singh, Principal Scientist, DM Division, for their expertise, technical guidance, emphatic help, pertinent suggestions in planning and critical amendments made during the entire microbiological study of this work.

My profound admiration and sincere gratefulness is extended to Dr. J. P. Sehgal Principal Scientist, DCN Division; for his encouragement and precious, innovative and remedial suggestions during the course of this study.

I sincerely pay my regards to Dr. K.K. Singhal Head, DCN Division and Dr. S. K. Sirohi, Senior Scientist, DCN Division; for their suggestions and support during the entire study.

I am falling short of words to express my thanks to Mrs. Monica Puniya and Dr. Vinod Sidhu whose cooperation, suggestions, and moral support enabled me in many ways to complete this research work.

I extend my special thanks to Ravinder Nagpal, Malvika and Sanjay sir for their unconditional and timely help while carrying out the present study.

Neither my language nor space is sufficient to express my deepest and warm feelings to my batchmates Anamika, Ashok, Anil, Shalini, Parag, Brishketu, umedsingh and my seniors Mayank, Sanjay, Debanujit, Kadir velan and my friends Sandip, Kapil, vishal whose cooperation and company made my stay at this Institute a pleasurable one.

My warm thanks are expressed to Dr. S. Raju, Mr. V. P. Kalara and help rendered by Mr. Madan and Mr. Meena, during my research work is duly acknowledged. A formal line of appreciation is not sufficient to express my gratitude and indebtedness to them.

Financial assistance in the form of institute Fellowship (NDRI) is duly acknowledged.

Lastly, my deepest feelings of gratitude and love to my Dada, Akka, Balasaheb (brother), Suman (sister) and Sumit, Amit (nephew), Harshada (niece) for their encouragement, silent sufferings and moral support throughout my study.

Date: June 13th 2007


(Shelke Sachin Karbhari)

ABSTRACT

The present study was designed to investigate the influence of administration of zoospores of anaerobic fungi *Neocallimastix sp.* GR1, *Piromyces sp.* WNG12 and *Orpinomyces sp.* C14 on *in vitro* digestibility of sugarcane bagasse and sugarcane bagasse based ration. Sugarcane bagasse based total mixed ration was prepared by mixing sugarcane bagasse, wheat straw, concentrate mixture in the ratio of 20:30:50 having CP 13% and TDN 55%. Ruminal fungal isolates i.e. *Orpinomyces sp.* C-14, *Piromyces sp.* WNG-12 and *Neocalamastix sp.* GR1 were revived using Hungate's roll tube techniques and checked for purity based on their morphological characteristics. Attempt were made to promote the zoosporogenesis, so as to get more number of zoospores/ zoosporangium either using media deficient in essential nutrients like cellobiose or by using complex substrate like sugarcane bagasse instead of cellobiose. However, after 96 h of incubation, normal media contained more number of zoospores (2×10^3 zoospores/ ml) of *Neocallimastix sp.* GR-1 and *Piromyces sp.* WNG-12 as compared to that of media containing deficient/ complex substrates (0.8×10^3 to 1.3×10^3 zoospores/ ml) and *Orpinomyces sp.* C-14 (0.7×10^3 zoospores/ ml). Hence, the standard media was used for the growth of selected ruminal fungi in order to obtain the maximum number of zoospores and for *in vitro* digestibility of sugarcane bagasse (SB).

Neocallimastix GR1 and *Piromyces* WNG12, increased *in vitro* digestibility of DM, NDF, and ADF of SB and SB based rations significantly ($P < 0.01$). *Neocallimastix* GR1 and *Piromyces* WNG12, also increased TVFA and hydrolytic enzymes (CMCase, FPase and Xylanase) significantly ($P < 0.05$) under *in vitro* studies of SB and SB based ration. Based on results obtained in this study, it may be stated that the zoospores of anaerobic fungi, *Neocallimastix* GR1, may be used as probiotic in ruminants to improve the nutritive value of high fibrous based diets, ultimately leading to increased ruminant productivity.

सारांश

वर्तमान अध्ययन की योजना अवायुवीय कवको. निओक्यालिमैस्टीक्स जाति जी. आर.-1, पाइरोमायसीज जाति डब्ल्यू. एन जी. 12 और ओरपीनोमायसीज सी. 14 जाति के जूस्पोर्स के उपयोग के प्रभाव का गन्ने की खोई और गन्ने की खोई पर आधारित खुराक के अन्तः पात्र पाचन को खोज करना था. गन्ने की खोई की पर आधारित सम्पूर्ण खुराक जिसमें 13 प्रतिशत सी. पी. तथा 55 प्रतिशत टी.डी. एन. या को गन्ने की खोई 20 भाग, गेहूँ का भूसा 30 भाग तथा दाना 50 भाग से तैयार किया गया। रूमिनल कवक पृथक जोकि ओरपीनोमाइसीज जाति सी-14 पाइरोमायसीज जाति डब्ल्यू. एन जी. 12 तथा निओक्यालिमैस्टीक्स जाति जी. आ.1 को हंगेट गोल नालिका विधी द्वारा पुर्न उदपादित किया गया और आकाशिय लक्षणो के आधार पर सुदघता के लिये जांचा गया। अधिक जुस्पोर उत्पादन के लिए माध्यम को आवश्यक तत्वो की कर्मा करके जिसमे की सैलोबायोज या गन्ने की खोई डालकर की गयी। परन्तु, 96 घंटे के बाद भी सामान्य मध्यम अन्तर्विश्ट में अधिक जुसपोर (2×10^3 जुस्पोर/एम.एल) निओक्यालिमैस्टीक्स जाति जी. आर.-1 तथा पाइरोमाइसीज जाति डब्ल्यू. एन. जी.12 पाये गए जबकि आवश्यक तत्वो की कमी वाले मध्यम में (0.8×10^3 से 1.3×10^3) जुस्पोर/एम.एल.) तथा ओरपीनोमाइसीज जाति सी -14 (0.7×10^3 जुस्पोर/एम.एल) कम जुस्पोर पाये गये। इसलिए अधिक जुस्पोर व अन्तः पात्र पाचकता प्राप्त करने के लिए सामान्य मध्यम का उपयोग किया गया।

निओक्यालिमैस्टीक्स जाति जी. आर.1 एवं पाइरोमाइसीज जाति डब्ल्यू. एन. जी.12 के कवको ने गन्ने की खोई और गन्ने की खोई आधारित खुराक के डि.म., ऐन.हि.एफ., और एन्जाईम कार्यकुशलता अन्तः पात्र पाचकता को सार्थक रूप से बढ़ा दिया।

इस अध्ययन के आधार पर यह कहों जा सकता है कि अवायुविय कवको के जुस्पोरो को (निओक्यालिमैस्टीक्स जाति जी.आर.1 एवं पाइरोमाइसीज जाति डब्ल्यू.एन.जी.12) अधिक रेशें वाले रोमन्थी पशुओं के खुराक की पोशक मानता को बढ़ावा के लिए प्रोबायोटिक्स की तरह उपयोग मे लाकर रोमन्थी उतपादकता को बढ़ाया जा सकता है।

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LIST OF ABBREVIATIONS

| | | |
|---------|---|---|
| ADF | = | Acid detergent fibre |
| ADL | = | Acid detergent lignin |
| ATP | = | Adenosine triphosphate |
| BIS | = | Bureau of Indian Standards |
| CF | = | Crude fibre |
| CFU | = | Colony forming unit |
| CMC | = | Carboxymethyl cellulose |
| CP | = | Crude protein |
| CRF | = | Clarified rumen fluid |
| DCP | = | Digestible crude protein |
| DM | = | Dry matter |
| DMI | = | Dry matter intake |
| EDTA | = | Ethylene diamine tetra acetic acid |
| EE | = | Ether extract |
| FAO | = | Food and Agriculture Organization |
| g | = | Gram |
| h / hrs | = | hour(s) |
| IU | = | International unit |
| IVADFD | = | <i>In vitro</i> acid detergent fiber digestibility |
| IVDMD | = | <i>In vitro</i> dry matter digestibility |
| IVOMD | = | <i>In vitro</i> organic matter digestibility |
| IVNDFD | = | <i>In vitro</i> neutral detergent fiber digestibility |
| kg | = | Kilogram |
| L | = | Litre |
| min | = | Minute |
| ml | = | Millilitre |
| mM | = | Millmole |
| NAD | = | Nicotinamide adenine dinucleotide |

| | | |
|------------|---|----------------------------|
| NDF | = | Neutral detergent fibre |
| NDS | = | Neutral detergent solution |
| NFE | = | Nitrogen free extractives |
| OM | = | Organic matter |
| RNA | = | Ribonucleic acid |
| sp. | = | Species |
| SRL | = | Strained rumen liquor |
| SSF | = | Solid state fermentation |
| TDN | = | Total digestible nutrients |
| TFU | = | Thallus forming unit |
| VFA | = | Volatile fatty acid |
| TVFAs | = | Total volatile fatty acids |
| $W^{0.75}$ | = | Metabolic body weight |
| °C | = | Degree centigrade |
| µg | = | Microgram |

INTRODUCTION

In India and many tropical countries, the majority of livestock subsist on poor quality native grasses, crop residues and agro industrial byproducts. In certain states, the crop residues are available in plenty, but many states are not self sufficient and situation aggravates during drought, which occurs in one or other states annually in India. Hence, alternate roughage sources need vigorous investigation for their incorporation in livestock feeding. Further, most of the crop residues are lignified and fibrous in nature and being poor in their nutritive value need certain treatments to raise their palatability and feed value. In this direction many efforts have been made in the past and researchers have shown interest in physical and chemical treatments but these treatments are not applicable at farm level, as a common dairy farmer is not aware of the technologies for improving the quality of unconventional roughages in order to prove their worth in raising the animal productivity.

Complete feeding system of ruminants in which roughage and concentrate mixture are mixed homogeneously in a particular ratio to provide the animals a nutritionally balanced diet. Complete feed is being manufactured commercially in India, using wheat straw as a source of roughage. However, seasonal availability and soaring price of wheat straw is causing concern to dairy farmers and forcing them to feed alternate sources of roughages like sugarcane bagasse to their livestock.

India is the second largest sugarcane (*Saccharum officinarum*) producers in the world after Brazil. Here 4.63 mha of land is under sugarcane with about 333.3 million tones of annual production (Techno economic assessment of bioenergy in India, 2006) and availability of sugarcane bagasse, a byproduct from sugar industry is about 95.5 million tones annually (assuming 30 percent of sugarcane, ISMA 2006). Sugarcane bagasse is mainly used as a source of fuel in sugar industry itself and partly it is being utilized by paper and board industry as it is a good source of fiber. Abundant availability

and low cost, demands its use as animal feed in view of the deficiency of feed resources in the country. Traditionally, sugarcane bagasse is not fed to the animals because of its low palatability. Fresh sugarcane bagasse (moisture about 40-60 percent) from sugar industry is a mixture of fine particles, which is different than that available from small scale sugarcane crushers units dotting the country side. Sugarcane bagasse from the sugar mills, in roughage deficient states such as Maharashtra and Gujarat, may be utilized in the animal ration. Sugarcane bagasse enriched with urea and molasses successfully tackled the drought situation in Maharashtra during 1977. Palatability problem of sugarcane bagasse can be overcome by using it at a lower level along with concentrate mixture (Reddy *et al.*, 1993; Reddy *et al.*, 2002).

However sugarcane bagasse is poor in nutritional quality because of its low protein and high lignin contents. But, it is a potential source of energy due to the presence of cell wall polysaccharides, such as cellulose and hemicellulose. High lignin content of sugarcane bagasse reduces its digestible energy because lignin encrusts cell wall and develops ester covalent bonds with hemicelluloses. In particular lignin prevents close contact between the cell wall polysaccharides and the rumen microorganisms. Thus upgradation of sugarcane bagasse is important issue for improving ruminant livestock production. In this direction many efforts have been done in past to enhance the digestibility of poor quality lignocellulosic feeds by the addition of various microbial or chemical feed additives. Among microbial additives, there are evidences of definite positive relationships between anaerobic fungi in the rumen and the increased voluntary intake of low digestible fibrous feeds (McAllister *et al.*, 1994; Ha *et al.*, 1994; Dey *et al.*, 2004). Administration of elite ruminal fungi isolates from goat, cattle and buffaloes has increased the digestibility and nutritive value of wheat straw based rations (Tripathi *et al.*, 2007; Swati *et al.*, 2006).

Manikumar *et al.* (2004) showed the ability of different fungal species on fiber digestibility *in vitro* and reported that *Orpinomyces* sp. (C-14) is a most promising isolate for degradation of wheat and paddy straws. Tripathi *et al.* (2007) isolated and identified *Piromyces* sp WNG-12 as elite fungus that has higher CMCase, cellulobiose and xylanase activity than other 11 fungal species isolated from the faeces of the blue bull. *Piromyces*

sp. WNG-12 culture administration increased the growth rate, percent feed efficiency and nutritive value of wheat straw based ration in buffalo calves compared to control diet.

Debanujit *et al.* (2006) concluded that *Neocallimastix* sp. GR-1, isolated from goat, was a promising anaerobic fungi to act as a probiotic in the rumen of buffalo calves to improve the nutritive value of wheat straw based complete feed diets. Swati *et al.* (2006) showed that *Piromyces* sp. WNG-12 could be a potential anaerobic fungi that could be exploited as probiotic in the rumen of lactating buffaloes fed on a wheat straw based total mixed ration for enhancing milk production and its composition. Keeping this in mind the present study has been designed to maximize the Zoosporogenesis of elite fungal isolates i.e. *Neocallimastix* sp. GR-1, *Piromyces* sp. WNG-12 and *Orpinomyces* sp. C-14 and conduct *in vitro* studies on nutrient utilization of sugarcane bagasse based rations with or without administration of superior anaerobic fungal zoospores separately with the following objectives:

1. Revival and purification of existing ruminal fungi in order to develop their zoospores.
2. To monitor the *in vitro* digestibility and rumen fermentation pattern of sugarcane bagasse based ration containing fungal zoospores.

2. REVIEW OF LITERATURE

Sugarcane bagasse (SB), a byproduct of sugar industry, consists of fibrous residue of sugarcane stalks after the removal of cane juice. The sugarcane bagasse is poor in nutritional quality because of low protein and high lignin content, but it is a potential source of cell-wall polysaccharides, such as, cellulose and hemicelluloses. The high lignin and silica contents may reduce its digestive energy content. In the past, many efforts have been made to enhance the digestibility of lignocelluloses containing feeds (cereal straws) by the addition of various biological or chemical feed additives. Among biological additives, anaerobic rumen fungi have attracted considerable attention globally because of their ability to attack, penetrate and utilize the plant biomass. It is also seen that the fungus has a preference for colonizing the more recalcitrant lignified plant tissues by attacking sclerenchyma and fibers of vascular bundles (Akin *et al.*, 1983; Grenet *et al.*, 1989). The fungi are able to penetrate intracellular barriers within the plant particles thus enabling them to utilize substrates that are less accessible to other rumen microorganisms. In the following text, an attempt has been made to compile the related information on sugarcane bagasse, anaerobic fungi and their role in ruminant nutrition.

2.1. PHYSICAL AND CHEMICAL CHARACTERISTICS OF SUGARCANE BAGASSE

2.1.1. Bulk density of sugarcane bagasse

The density levels, in which the straws and stovers can be compacted, determine the economy of storage and transportation of these materials. Crop residues are usually bulky in nature and their bulk density has large variations. Samanta *et al.* (2004) reported that bulk density of wheat straw (WS), rice straw (RS) (10-15 cm), natural grass (10-15 cm), and sorghum stover (10-12 cm) was found to be 93.99, 24.75, 25.70 and 42.40 kg/ m³. Several reports indicated that bulk density of roughages increased following their densification. Bulk density of SB increased from 61.0 to 567.5 kg/ m³ following its densification (Singh *et al.*, 2004). Kundu *et al.* (1998) reported that bulk density of WS based complete feed in mash form increased from 130 kg/ m³ to 440 kg/ m³ following its

densification. Fardin (2005) reported that bulk density of SB and WS (0.5 to 3 cm) was 81.27 and 68.32 kg/ m³ respectively.

2.1.2. Chemical composition of sugarcane bagasse

Various roughages such as SB, WS, sorghum stover and cottonseed hulls have considerable variation in chemical composition particularly in their cell wall composition. Reddy *et al.* (2002) reported that OM, CP, EE, NFE and CF contents of SB were 94.71, 3.20, 1.35, 48.06 and 41.70 percent, respectively. However, Tinh *et al.* (2001) reported 1.40 percent CP and 49.66 percent CF in sugarcane bagasse.

NDF content of SB was reported to be in the range of 69.77 to 90.16 percent which appeared to be higher than WS (Rangneker *et al.*, 1982; Kaur and Kaushal, 2001; Tinh *et al.*, 2001; Reddy *et al.*, 2002). Other cell wall constituents particularly lignin was reported to be higher in SB than in WS (Table 2.1)

Table 2.1 Chemical composition of fibrous feeds (% DM basis)

| | Bagasse | Wheat straw | Sorghum stover | Cottonseed hulls |
|--------|----------------|--------------------|-----------------------|-------------------------|
| OM | 92-97.50 | 85-94 | 85-90 | 96.50 |
| CP | 1.3-3.2 | 2.5-5.0 | 3.5-4.0 | 3.0-12.4 |
| CF | 41-50 | 40-44 | 32-35 | 37-48 |
| NFE | 45-49 | 45-46 | 52 | 41-53 |
| NDF | 69-85 | 70-83 | 72-74 | 73-94 |
| ADF | 41-44 | 45-64 | 42-48 | 57-71 |
| Lignin | 3-19 | 8-15 | 7-8 | 16-27 |
| Ca | 0.51-0.90 | 0.16-0.22 | 0.7 | 0.2 |
| P | 0.23-0.29 | 0.05-0.14 | 0.2 | 0.6 |

Ca and P contents of SB varied in the range of 0.51-0.90 and 0.23-0.29 percent, respectively, which were higher than that reported for WS (Donald *et al.*, 1995; Reddy *et al.*, 2002; Siulapwa and Simukoko, 2003). Fardin (2005) analyzed SB and WS for proximate analysis and cell wall constituents (Table 2.2).

Table 2.2 Chemical composition of SB and WS (% DM basis)

| Particulars | SB | WS |
|--------------------|-----------|-----------|
| DM | 95.88 | 95.27 |
| OM | 97.09 | 92.70 |
| CP | 2.98 | 3.41 |
| CF | 45.09 | 41.30 |
| Ash | 2.91 | 7.30 |
| NDF | 84.61 | 81.75 |
| ADF | 53.41 | 51.63 |
| Hemi-cellulose | 31.20 | 30.12 |
| Cellulose | 40.33 | 39.45 |
| ADL | 10.79 | 7.46 |
| Ca | 0.49 | 0.18 |
| P | 0.22 | 0.11 |

2.2. SUGARCANE BAGASSE AS A ROUGHAGE SOURCE

Sugarcane is a major cash crop grown in many parts of India. It is popular with farmers in many regions for its ability to withstand extremes of environmental conditions and for its assured market price. The crop is primarily grown for sugar, but sugarcane bagasse, cane tops and molasses are important byproducts. Sugarcane bagasse has an industrial use like manufacture of paper and board, which can also be used as animal feed.

A variety of methods have been used to improve the nutritive value of sugarcane bagasse. Urea when used for treatment of sugarcane bagasse enhances the nitrogen/protein content, it also increases palatability and improves digestibility of cell wall contents. Bagasse is commonly not palatable, if fed alone but total mixed rations or complete feed containing 40% sugarcane bagasse is quite palatable to animals (Reddy *et al.*, 2002).

2.3. TO IMPROVE THE INTAKE AND UTILIZATION OF SUGARCANE BAGASSE AND OTHER POOR QUALITY ROUGHAGES.

Poor nutritive value and coarse physical form of SB and other poor quality roughages limit the activity of rumen microorganisms resulting in their low rate of passage through the digestive tract and lead to low voluntary intake. The higher DM intake has been reported following NPN and molasses supplementation in animals fed on poor quality roughages (Singh *et al.*, 1990; Dahiya and Mudgal, 1991; Prakash *et al.*, 1996; Dahiya *et al.*, 1998). Therefore, fortification of roughages with energy, protein and minerals is a sustainable way for improving their feed value.

Improvement in the intake of DM and TDN from WS (Verma *et al.*, 1996) and SB (Castro and Machado, 1990) was noticed when fed as complete diet. Singh *et al.* (2004) compared complete feed (CP 11.43- 12.23%) containing WS with SB based complete feed (R: C, 50:50) and reported significantly higher DMI (101.5 vs. 76.6 g/w^{0.75}), DM digestibility (56.26 vs. 33.60%), NDF digestibility (56.49 vs. 50.80%) and nitrogen balance (47.60 vs. 33.40 g/d) in WS based diet than in SB based diet.

Fardin (2005) concluded that 40% replacement of WS by SB yielded higher degree of densification of feed blocks and feeding of such blocks did not affect feed intake, nutrient utilization and growth of crossbred calves.

2.4. SUGARCANE BAGASSE BASED COMPLETE FEEDS

Palatability and nutrient utilization of SB can be improved by its incorporation into complete feed (Reddy *et al.*, 1993). Reddy *et al.* (2002) compared the feeding of SB in calves in the form of complete diet and conventional diet (concentrate and SB fed to animal, separately in a 60:40 ratio). They reported that incorporation of SB in complete diets did not affect the voluntary DM intake. However, the digestibility of DM, OM (P<0.01), CP, NFE (P<0.05) and nutritive value in the terms of DCP and TDN contents (P<0.05) improved by incorporating 40 percent SB in complete feed. They also reported that the average daily gain (ADG) in calves fed on complete feed was higher than that on conventional diet.

Voluntary intake of roughages by ruminants is often related to their digestible energy and the quality of feed may also attribute to its consumption. Feed intake is negatively correlated with retention time and organic matter digestibility (Forbes, 1995). Voluntary intake and digestion of crop residues are generally poor because rumen microbes degrade their fibre content at a relatively slow rate and only to a limited extent. This is partly an intrinsic characteristic of their fiber, which results in unbalanced nutrient availability for microbial growth. Although, the TDN content of SB and cereal straws was reported to be approximately 44 percent on DM basis (Bath *et al.*, 1995) but the extent of energy utilization depends primarily on microbial degradation of cell wall components in the rumen. Fibrous nature of these roughages may limit their degradation by ruminal microbial enzymes.

The digestibility of DM or OM reflects the amount of energy an animal can derive from the feed and this is an important criterion for roughages based diet. Generally, there is a positive relation between roughage intake and their digestibility. Van Soest (1994) reported a correlation ($r^2=0.60$) between *in vivo* digestibility and voluntary intake for a wide range of diets. Wongsrikreu and Wanapat (1985) observed that cattle consuming rice straw alone @ 2% of BW, can fulfill their maintenance requirement, but they lost their body weight due to inadequate supply of other nutrients and because of the limited efficiency of absorbed energy utilized. The low N content in crop residues may be an important factor for their low OM digestibility and hence lowers the intake (Doyle and Pandey, 1990).

Acid detergent fiber (ADF) content correlates negatively with DM intake. Lignin content was significantly correlated (negatively) with *in vivo* digestibility of straw (Reid *et al.*, 1988). Saha (1997) also reported that fibrous fractions (NDF, ADF and cellulose) were negatively correlated with IVDMD of rice straw.

Fardin (2005) analyzed a WS based complete feed having roughage to concentrate ratio 60:40, where WS was replaced by SB by 20, 40, 60, 80 and 100 percent but maintaining the roughage to concentrate ratio of 60:40. These complete feeds were analyzed for their chemical composition and subjected to *in vitro* evaluation for DM and OM digestibility and found that replacement of WS by SB beyond a level of 40%

resulted in reduced DM digestibility, revealing that 40 percent of WS in complete feed could be replaced by SB.

2.5. IMPROVEMENT OF NUTRIENT UTILIZATION OF POOR QUALITY ROUGHAGES BY ANAEROBIC RUMEN FUNGI

Some success has already been achieved in increasing the fiber degradability and productivity in sheep, where the sheep rumen dosed with superior fungi resulted in increased intake of poor quality feeds by about 7 percent (Gordon and Phillips, 1989) and also led to increase in the fiber degradation and overall productivity. *In vitro* studies with different fungal species improved the dry matter digestibility and cell wall constituents of cereal straws (Manikumar *et al.*, 2004). Incorporation of fungus increased growth rate, rumen fermentation, nutrient digestibility and nitrogen retention in sheep (Lee *et al.*, 2004) and crossbred calves (Dey *et al.*, 2004).

2.6. RUMEN FUNGI, THEIR DISCOVERY, SYSTEMATICS AND ISOLATION.

2.6.1. Rumen fungi

Rumen, the anaerobic chamber of ruminant digestive system and place for microbial-based fermentation of feed, contains mainly four kinds of microbes-

a) bacteria, b) protozoa, c) fungi d) bacteriophages. The fungi found in rumen are mainly of two kinds- aerobic and anaerobic. Clarke and DiMenna (1961) reported that yeast and aerobic fungi are normal inhabitants of rumen and intestines, but they are transient, non-functional and enter the rumen along with the feed. Brewer *et al.* (1972) suggested that nine species of yeast of four genera (Candida, Trichosporon, Rhodtoirula and Saccharomyces) are found in rumen. Some species of yeast and aerobic fungi like *Aspergillus fumigatus*, *Mucor rouxii*, *Rhizopus sp.* and *Sporormia minima* are the causative agent of novine ill thrift and could survive in the rumen.

2.6.2. Discovery of anaerobic fungi

Earlier the zoospores of the anaerobic fungi were traced from rumen liquor, but at that time they were considered to be as protozoa. Liebetanz (1910) designated uniflagellate organisms from ruminants as protozoa though they were smaller in size than

usual protozoa. Finally, Orpin (1975) identified anaerobic fungi in sheep rumen, which had a motile stage (zoospore) and a non-motile stage (zoosporangium).

2.6.3. Systematics of rumen anaerobic fungi

Total six genera under the family Neocallimasticaceae and class Chytridiomycetes have been identified till date and the latest genus discovered was *Cyllamyces* sp. (Ozokose *et al.*, 2001). Earlier, Li and Heath (1990) and Li *et al.* (1993) proposed to elevate the family Neocallimastigaceae to the new order Neocallimasticales. Systematics of rumen anaerobic fungi under the phylum- Neocallimastigomycota are:

- Division : Eumycota
- Subdivision : Mastigomycotina
- Phylum : Neocallimastigomycota
- Class : Neocallimastigomycetes
- Order : Spizellomycetales
- Family : Neocallimasticaceae
- Genera (6) : *Neocallimastix* sp, *Piromyces* sp , *Caecomyces* sp

Anaeromyces sp, *Orpinomyces* sp, *Cyllamyces* sp.

2.6.4. Isolation of anaerobic fungi

Different workers adopted different procedures for isolation of rumen fungi. Orpin (1975, 1977) and Joblin (1981) showed that rumen anaerobic fungi could be directly isolated from rumen fluid. Joblin (1981) adopted and developed the roll tube method of Hungate for culturing of rumen anaerobic fungi using antibiotics as growth inhibiting agents for bacteria.

Orpin (1981) did microscopic examination of horse caecum contents and revealed vegetative growth of phycomycete fungi on particles of digesta and uniflagellated cells similar to fungal zoospores in the liquid phase. Three

morphologically distinct isolates of strictly anaerobic phycomycete fungi were obtained from the caecal contents and later they were cultured *in vitro*. Trinci *et al.* (1988) showed that anaerobic rumen fungi could be isolated from saliva of ruminants kept at 39°C upto 8 hrs after collection. They also showed that anaerobic rumen fungi could also be isolated from faeces of sheep air-dried at 20°C/ 39°C for 128 days but in that case zoospore in faeces could not be traced. However rhizomycelium could be detected.

Thodorou *et al.* (1990) observed that faeces contained a large population of anaerobic fungi which declined very slowly and faeces could be dried in such a way that organisms could be cultured even 10 months later. Sijtsma *et al.* (1993) showed rumen fungi could be isolated from fresh faeces of wild animals and they isolated these from llama and yak. Paul *et al.* (2004) and Tripathi *et al.* (2007) showed that anaerobic rumen fungi could be isolated from wet faeces of blue bull.

2.7. IDENTIFICATION AND CLASSIFICATION OF ANAEROBIC CHYTRIDIOMYCETE FUNGI

Trinci *et al.* (1994) proposed that identification of fungus up to genus could be done by comparing ultra structure of zoospore, structure of sporangia, nature of rhizomycelium, chemistry of cell wall, metabolic characteristics, rRNA analysis, structure and distribution of kinetosomes, hydrogenosomes, guanine-cytosine content of DNA. Complex chemical analysis and electron microscopy are necessary steps for identification of anaerobic fungi up to species level.

Munn *et al.* (1994) examined the zoospores and vegetative growth phases of three cellulolytic rumen chytridiomycetes i.e. Piromonas, Sphaeromonas and NF1 by electron microscopy. The four genera had some 16 distinctive ultrastructural features in common, which collectively were used to define the group. Some of the common features were the presence of hydrogenosomes at all stages of the life cycle, the presence of rhizoid and sporangia of characteristic crystals coated with hexagonal arrays of particles, and in zoospores, the presence of distinct surface layers on the motility organelles and cell body.

2.7.1. Morphological characteristics of different genera

| Genera | Zoospores | Rhizomycelium | Sporangium |
|----------------|---|----------------------------------|-------------|
| Neocallimastix | Polyflagellated | Extensive filamentous | Monocentric |
| Piromyces | Uniflagellated | Filamentous/Highly branched | Monocentric |
| Caecomyces | Uniflagellated (One or two flagella) | Globular/bulbar Rhizoids | Monocentric |
| Anaeromyces | Uniflagellated | Filamentous/ Tubular branched | Polycentric |
| Orpinomyces | Polyflagellated | Filamentous | Polycentric |
| Cyllamyces | Uniflagellated | Bulbous Rhizoid | Polycentric |

Guillot *et al.* (1990) established a technique based on fluorescein-linked lectins to determine the cell wall structure of anaerobic rumen fungi belonging to genera *Neocallimastix*, *Piromonas* and *Sphaeromonas*. Further, this technique showed differences of cell wall composition between different parts of the thallus (spores, sporangia, rhizoids).

Brookman *et al.* (2000) obtained two new isolates of the gut fungi from the rumen digesta and faeces of cow. These isolates were designated as *Anaeromyces* following rDNA typing. Ozkose *et al.* (2001) isolated anaerobic gut fungi (*Cyllamyces aberensis*) from fresh cattle faeces with bulbous holdfast, branched sporangiophores and limited polycentric thallus. Due to its unique characteristics this was put under a new genus *Cyllamyces*.

Fliegerova *et al.* (2002) reported that six polycentric anaerobic fungi isolated from cow, lama and bison were of the *Anaeromyces* strain and analyzed them for variability in internal transcribed spacers and fibrolytic activities. First time they

observed the production of xylanase, beta - glucosidase and beta – endoglucanase in *Anaeromyces* isolate.

Farkas (2003) observed that the fungal cell walls possess a characteristic chemical composition differentiating fungal cells from other cell types. For this reason, the mechanisms involved in cell-wall formation represent a potential target for selective antifungal drugs.

Paul *et al.* (2004) isolated 17 strains of anaerobic fungi from fresh fecal samples of wild ruminants and 15 strains of anaerobic fungi from rumen liquor of domestic animals. All the isolates belonged to genera i.e *Piromyces*, *Anaeromyces*, *Neocallimastix* and *Orpinomyces*. Tripathi *et al.* (2007) isolated 12 fungal isolates from the faeces of the wild blue bull. These were identified up to genus level on the basis of their morphological characteristics such as zoospores flagellation (mono- or poly-flagellated), shape of sporangia and presence or absence of sporangial stalk and found all the isolates belonged to four genera i.e *Piromyces*, *Anaeromyces*, *Neocallimastix* and *Orpinomyces*. Thareja *et al.* (2006) isolated 40 fungal isolates from grazing sheep rumen and grazing and non-grazing goat rumen liquor and faeces and found they belonged to four genera *Piromyces*, *Anaeromyces*, *Neocallimastix* and *Orpinomyces*.

2.8. DISTRIBUTION OF ANAEROBIC FUNGI IN DIFFERENT SPECIES OF LIVESTOCK AND THEIR SURVIVABILITY IN DIFFERENT HABITATS.

Anaerobic fungi have been isolated from both ruminants and monogastric herbivores. They are comprised of 6 genera and 18 species (Table 2.8.1).

2.8.2. Survivability of anaerobic fungi in different habitats

Presence of anaerobic zoosporic fungi in saliva and faeces suggested that saliva and faeces were possible routes of transfer of fungi (Lowe *et al.*, 1987; Theodorou *et al.*, 1990). Wubah *et al.* (1993) isolated species of *Neocallimastix*, *Piromyces*, *Orpinomyces*, and *Caecomyces* from fresh and dry faeces and rumen liquor of a cow. Each of these isolates produced a resting stage in vitro and similar melanized sporangia were observed in the faecal smears from which they were obtained. It showed that anaerobic fungi could survive outside the host animal environment also.

2.8.1 Rumen fungi from different animals (6 genera and 18 species)

| Genus | No. of Species | Source of isolate | References |
|---|----------------|---|---|
| <i>Caecomyces</i> (Previously known as <i>Spheromyces</i>) | Two | Sheep, Horse | Gold <i>et al.</i> (1988) |
| <i>Piromyces</i> (previously known as <i>Piromonas</i>) | Seven | Sheep, Horse, Cow, buffalo, Elephant, Ass, Deer, Goat, Kangaroo, Blue bull, Hog deer, Blackbuck, spotted deer | Li <i>et al.</i> (1990), Ho <i>et al.</i> (1993), Samanta and Walli. (1999), Singhal (2000) Paul <i>et al.</i> (2004) Thareja <i>et al.</i> (2006) |
| <i>Neocallimastix</i> | Four | Sheep, Cow, Deer ,goat | Heath <i>et al.</i> (1983) Orpin and Mann (1986) Ho <i>et al.</i> (1993) Thareja <i>et al.</i> (2006) |
| <i>Anaeromyces</i> (Previously known as <i>Ruminomyces</i>) | Two | Sheep, Cow | Breton <i>et al.</i> (1990) Tripathi <i>et al.</i> (2007) |
| <i>Orpinomyces</i> | Two | Sheep, Cow Buffalo | Ho <i>et al.</i> (1994), Singhal <i>et al.</i> (2000) Thareja <i>et al.</i> (2006) Tripathi <i>et al.</i> (2007) |
| <i>Cyllamyces</i> | One | Cattle | Ozokose <i>et al.</i> (2001) |

Wubah *et al.* (1993) isolated species of *Neocallimastix*, *Piromyces*, *Orpinomyces*, and *Caecomyces* from fresh and dry faeces and rumen liquor of a cow. Each of these isolates produced a resting stage in vitro and similar melanized sporangia

were observed in the faecal smears from which they were obtained. It showed that anaerobic fungi could survive outside the host animal environment also.

2.9. ENUMERATION AND LIFE CYCLE OF ANAEROBIC FUNGI

2.9.1. Enumeration of anaerobic fungi

Gordon *et al.* (1998) proposed that fungal isolates could be enumerated by microscopy or culture method or through fungal biomass measurement. Zoospore or sporangia could be enumerated by microscopy method. Roll tube technique (Joblin 1981) and MPN count can be used in culture method. Fungal biomass measurement method involved use of chitin (poly-N-acetylglucosamine) as chemical marker. Fungal biomass was measured to be about 8% of total microbial biomass in rumen by using chitin as chemical marker (Akin *et al.*, 1990). MPN count involved dilution of whole rumen digesta serially and sets of culture tubes were inoculated with each dilution of digesta. By determining the number of tubes showing fungal growth at each dilution and consulting statistical tables was possible to determine a probable number of fungi in original sample (Theodorou *et al.*, 1990; Mc Granaghan *et al.*, 1999).

Samanta *et al.* (1999) enumerated anaerobic fungal population in the faeces of riverine buffalo and crossbred cattle through culturing and counting by roll tube technique. The animals were maintained on wheat straw and oat fodder given in the ratio of 50:50. The values (number /g of faecal dry matter) were 6.5×10^3 in riverine buffalo while in cattle it was 5.3×10^3 to 5.6×10^3 cfu.

Dey *et al.* (2004) and Tripathi *et al.* (2007) counted fungal zoospores by direct microscope after taking fresh rumen liquor sample from growing calves and buffalo calves respectively, using 1 cm^2 smear of rumen fluid (0.01 ml). The smears were stained with crystal violet for one minute and the spores present in 10 fields were counted based on their morphological characteristics, using compound microscope. The average count was taken and final results of zoospore count per ml of rumen fluid were calculated by taking into account the dilution factors. Further Dey *et al.* (2004) reported that the number of zoospores/ml was approximately 2.42×10^5 for superior anaerobic fungi *Orpinomyces sp.* C-14 (isolated from cow) administered group against 1.08×10^5 in control group.

2.9.2. Life cycle of anaerobic fungi

Due to long survivability of fungi in dry faeces, Theodorou *et al.* (1990) proposed three stages of life cycle of fungi– a) Motile zoospore, b) Vegetative thallus, c) Aerotolerant survival stage.

In vitro and *in vivo* studies conducted to observe the life cycle of fungi indicated that it takes between 24-32 hours for completion of life cycle, although in continuously fed animal, the time span may reduce to 8 hours. (Joblin, 1981; Lowe *et al.*, 1987; Wubah *et al.*, 1993). Attachment and encystment of zoospore to feed particles occurs within 15 to 30 minutes of feeding (Akin, 1988; Gordon, 1993)

Zoospores are released after 15-30 minutes of feeding and by chemotactic response they get attached to the damaged surface, stomata and highly lignified portion of feed/plant particles. After attachment they start encystment and development of thallus takes place. Penetration and damage of cell wall of feed/plant particle occurs. Zoosporogenesis starts at sporangia parts. Zoospores are released again after feeding.

Lowe *et al.* (1987) reported that the life cycle of an anaerobic fungus isolated from the rumen of sheep was similar to that of the chytrids. The organism was monocentric. At 39°C, the duration of the life cycle varied between 26 to 32 h. The zoospores were spherical or oval in shape and were polyflagellated. During the first 6.5 h of growth, there was a rapid development of an extensive, non separable, highly branched rhizoidal system and during this period the main rhizoid increased in length. They also observed that after zoosporogenesis, zoospores were liberated through a pore formed in the zoosporangial wall opposite the main rhizoid. Zoosporangia varied in shape from spherical to columnar, and some columnar zoosporangia were observed to become spherical.

The time span of typical fungal growth cycle in rumen is likely to be similar to the mean retention time of digesta particles in the rumen i.e. about 18-24h (Theodorou *et al.*, 1990). Higher population density of zoospores was found 15-30 minutes after feeding (Orpin, 1975) but with fungi *Sphaeromonas* and *Piromonas*, the peak occurred after 1 hour of feeding (Ho *et al.*, 1991).

Fungal zoospores swim freely in the rumen fluid, locate freshly ingested plant fragments by chemotaxis of soluble carbohydrates diffusing from the damaged plant

tissues. Zoospores of *N.frontalis*, *P.communis*, *O.joyonii* and *Anaeromyces spp.* also show chemotactic response to phenolic acids such as p-coumaric acid, ferulic acid syringic acids, often found in lignified tissue of plant (Wubah and Kim 1996). That's why rumen fungi attack highly lignified tissue (which other microorganisms can not attack) or damaged tissues/cut surfaces of plant from where soluble sugar diffuses.

2.10. Role of rumen fungi in fibre degradation

The crop residues are the most abundant of the renewable resources having potentiality as animal feeds. Out of these, cereal straws are the most important byproducts that are available in larger quantities. These straws are characterized by low protein, low energy, low mineral and low vitamins, but contain a potential source of digestible energy due to the presence of cell wall carbohydrates such as cellulose and hemicellulose. These polysaccharides are not available to the ruminants because of the presence of high lignin. High lignin content has been implied to be a major hindrance to unlock energy from cellulose (VanSoest, 1981).

Cellulose is a linear polymer of D-anhydro glucopyranose units linked by β -1, 4 glucosidic bonds. Sehgal and Punj (1983) reported cellulose contents of wheat straw as 40.3 percent. Cellulose contents of wheat, barley and oat straw as 40, 41.3 and 42 percent, respectively. Although the percentage of cellulose depends upon the varieties and proportion of leaves in straws (Givens *et al.*, 1989).

Hemicelluloses are chemically the most complex plant polysaccharides and consist largely of heteropolymers of xylose and smaller quantities of arabinose, glucose, galactose, mannose and rhamnose. The bulk of hemicelluloses are based on a linear chain of β -1,4 linked D-xylopyranose units with varying amounts of side branches containing other sugars. Sehgal and Punj (1983) reported that wheat straw contained 26.9 percent hemicellulose. Givens *et al.* (1989) also reported the hemicellulose contents of wheat, barley and oat straws as 31.4, 29.6 and 24.3 percent, respectively.

Lignin is a major component of straws, apart from the structural carbohydrates. It is a complex three-dimensional polymer arising from an enzyme-initiated dehydrogenative polymerization of three primary precursors i.e. trans-p-coumaroyl alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol. Sehgal and Punj (1983)

reported that wheat straw, maize and alfalfa hay contained 12.3, 1.2 and 8.0 percent acid detergent lignin, respectively. According to Givens *et al.* (1989), the lignin content of wheat straw was 9.9 percent. It has been reported that the presence of 5.8 to 15.0 percent lignin limits the voluntary dry matter intake as well as digestibility of cereal straws (Kundu and Sharma, 1995), because the lignin prevents close contact between the cell walls and the rumen microorganisms. Rumen anaerobic fungi help in degrading the fibre portion of low grade cereal straws.

2.11. ROLE OF RUMEN FUNGI IN LIGNIN DEGRADATION

Rumen fungi colonize on thick walled sclerenchyma and vascular tissues and other lignified tissues (Bauchop, 1979 ; Ho *et al.*, 1996). They solubilize lignin but can not ferment it. They produce feruloyl and p-coumaroyl esterase's and breaks ester linkages between lignin and hemicelluloses (Borneman *et al.*, 1991) but, unable to break either the ether bonds between the phenolic moities and polysaccharides or the interphenolic bonds found in the lignin complex (McSweeney *et al.*, 1994). Selected strains of anaerobic fungi were found to solubulize about 35% of the labelled C-14 (lignin) lignocellulose preparation (Gordon and Philips 1989). More than 34% of the lignin component of sorghum stem was removed by the anaerobic fungus *N.patriciarum* (McSweeney *et al.*, 1994)

Zoospores of *N.frontalis*, *P.communis*, *O.joyonii* and *Anaeromyces spp.* also show chemotactic response to phenolic acids such as p-coumaric acid, ferulic acid syringic acids, often found in lignified tissue of plant (Wubah and Kim 1996). Perhaps this is the answer why rumen fungi attack highly lignified tissue.

2.12. INFLUENCE OF SUPERIOR RUMINAL ANAEROBIC FUNGI ON *IN VITRO* DIGESTIBILITY OF CEREAL STRAWS

Gordon and Phillips (1989) cultivated three different ruminal fungi i.e. *Neocallimastix sp.*, *Piromonas sp.*, and *Sphaeromonas sp.* under anaerobic conditions in liquid media which contained a suspension of either 1% (w/v) purified cellulose or finely milled wheat straw as the source of fermentable energy. All the three anaerobic fungi grew in the straw containing medium and loss of dry weight of substrate from the cultures indicated degradation of straw to various degrees (*Neocallimastix* >*Piromyces* >*Sphaeromyces*). The total fiber and the cellulose component of the straw were

degraded in similar proportions, but the lignin component remained undegraded by any of the fungi. Kurar *et al.* (1995) reported that protozoa and fungi have got a vital role in digestion of DM and NDF and fungi from buffalo were more active than from cattle in DM digestibility. However, Ito *et al.* (1994) studied sheep rumen fungi for digestibility of rice straw and found that there was a significant decrease in lignin residue content resulting in increased digestibility of rice straw. Percent in vitro dry matter, organic matter and neutral detergent fibre digestibility were found to be higher with SRL containing all rumen microorganism than protozoa or fungi alone (Malakar and Walli, 1995). Further, Samanta *et al.* (1999) reported that incubation of straws with fungal inocula having mixed rumen cultures from rumen liquor from cow increased percent IVDMD, percent IVOMD, TVFA concentration (mmol / ml SRL) and molar proportions of acetate.

Sehgal *et al.* (2002) and Manikumar *et al.* (2004) incubated wheat and paddy straws with different fungal isolates from cattle and buffalo and found an increase in IVDMD of wheat straw from 34.0 to 54.0 percent with addition of *Orpinomyces* sp C-14 from cow, and 31.4 to 35.5 percent with addition of *Orpinomyces* sp B-13 from buffalo @ 103 cfu/ml. Increase in percent IVDMD of wheat straw from 37.9 to 55.3 and of paddy straw from 33.7 to 44.8 percent, decrease in NDF percent from 79.9 to 64.1 in wheat straw and 72.6 to 57.1 in paddy straw, decrease in ADF percent from 41.1 to 27.1 in wheat straw and 39.1 to 27.4 in paddy straw, decrease in ADL percent 12.3 to 6.25 in wheat straw and 10.9 to 5.9 in paddy straw by addition of *Orpinomyces* sp c-14 from cow @106 cfu/ml. It was informed that *Orpinomyces* sp C-14 from cow was most promising isolate than other isolated fungi studied from two species of livestock.

Paul *et al.* (2003) isolated various fungi from different wild and domestic animals and compared their degradation and enzymatic activities and concluded *Piromyces* sp isolated from nil gai was the promising one.

Dayananda (2005) compared *Piromyces* sp. (WNG-12) from nil gai with *Orpinomyces* sp.(c-14) isolated from dairy cattle for *in vitro* biodegradation of urea –

ammonia treated wheat straw and found *Piromyces* sp.(WNG-12) from nil gai was most effective in enhancing digestibility of nutrients and TVFA production.

Thareja *et al.* (2006) isolated 40 fungal isolates from sheep and goat and compared their enzymatic activity. They found *Anaeromyces* sp. SR4 isolated from sheep rumen and *Neocallimastix* sp GR1 isolated from grazing goat, were most efficient in enzymatic activity. They also compared these with previously isolated *Piromyces* sp. WNG-12 from nil gai through *in vitro* digestibility study and concluded that *Neocallimastix* sp.- GR1 was the elite fungi to digest fibre and even better than *Piromyces* sp. WNG-12.

2.13. EFFECT OF ORAL ADMINISTRATION OF SUPERIOR RUMEN ANAEROBIC FUNGI ON *IN VIVO* DIGESTIBILITY OF LOW GRADE ROUGHAGE TO ENHANCE THE LIVESTOCK PRODUCTIVITY.

Lee *et al.* (2000) isolated *Orpinomyces* sp from Korean native goat and dosed daily with 200 ml culture @ 10^6 cfu/ml to 4 sheep intraruminally , 4 sheep each dosed with 200ml broth (control) and 4 sheep given 200ml autoclaved culture (containing fungal enzymes only) per day. The sheep were fed with orchard grass hay 560 gm/day and concentrate mixture 240 g/day (roughage: conc: 80:20). Fungal culture administration showed an increased nutrient digestibility (DM from 71.5 to 75.2%, NDF from 65.1 to 68.9%; ADF from 57.3 to 62.9%), nitrogen retention, increased population of total bacteria (2 fold), cellulolytic bacteria (1.3 fold) and fungi (two-fold), increased ruminal TVFA concentration (TVFA concentration increased from 6.57 to 8.33 mM) and fibrolytic enzyme activities but fungal enzyme administration could not show any effect as they being proteins rapidly degraded in rumen without any effect.

Dey *et al.* (2004) dosed *Orpinomyces* sp. C-14 isolated from cow to 6 crossbred calves of 10 months of age @ 160 ml (10^6 cfu/ml)/animal/week. The calves were fed wheat straw: concentrate mixture in the ratio 50:50 on DM basis with 1 kg green oats /animal /day. They found that average daily gain was significantly higher (15.37%); FCR and percent feed efficiency also increased significantly. Nutrient digestibility increased significantly for CF, NDF, and ADF after fungal administration. DE value also increased in terms of TDN. The pH and $\text{NH}_3\text{-N}$ were lower whereas TVFA, Total -N, TCA-N and number of zoospores were increased significantly in rumen of fungi administered group.

Paul *et al.* (2004) dosed *Piromyces* sp. isolated from blue bull to 8 growing male buffalo calves of 16 months of age @ 200 ml culture having 10^6 cfu/ml by daily mixing with concentrate mixture for a period of 26 days. The calves were given a diet consisted of wheat straw: concentrate mixture: 50:50. Except CP and EE, digestibility of all other proximate principles and cell wall constituents increased significantly.

Tripathi *et al.* (2007) isolated elite fungi *Piromyces* sp. WNG-12 from blue bull and compared this with previously isolated *Orpinomyces* sp. C-14 from dairy cow dosing these two separately in male growing buffalo calves of 8-12 months age @ 160 ml (10^6 cfu/ml) per animal at alternative days upto 45 days and thereafter on every 4th day upto 150 days of experimental feeding. The calves were fed with roughage: concentrate mixture -50:50. Roughage constituted wheat straw and 2 kg green oats/maize daily. They found although in both fungal culture administered groups, nutrient digestibility, Total-N, TCA-N, zoospore counts, average daily wt gain, FCR and percent feed efficiency increased significantly but *Piromyces* sp. from blue bull was found to be more efficient than *Orpinomyces* sp. from cow.

Debanujit *et al.* (2006) dosed *Neocallimastix* sp. GR1 isolated from grazing and browsing goat to 12 female murrah buffalo calves (5-7 months of age, average body weight 122 kg) @ 250 ml (10^6 cfu/ml). The calves were fed wheat straw: concentrate mixture -50:50 on DM basis with 2 kg green oats /animal /day. They found that an increase of 26.84 percent in body weight gain of calves (659.81 g/day) of fungal culture administered group (FCA) over the control group (520 g/day) with similar feed intake. Percent feed efficiency also enhanced by 28.7 percent. The digestibility of DM, OM, CP, CF, NDF, ADF and cellulose content of feed increased significantly ($P < 0.05$) in FCA group. The digestible energy value in terms of percent TDN also enhanced significantly ($P < 0.01$). The pH, $\text{NH}_3\text{-N}$ and protozoal count/ml were lowered, whereas TVFA, Total N, TCA-N and number of zoospore increased significantly in rumen of fungi administered group.

Swati *et al.* (2006) concluded that *Piromyces* sp. WNG- 12 could be promising anaerobic fungi that can be exploited as probiotic in the rumen of lactating buffaloes fed on wheat straw based total mixed ration for enhancing milk production and composition.

The review of literature suggested that administration of elite anaerobic fungi with straw based ration increased the *in vitro* DM, NDF and ADF digestibility significantly and also *in vivo* increased the growth rate of calves and milk production as well as milk composition in milking animals. Thus, there is a need to study the useful attributes of rumen anaerobic fungal zoospores for setting better utilization of low grade SB based ration so that these fungal zoospores are easily added in feed blocks / TMR without maintaining anaerobic conditions and act as feed additives.

3. MATERIALS AND METHODS

The research methodology has been divided into two phases.

PHASE-1

3.1. PROCUREMENT OF SUBSTRATE AND REVIVAL OF EXISTING RUMINAL FUNGI

3.1.1. Procurement of substrate

Sugarcane bagasse, a by-product from sugar industry was used as substrate. It is soft and white in colour. The bagasse consists of particles of outermost hard covering of the sugarcane and the pith portion. Sugarcane bagasse used in the present study was procured from Karnal co-operative sugar mill, Meerut road, Karnal. Three representative samples were collected.

3.1.1.1. Proximate analysis of sugarcane bagasse

Dried and ground samples of sugarcane bagasse were analyzed for proximate analysis, viz., dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), crude fibre (CF) and total ash as per standard procedures of Association of Official Analytical Chemists (AOAC, 2000) as given below.

i) Dry matter (DM)

A known quantity of ground samples were taken in pre-weighed moisture cups; and these cups were placed in a hot air oven at $100 \pm 2^{\circ}\text{C}$ for 24 hours. The loss in moisture content after drying was estimated and dry matter was calculated as follows:

$$\%DM = \frac{\text{Weight of sample after drying (g)}}{\text{Weight of sample taken (g)}} \times 100$$

ii) Total ash

A known quantity of sample was taken in pre-weighed China crucible. After charring the sample till the smoke disappeared, the crucible was kept in muffle furnace for ignition at 550°C for 2-3 hours. The ash content was calculated as follows:

$$\text{Total ash (g/100 g sample)} = \frac{\text{Weight of ash (g)}}{\text{Weight of sample (g)}} \times 100$$

iii) Organic matter (OM)

It was determined by subtracting the total ash content from 100.

$$\% \text{ OM} = 100 - \text{Total ash (percent)}$$

iv) Crude protein (CP)

Crude protein was estimated as per Kjeldahl's method. A known quantity of ground sample was taken in Kjeldahl flask and digested with concentrated H₂SO₄ and 2-3 g of digestion mixture (K₂SO₄ and CuSO₄ in the ratio of 9:1) till the solution became colorless. After digestion, the contents were cooled and distilled in kjeltech distillation apparatus. Distillate was collected into a conical flask containing 35-40 ml of 4 percent boric acid solution having mixed indicator (0.1% methyl red and 0.1% bromocresol green in the ratio of 2:1 in absolute alcohol). The distillate was then titrated against standard sulphuric acid solution (N/10)

$$N \text{ (g/100 g sample)} = \frac{0.014 \times \text{vol. of acid used} \times \text{Normality} \times \text{Vol. made}}{\text{Aliquot taken} \times \text{Sample taken (g)}} \times 100$$

The crude protein of sample was calculated by multiplying the N-content with factor 6.25.

v) **Ether extracts (EE)**

A known quantity of ground sample was taken in a thimble and extracted for 16 h with petroleum ether (40-60°C) in Soxhlet's extraction apparatus having a pre-weighed flask. The flask was removed after evaporating the excess of ether and dried in oven for overnight. The flask was cooled in a desiccator and the weight was taken. The difference in two weights gave the amount of ether extract in the sample.

$$EE \text{ (g/100 g sample)} = \frac{\text{Weight of ether extract}}{\text{Weight of sample taken}} \times 100$$

vi) **Crude fibre (CF)**

A known quantity of ground sample was taken in a spoutless beaker of 1 L capacity previously marked to 200 ml. Then, 25 ml of 10 percent H₂SO₄ (w/v) was added and volume was made up to 200 ml with water from the sides of the beaker to have 1.25 percent acid solution in the beaker. The contents of beaker were refluxed for 30 minutes. The contents were then filtered through muslin cloth with the help of buchner funnel under vacuum. After repeated hot water washings, the residue left on muslin cloth was transferred to the same spoutless beaker with smooth steel spatula, followed by little washing of muslin cloth.

Then 25 ml of 10 percent NaOH (w/v) was added in the beaker containing residue and volume was made up to 200 ml with water to have 1.25 percent alkali solution. The contents were refluxed for 30 minutes and filtered through the muslin cloth and washed with hot water. The residue left on muslin cloth was transferred to a clean silica crucible with the help of steel spatula and little water washing was done. The contents were dried in hot air oven at 100 ± 2°C and weighed. Silica crucible containing dried residue was kept in a muffle furnace at 550°C for 2 h for ashing and again weighed next day. The crude fiber content of sample was estimated as follows:

$$\text{Crude fibre (g/100 g sample)} = \frac{\text{Wt. of dried residue} - \text{Wt. of ash}}{\text{Wt. of sample taken}} \times 100$$

vii) Nitrogen free extract (NFE)

The nitrogen free extractive (NFE) of sample was estimated as follows:

$$\text{NFE (g/100 g sample)} = 100 - [\text{CP (\%)} + \text{EE (\%)} + \text{CF (\%)} + \text{Total ash (\%)}]$$

3.1.1.2. Estimation of cell wall components and cell solubles of sugarcane bagasse (VanSoest Analysis)

Cell wall components and cell solubles of sugarcane bagasse were estimated by the method of (Goering and VanSoest, 1970) as given below.

a) Neutral detergent fiber (NDF)

Reagents

| | |
|--|---------|
| Sodium lauryl sulphate | 30 g |
| Disodium ethylene diamino tetra acetate (EDTA) dehydrate | 18.61 g |
| Sodium borate decahydrate | 6.81 g |
| Disodium hydrogen phosphate (anhydrous) | 4.56 g |
| 2 Ethoxy ethanol | 10 ml |

Solution preparation

EDTA and sodium borate decahydrate were put together in a large beaker with some distilled water and heated on hot plate until dissolved. The solution containing sodium lauryl sulphate and 2 ethoxy ethanol was added. Sodium hydrogen phosphate was taken in another beaker and some amount of distilled water was added and the contents were heated until dissolved. Then, it was added to solution containing other ingredients and volume was made up to one litre.

Procedure

1 g of sample was taken in 1 L spoutless beaker. To this, 100 ml of neutral detergent solution was added. Decalin was avoided because the fat content of samples was very low. The contents in beaker were refluxed for one hour. After refluxing, the sample was filtered through pre-weighed 50 ml capacity sintered glass crucible grade-I using oil-free vacuum pump. Material was washed with hot boiling water and then with

acetone to remove all salts and moisture. The sintered crucible containing residue was dried in hot air oven at 100°C and weighed.

The NDF was determined as follows:

$$NDF \% = \frac{\text{Wt. of crucible + cell wall constituents} - \text{Wt of empty crucible}}{\text{Wt. of sample taken}} \times 100$$

b) Acid detergent fibre (ADF)

Acid detergent solution: 20g cetyl-trimethyl-ammonium bromide (CTAB) was added to one litre of 1 N H₂SO₄ (26.7 ml H₂SO₄ in 1 litre distilled water).

Procedure

One g of sample was taken in a spoutless beaker of 1 L capacity. To this, 100 ml acid detergent solution was added and the contents were refluxed for exactly 1 hour. After refluxing the residue was filtered through pre-weighed sintered glass crucible grade-I using vacuum pump, washed with hot water and then acetone was used to washout all the salts. The residue was dried in hot air oven at 100°C and weighed.

The ADF was determined as follows:

$$ADF \% = \frac{(\text{Wt. of crucible + fibre}) - \text{Empty wt of crucible}}{\text{Wt. of sample taken}} \times 100$$

c) Acid detergent lignin

Sintered glass crucible grade G-I containing ADF contents was placed in enamel tray in such a manner that one end of the enamel tray was about 2 cm high than the other end, so that acid could drain away from the crucible. The crucible was then filled with 72 percent H₂SO₄ (w/w) and the contents were stirred with glass rod to break all the lumps. The crucible was refilled with 72 percent H₂SO₄ after 1 hour interval. After 3 hours, the crucible was removed from the tray and filtration of acid was done by using vacuum pump. The material was washed with hot water until free from acid and kept in oven at

100°C overnight and weighed. Crucible was then kept in muffle furnace for ashing. The acid detergent lignin was calculated as follows:

$$ADL \% = \frac{Wt. \text{ of lignin} - Wt \text{ of ash}}{Wt. \text{ of sample taken}} \times 100$$

d) Hemicellulose

Hemicellulose was calculated by subtraction of ADF from NDF as per the procedure given below.

$$Hemicellulose (\%) = NDF (\%) - ADF (\%)$$

e) Cellulose

Cellulose was calculated as follows:

$$Cellulose (\%) = ADF (\%) - Lignin (\%)$$

3.1.2. Revival and purification of existing ruminal fungi

Three anaerobic ruminal fungal isolates viz. *Neocallimastix* sp. GR1 (Thareja *et al.*, 2005), *Orpinomyces* sp. C-14 (Manikumar *et al.*, 2004) and *Piromyces* sp. WNG-12 (Tripathi *et al.*, 2005) were procured from Fungal Biotechnology Laboratory, Dairy Microbiology Division, NDRI, and were revived using Hungate's roll-tube technique employing standard anaerobic procedures as described by Joblin *et al.* (1981).

a) Preparation of anaerobic media

The composition of the anaerobic medium used in this study has been presented in Table 3.1

Table 3.1 Anaerobic media for rumen anaerobic fungi (100ml):

| Ingredients | Quantity (for 100 ml) |
|---------------------------|-------------------------------|
| 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 | 43.5 ml |
| Agar | 2.0 g |

With the exception of reducing agents (Sodium bicarbonate and L-cystein hydrochloride), all the quantified ingredients of the medium were taken in 250 ml flask. The pH was adjusted to 6.9 ± 0.1 . The medium was boiled to free it from dissolved oxygen and cooled under the continuous flow of CO₂, supplied through gassing manifold biosystem. Before solidification of media under continuous CO₂ flow, the quantified reducing agents were added, and the media was autoclaved. Filter-sterilized antibiotics solutions were added to the sterile media under strict aseptic conditions. The slight yellowish appearance of media was indicative of perfectly anaerobic condition.

b) Composition of salt solutions

The composition of salt solutions (Bauchop and Mountfort, 1981) are given in Tables 3.2 and 3.3

Table 3.2 Composition of salt solution A:

| Salt | Quantity (w/v) (%) |
|---|--------------------|
| KH ₂ PO ₄ | 0.30 |
| NaCl | 0.60 |
| (NH ₄) ₂ SO ₄ | 0.30 |
| CaCl ₂ | 0.03 |
| MgSO ₄ | 0.03 |

Table 3.3 Composition of salt solution B

| Salt | Quantity (w/v) (%) |
|---------------------------------|--------------------|
| K ₂ HPO ₄ | 0.30 |

c) 0.05 percent hemin solution

0.05 g hemin powder was dissolved in a 100 ml solution of 1:1 ethanol and 0.05 M sodium hydroxide solution (2 g NaOH dissolved in one litre distilled water).

d) Clarified rumen fluid (CRF) for the medium

The rumen liquor was collected at zero hour from fistulated crossbred male calf (app. 250 kg body weight, fed with a ration consisting of one kg concentrate mixture, 10 kg non-leguminous green fodder and. wheat straw ad lib) in thermos flask flushed with CO₂, strained through four layers of muslin cloth. The rumen liquor was clarified by

through centrifugation at 12,000 rpm for 10 minutes and kept at -5°C before being added to the medium. The clarified rumen fluid was used to provide additional nutrients for the growth of the anaerobic fungus.

3.1.2.2. Antibiotic solutions

a) Penicillin stock solution

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

b) Streptomycin sulphate stock solution

Streptomycin sulphate stock solution was prepared at a concentration of 2 mg/ml and sterilized through membrane filtration (0.22 μm pore diameter). This was gassed through oxygen free carbon dioxide before use and 0.25 ml of this was dispensed into each roll tube.

3.1.2.3. Culturing of fungal isolates using roll tube method

For roll-tube method, the media (7 ml) was first dispensed into roll-tubes (7.5 x 1.5 cm) flushed with CO_2 for 10-15 minutes under aseptic conditions and finally the roll tubes were fitted with butyryl rubber caps and autoclaved. Antibiotic solutions were added to the sterile roll tubes containing molten agar media under aseptic conditions. These roll tubes were then kept in water-bath ($45\text{-}50^{\circ}\text{C}$) before being used for culturing of anaerobic fungi.

The selected *Neocallimastix* sp. GR-1, *Orpinomyces* sp. C-14 and *Piromyces* sp. WNG-12 culture (0.5 ml inoculum) at desired level of dilution was added in sterile roll tubes containing molten agar media and antibiotic solutions. The tubes were then rolled over platform of cold water to solidify the media uniformly as a thin film on the inner wall of the roll tubes. Then, the oxygen free CO_2 was passed through the roll tubes from Gassing Manifold Biosystem. The tubes were then kept in CO_2 incubator at a temperature of 39°C . During incubation, the roll tubes were regularly inspected for appearance of colonies visible through naked eye and the numbers of colonies were counted.

3.1.2.4. Culture purity

The purity of cultures was examined regularly under phase-contrast microscope on the basis of morphological characteristics i.e. nature of growth, rhizomycelia, nature of stalk, shape of sporangia, zoospore flagellation and confirmed by re-isolation on solid media until single type of colony was obtained. The cultures were also checked for unwanted growth, if any, under aerobic conditions.

3.1.2.5. Preparation of media with altered composition

Anaerobic media (Table 3.1) formulations were modified for induction of sporulation in order to obtain more number of zoospores with change in concentrations of cellobiose (100 mg and 400 mg) and sugarcane bagasse (100 mg, 200 mg and 400 mg).

3.1.2.6. Inoculation of fungal culture in altered media

Attempts were made to promote the zoosporogenesis by inoculating fungal cultures (*Neocallimastix* sp. GR-1, *Orpinomyces* sp. C-14 and *Piromyces* sp. WNG-12) into media with changed composition of essential nutrients (cellobiose) and/ or by incorporating complex substrates (i.e. SB) for the growth of anaerobic fungal culture, so as to get more number of zoospores/ zoosporangium. Finally, a single media showing maximum/ significant efficiency to produce zoospores and/or growth in response to different nutrients in media was selected and used for further *in vitro* studies.

PHASE – II

3.2. IN VITRO DIGESTIBILITY OF SUGARCANE BAGASSE AND SUGARCANE BAGASSE BASED TOTAL MIXED RATION (TMR)

3.2.1. Preparation of total mixed ration.

Total mixed ration was prepared by mixing sugarcane bagasse, wheat straw, concentrate mixture in the ratio of 20:30:50 having CP 13% and TDN 55%.

3.2.2. McDougall's buffer

The McDougall's buffer solution was prepared by mixing the ingredients given in the table 3.4, except CaCl₂ which was added at the end. Thereafter, buffer was saturated with CO₂ until became clear (pH, 6.9)

Table 3.4 Composition of McDougall buffer*

| | |
|---|---------------|
| Sodium hydrogen carbonate (NaHCO ₃) | 9.80 g |
| Disodium hydrogen carbonate (Na ₂ HPO ₄) | 9.30 g |
| Sodium chloride (NaCl) | 0.47 g |
| Potassium chloride (KCl) | 0.57 g |
| Calcium chloride (CaCl ₂) | 0.04 g |
| Magnesium sulphate (MgSO ₄ .7H ₂ O) | 0.12 g |
| Distilled water | Up to 1 liter |

*McDougall (1948)

3.2.3. Strained rumen liquor (SRL)

Rumen liquor was collected from two male crossbred bulls fitted with permanent rumen fistula. It was stirred for few minutes and strained through 4 layers of muslin cloth and used subsequently as a source of inoculum.

3.2.4. Sterile strained rumen liquor (Sterile SRL)

SRL was sterilized in autoclave at a pressure of 15 psi and temperature 121⁰C for 15 minutes.

3.2.5. *In vitro* digestibility experiments

In vitro digestibility of sugarcane bagasse and sugarcane bagasse based total mixed ration (TMR) will be carried out by Tilley and Terry (1963) method.

a) *In vitro* digestibility of sugarcane bagasse

1g sugarcane bagasse was taken in incubation flasks of 100 ml capacity along with 40 ml McDougall's buffer, 10 ml SRL and 5ml of selected anaerobic fungal zoospores (2 x 10³ zoospores/ ml). The following treatments were made.

- **T1(C)** - sugarcane bagasse + buffer + SRL + 5 ml broth without fungal zoospores

- **T1(A)** - sugarcane bagasse + buffer + SRL + 5 ml broth containing zoospores of *Neocallimastix* sp. GR1
- **T1(B)** - sugarcane bagasse + buffer + SRL + 5ml broth containing zoospores of *Piromyces* sp. WNG 12

b) *In vitro* digestibility of sugarcane bagasse based total mixed ration

Total mixed ration (1 gm) was taken in incubation flasks (100 ml capacity) along with 40 ml McDougall's buffer, 10 ml SRL and 5ml of selected anaerobic fungal zoospores ($\approx 10^6$ zoospores/ml). The following treatments were made.

- **T2(C)** - TMR + buffer + SRL + 5 ml broth without fungal zoospores
- **T2(A)** - TMR + buffer + SRL + 5 ml broth containing zoospores of *Neocallimastix* sp. GR1
- **T2(B)** - TMR + buffer + SRL + 5ml broth containing zoospores of *Piromyces* sp. WNG 12

3.2.6. Incubation period and further processing

In the above experiments, three replicates were taken. Before incubation, all flasks were flushed thoroughly with CO₂ gas and flasks were sealed immediately with Bunsen valve. The flasks were then placed in an incubator maintained at 39 °C. All the flasks were incubated for 48 h. at the end of incubation saturated mercuric chloride was added to each flask to arrest the microbial activity. The content of flask was centrifuged and supernatant was used for estimation of TVFA and activity hydrolytic enzymes.

3.2.7. Estimation of total volatile fatty acids (TVFA)

TVFA concentration was estimated by the method of (Barnett and Reid, 1957). One ml of sample taken into Markham's distillation apparatus was steam distilled with 1 ml of oxalate buffer containing 10 percent potassium oxalate and 5 percent 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 as an end point.

$$\text{TVFA (mM/100 ml SRL)} = \frac{\text{Vol. of N/100 NaOH used} \times \text{strength of NaOH}}{\text{Vol. of SRL taken}} \times 100$$

3.2.8. Enumeration of Zoospores

Zoospores were enumerated from the samples of fungal colonies in the roll-tubes and inoculated broth media using phase-contrast microscope, stained with lactophenol cotton blue.

3.2.9. Hydrolytic enzyme activities

3.2.9.1. Preparation of standard curve

For preparing standard curve for CMCase and FPase, known aliquots of 0.1, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/ml of glucose solution were taken, in duplicate, in test tubes and the volume was made to 1 ml with double distilled water. To each tube, 3 ml DNS reagent was added. This was kept in boiling water bath for 15 min for colour development. After cooling, the intensity of brownish red colour developed was measured 575 nm using a spectrophotometer. The absorbance was plotted against concentration of glucose ($\mu\text{g/ml}$) for obtaining standard curve.

For xylanase activity, similar concentration of xylose was taken instead of glucose and standard curve was drawn.

3.2.9.2. Units of enzyme activities

CMCase, FPase and xylanase activities were expressed in IU, i.e., μmol of glucose or xylose released per ml of culture filtrate (enzyme solution).

Figure 3.1 Standard curve for Glucose

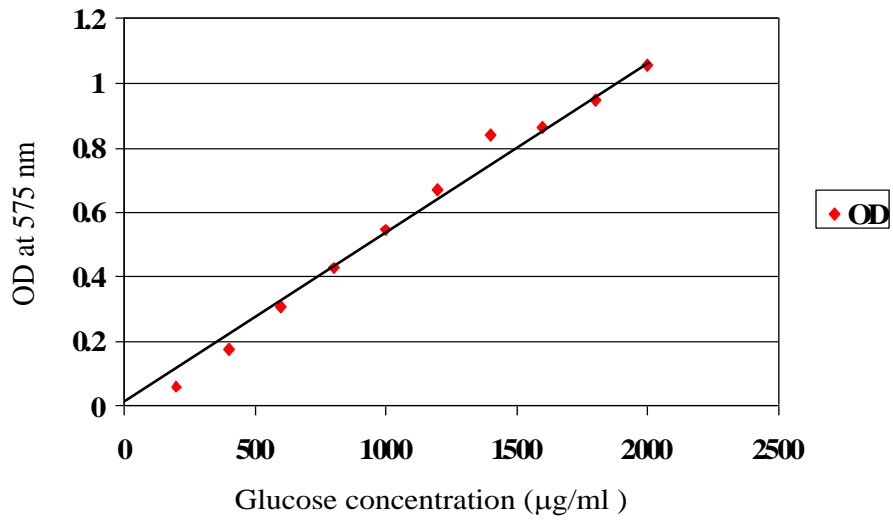
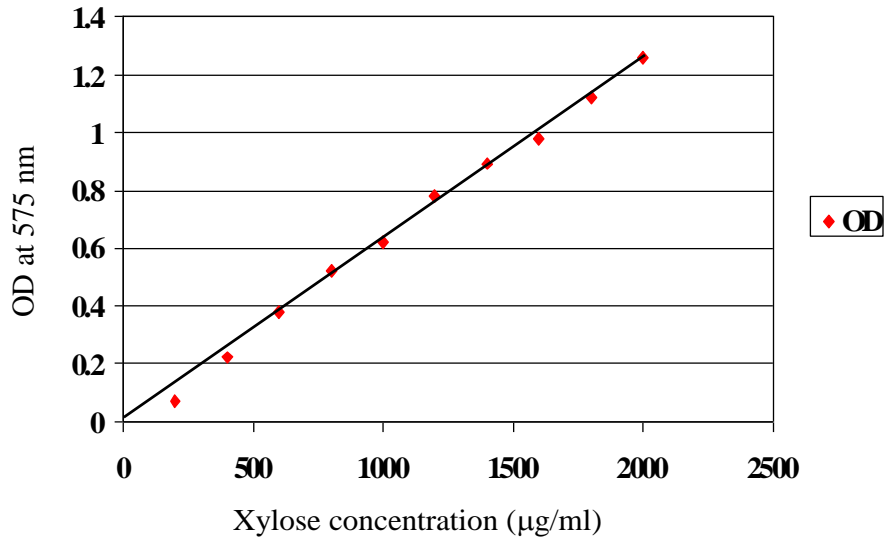


Figure 3.2 Standard curve for Xylose



3.2.9.3. Procedures for the estimation of enzyme activities

3.2.9.3.1. CMC_{Case} activity

In a test tube, 0.5 ml of supernatant (see 3.2.6) was mixed with 4.5 ml of 0.05 M citrate buffer containing 1 percent CMC. The mixture was incubated at 50⁰C for 30 min. One ml of this mixture was assayed for reducing sugars (Mandal *et al.*, 1976; Srinivasan *et al.*, 2001).

3.2.9.3.2. FPase activity

Add 1 ml of 0.05 M citrate buffer in 0.5 ml of supernatant (see 3.2.6). Then 1 x 6 cm strip (50 mg) of filter paper (Whatman No. 1) was placed in the tubes and incubated at 50⁰C for 30 min. One ml of this mixture was assayed for reducing sugars (Mandal *et al.*, 1976; Srinivasan *et al.*, 2001).

3.2.9.3.3. Xylanase activity

One ml of supernatant (see 3.2.6) was mixed with 2.5 ml of 0.5 percent xylan solution and 2.5 ml of 0.05 M acetate buffer. The mixture was incubated at 50⁰C for 30 min. One ml aliquot from this mixture was assayed for reducing sugars (Kawaminami and Iizulka, 1970; Srinivasan *et al.*, 2001).

3.2.9.4. Assay for reducing sugars (DNS method)

CMC_{Case}, FPase and xylanase activities were assayed for reducing sugars by DNS method. DNS reagent (3ml) was added to 1 ml of the reaction mixture in a test tube. This was then kept in boiling water bath for 15 min for colour development. After cooling, the color intensity was measured at 575 nm. The concentration of the product (reducing sugar) formed was read from the standard curve.

3.2.10. Estimation of *in vitro* dry matter digestibility (IVDMD)

After the incubation of substrates for 48 h, samples from each trial were centrifuged at 5000 rpm for 10 minutes the pellets were dried at 100⁰C for 24 h (Tilley and Terry, 1963)

3.2.12. Estimation of *in vitro* neutral detergent fiber digestibility (IVNDFD)

The sample obtained after IVDMD was taken into a 1000 ml spout-less beaker. To this, 100 ml neutral detergent solution, 2 ml decalin and 0.5 g sodium sulphate were added and the contents of spout-less beaker were refluxed on hot plate for one hour. After refluxing, the sample was filtered through pre-weighed 50 ml capacity sintered glass crucible grade-I using vacuum pump. Residue was washed with hot boiling water and then acetone to remove all salts and moisture. The sintered crucible containing residue was dried in hot air oven at 100 °C and weighed.

The NDF was determined as follows:

$$NDF \% = \frac{(Wt. \text{ of crucible } + \text{ cell wall constituents }) - Wt \text{ of empty crucible}}{Wt. \text{ of sample taken}} \times 100$$

3.2.13. *In vitro* acid detergent fiber digestibility (IVADFD)

The sample obtained after IVDMD treatment was taken in a spout-less beaker of 1 L capacity. To this, 100 ml acid detergent solution and 2 ml decalin were added and the contents were refluxed on hot plate for 1 hour. After refluxing the residue was filtered through pre-weighed sintered glass crucible grade-I using vacuum pump, washed with hot water and then with acetone to remove all the salts. Then the residue was dried in hot air oven at 100°C and weighed.

The ADF was determined as follows:

$$ADF \% = \frac{(Wt. \text{ of crucible } + \text{ fibre }) - \text{Empty wt. of crucible}}{Wt. \text{ of sample taken}} \times 100$$

3.2.14. Statistical Analysis

All the data was subjected to the statistical analysis as per (Snedecor and Cochran, 1980).

4. RESULTS & DISCUSSION

In present study the fungal cultures i.e. *Neocallimastix sp.*, GR1; *Piromyces sp.*, WNG12 and *Orpinomyces sp.*, C14 isolated from grazing and browsing goat, cattle and wild blue bull, respectively were grown and checked for purity before being used further in the experiment. These cultures were also attempted to grow in stressed conditions so as to initiate the maximization of zoosporegenesis. Finally these zoospores were administered to see their effect on *in vitro* digestibility of sugarcane bagasse and sugarcane bagasse based total mixed rations.

4.1. PROCUREMENT OF SUBSTRATE AND REVIVAL OF EXISTING RUMINAL FUNGI

4.1.1. Chemical composition of sugarcane bagasse and total mixed ration

Results of proximate principles as well as cell wall constituents of SB and TMR on DM basis have been presented in Table 4.1.

4.1.1.1. Proximate analysis of sugarcane bagasse and total mixed ration

The organic matter content of SB was 97.79 ± 1.29 , that was similar to that of SB reported by Huque and Rahman (2002) and Ready *et al.* (2002). Average CP content of SB was 3.06 ± 0.25 percent; indicating that SB as a source of roughage was low in protein content. Similar CP content of SB (Amjed *et al.*, 1992; Kaur and Kaushal, 2001; Reddy *et al.*, 2002) has been reported. The EE and NFE contents of SB were 1.69 ± 0.02 and 50.11 ± 0.45 respectively. Similarly OM, CP, EE, NFE and CF contents of WS that were 92.73, 3.88, 2.14, 41.16 and 37.75 percent (Tyagi and Singhal, 2001). This showed that WS and SB both are low in protein content.

Average crude fiber of SB was 43.38 ± 0.62 , where as WS contained 41.30 percent of CF (Fardin 2005), showing that SB contained higher crude fiber than that of WS. However, Reddy *et al.* (2002) and Tinh *et al.* (2001) reported 41.70 and 49.66

percent crude fiber in SB. The variation in present values and reported values may be attributed to the differences in climate, plant variety and processing method.

A TMR consisted of concentrate mixture 50, wheat straw 30 and sugarcane bagasse 20 percent was used for the *in vitro* digestibility studies. The OM content of TMR was 92.75 ± 0.65 percent. TMR contained higher level of CP (12.68 ± 0.25) and EE (3.33 ± 0.16) as compared to SB. The CF and NFE content of TMR was 26.79 ± 0.66 and 49.95 percent respectively. Thus total mixed ration supplied higher level of nitrogen as well as readily available energy to the microbes for rapid growth.

4.1.1.2. Cell wall constituents of sugarcane bagasse and total mixed ration

Neutral detergent fiber (NDF) content of SB and TMR was 89.51 ± 0.75 and 62.51 ± 0.37 percent, respectively (Table 4.1). The NDF content of SB was higher than that of TMR. Amjed *et al.* (1992) and Kaur and Kaushal (2001) reported similar NDF content

Table 4.1 Chemical composition of sugarcane bagasse and total mixed ration (%DM basis)

| Particulars | SB | TMR |
|----------------|------------------|------------------|
| DM | 92.02 ± 1.29 | 89.16 ± 0.45 |
| OM | 97.79 ± 0.18 | 92.75 ± 0.65 |
| CP | 3.06 ± 0.25 | 12.68 ± 0.25 |
| CF | 43.38 ± 0.62 | 26.79 ± 0.66 |
| EE | 1.69 ± 0.02 | 3.33 ± 0.16 |
| Ash | 2.21 ± 0.18 | 7.25 ± 0.20 |
| NFE | 50.11 ± 0.45 | 49.95 |
| NDF* | 89.51 ± 0.75 | 62.51 ± 0.37 |
| ADF* | 52.65 ± 0.45 | 35.08 ± 0.27 |
| ADL | 10.42 ± 0.52 | 6.32 ± 0.25 |
| AIA | 1.65 ± 0.12 | 2.96 ± 0.32 |
| Hemi-cellulose | 36.86 | 27.43 |
| Cellulose | 40.58 | 25.8 |

* Determined on ash-free basis

in SB. NDF in SB has been reported in the range of 69.77 (Reddy *et al.*, 2002) to 90.16 percent (Tinh *et al.*, 2001), indicating that NDF content in SB varied significantly and influenced largely by the type of processing method and variety of sugarcane used for the sugar extraction. The NDF content of WS has been reported to vary in the range of 74.28 to 83.50 percent (Sahoo, 2002).

Acid detergent fiber (ADF) of SB and TMR showed the similar trend as recorded for NDF content. The ADF content of SB and TMR was 52.65 ± 0.45 and 35.08 ± 0.27 percent, respectively. Fardin (2005) reported that ADF content of SB was 53.41 percent.

Hemi-cellulose and cellulose contents of SB were 36.86 and 40.58 percent, respectively. Corresponding values for TMR were 27.43 and 25.8. However, hemi-cellulose and cellulose contents of WS were 30.12 and 39.45 (Fardin 2005). These observations indicated that WS and SB both were having similar hemi-cellulose and cellulose contents as reported by Rangnekar *et al.* (1982). However, Reddy *et al.* (2002) reported lower value and Tinh *et al.* (2001) recorded higher value for SB than reported in present study. These observations indicated that SB was having higher hemicellulose as well as cellulose contents than TMR.

Acid detergent lignin (ADL) content (%) of SB and TMR was 10.42 ± 0.52 and 6.32 ± 0.25 , respectively. However, ADL content of WS was 7.46 (Fardin *et al.*, 2005), showing higher lignin content in SB than WS and TMR. Lignin content of SB was similar to the value reported by Rangnekar *et al.* (1982) and Kaur and Kaushal (2001).

4.1.2. Revival and maintenance of anaerobic rumen fungal isolates

Three anaerobic ruminal fungal isolates viz. *Neocallimastix* sp. GR1 (Thareja *et al.*, 2006), *Orpinomyces* sp. C-14 (Singhal *et al.*, 2000) and *Piromyces* sp. WNG-12 (Tripathi *et al.*, 2007) were obtained from Fungal Biotechnology Laboratory, Dairy Microbiology Division, NDRI, and were revived using Hungate's roll-tube technique as described by Joblin (1981). These three cultures were tested for identical morphology based on their zoospores flagellation, rhizomycelium and shape of sporangium as characteristics of parent cultures.

Neocallimastix sp. GR1 had monocentric thallus and polyflagellated zoospores. Sporangium was globular in shape with stalk at the base. Rhizomycellia were thick and unsegmented (Plate 4.1). *Piromyces* sp. WNG12 had monocentric thallus and uniflagellated zoospores. Sporangium was ellipsoidal pyriform (Plate 4.2). *Orpinomyces* sp. C14 showed polycentric thallus and globose sporangium, with unsegmented rhizoids (Plate 4.3). The microscopic view of zoospores released in culture media is shown in Plates 4.4, 4.5 and 4.6.

These three fungal cultures were revived by inoculating in screw capped tubes (20 ml capacity) containing sterilized anaerobic fungal media (Joblin, 1981) along with required quantity of antibiotic solution to prevent bacterial contamination. Rees *et al.* (1998) reported that a minimum of 7 percent CO₂ in the gas phase was needed for optimum growth of rumen fungi and this also increased the acetate, lactate and hydrogen production, which enhanced the fungal population. Along with the concentration of CO₂, clarified rumen liquor (CRL) was used in the culture medium to provide unidentified growth factors to these anaerobic fungi. The mother cultures were maintained by inoculating them into fresh autoclaved anaerobic media at every 4th or 5th day.

4.1.2.1. Inoculation of fungal cultures in media with altered composition

The deficient anaerobic media was prepared for induction of sporulation in order to obtain more number of zoospores by changing the quantity of cellobiose @ 100 mg and 400 mg instead of 200 mg/ 100ml and also by adding sugarcane bagasse (@ 100, 200 and 400 mg/ 100ml) as a substrate instead of cellobiose.

Attempts were made to promote the zoosporogenesis by inoculating fungal cultures (*Neocallimastix* sp. GR-1, *Orpinomyces* sp. C-14 and *Piromyces* sp. WNG-12) into different deficient and normal media, for the growth of anaerobic fungal cultures so as to get more number of zoospores/ zoosporangium. After 96 hours of incubation, the zoospores stained with lactophenol cotton blue were counted using phase contrast microscope. It was found that after 96 hours normal media contained more zoospores as compared to that of modified media. This suggested that cellobiose (200 mg/ 100ml) as a substrate and a longer period of inoculation (96h) for enhancing the zosprogenesis were optimal. However, the normal media was used for further growth of fungal zoospores in

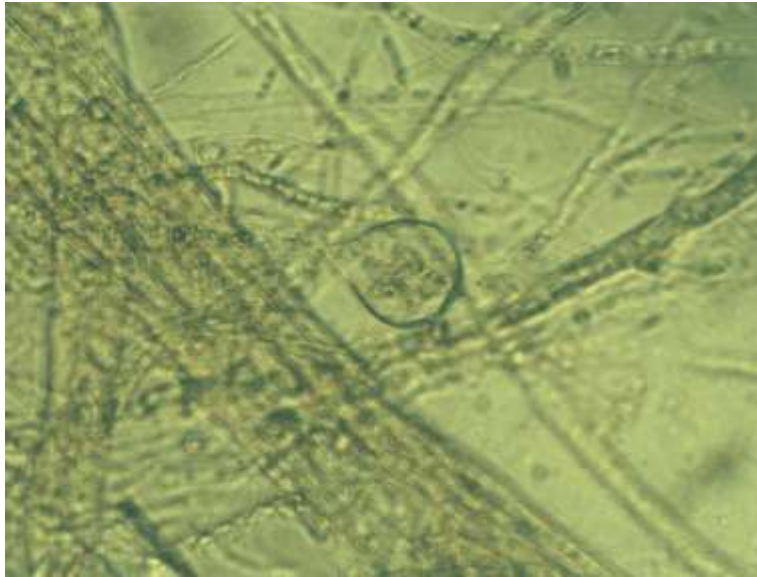


Plate 4.1 *Neocallimastix* sp. GR-1: Monocentric, globose ovoid sporangia, septed rhizoid.



Plate 4.2 *Piromyces* sp. WNG-12: Monocentric, ellipsoidal pyriform sporangia, non-frequent septum.

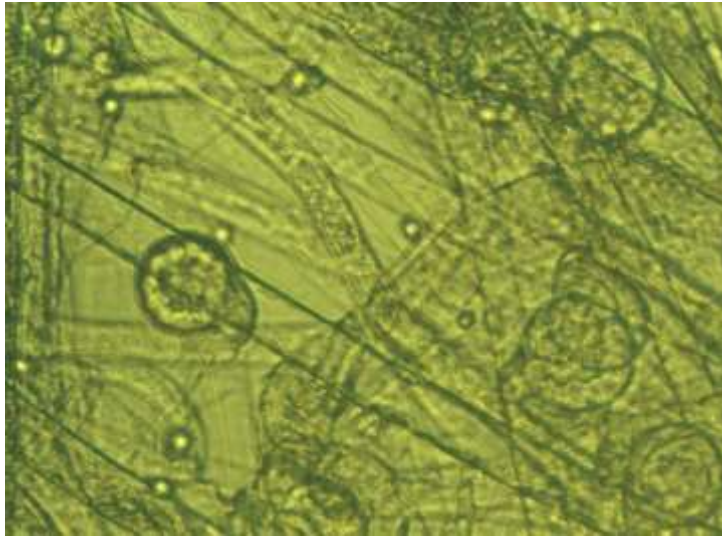


Plate 4.3 *Orpinomyces* sp. C-14: Polycentric, globose sporangia, unsegmented rhizoids.

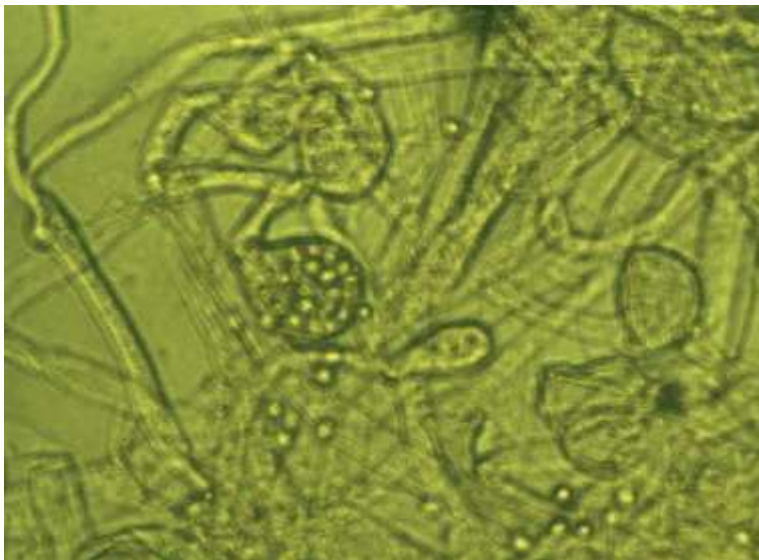


Plate 4.4 *Neocallimastix* sp. GR1 releasing zoospores.

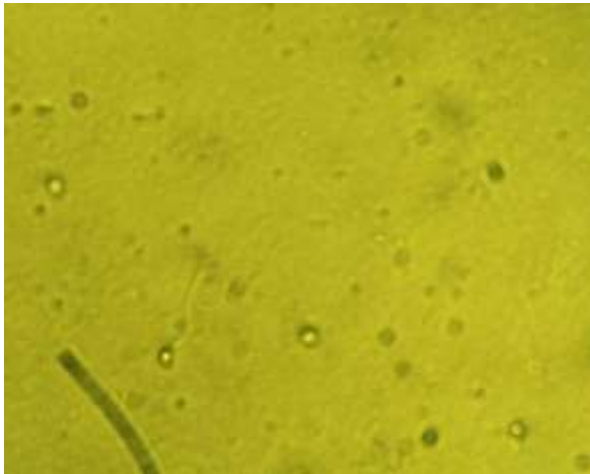


Plate 4.5 Released zoospores in the culture media.

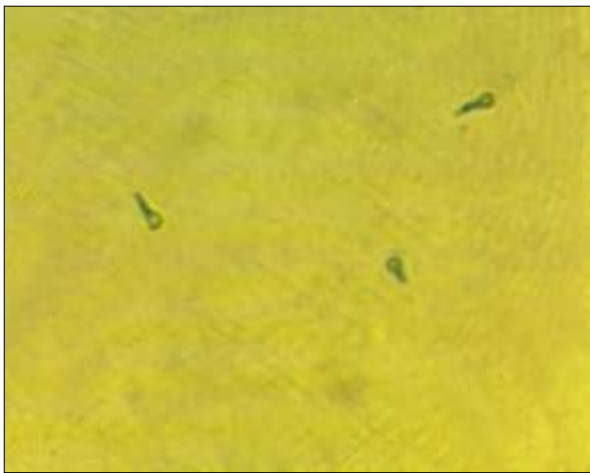


Plate 4.6 Stained zoospores released in the culture media.

substrate and a longer period of inoculation (96h) for enhancing the zosprogenesis were optimal. However, the normal media was used for further growth of fungal zoospores in bulk by incubating fungal cultures for 96 hours. The two fungal cultures i.e. *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12 produced more number of zoospores as compared to *Orpinomyces* sp. C-14. The fungi having polycentric sporangium like *Orpinomyces* sp. C-14 produced less number of zoospores as compared to monocentric fungi. Thus, two fungal cultures i.e. *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12 were used for *in vitro* digestibility studies using sugarcane bagasse and sugarcane bagasse based ration as substrates.

4.2. IN VITRO DIGESTIBILITY OF SUGARCANE BAGASSE AND SUGARCANE BAGASSE BASED TOTAL MIXED RATION (TMR)

4.2.1. In vitro dry matter digestibility (IVDMD)

Results of *in vitro* dry matter digestibility (IVDMD) are presented in Table 4.2. In treatment T1(C), where SB, SRL and 5 ml broth without fungal zoospores were added, digestibility of SB was 22.48 ± 0.54 percent, which after addition of zoospores of *Neocallimastix* GR1 i.e. T1(A) increased to statistically significant ($P < 0.01$) values of 27.98 ± 0.38 percent. In treatment T2(C), TMR, SRL and broth without fungal zoospores were incubated, digestibility of TMR was 47.99 ± 0.53 percent. After addition of zoospores of *Neocallimastix* GR1 i.e. T2(A), the IVDM digestibility increased significantly ($P < 0.01$) to 52.38 ± 0.54 percent (Table 4.2).

In treatment T1(C), where only SB, SRL and 5 ml broth without fungal zoospores were added, digestibility of SB was 22.82 ± 0.39 percent. After addition of zoospores of *Piromyces* WNG12 i.e. T2 (B), the IVDMD increased to 26.51 ± 2.92 percent. The differences were significant statistically. In treatment T2(C), TMR, SRL and broth without fungal zoospores were added. The IVDMD of TMR was 49.61 ± 0.63 percent. However, after addition of zoospores of *Neocallimastix* GR1 i.e. T2 (A), the dry matter digestibility increased to 52.84 ± 0.61 which was significantly ($P < 0.01$) higher than the control values. Thus, the results indicated that the incorporation of fungal zoospores led to a significant increase in the *in vitro* dry matter digestibility of sugarcane bagasse as well as sugarcane bagasse based total mixed rations. However, no significant differences were observed between the IVDMD by incorporating the zoospores of *Neocallimastix*

GR1 and *Piromyces* WNG12. The efficiency of degradation of DM was quite comparable with the earlier observations (Manikumar *et al.*, 2004) where the fungal cultures were administered. The administration of fungal zoospores seemed to be more feasible way to enhance the DM digestibility of fibrous roughages/ or roughage based rations.

Table 4.2 Effect of fungal zoospores on *in vitro* dry matter digestibility of sugarcane bagasse and sugarcane bagasse based ration.

| Treatment | Percent IVDMD | |
|--|---------------------------|------------------------|
| | Incubation Time (48 h) | |
| | <i>Neocallimastix</i> GR1 | <i>Piromyces</i> WNG12 |
| SB + Buffer + SRL + Broth without fungal zoospores | 22.48 ±0.54 | 22.82 ±0.39 |
| SB + Buffer + SRL + Broth containing fungal zoospores | 27.98 ±0.38** | 26.51 ±2.92** |
| TMR + Buffer + SRL + Broth without fungal zoospores | 47.99 ±0.53 | 49.61 ±0.63 |
| TMR + Buffer + SRL + Broth containing fungal zoospores | 52.38 ±0.54** | 52.84 ±0.61** |

** Significant (P<0.01)

4.2.2. Neutral detergent fiber (NDF)

Results of *in vitro* NDF disappearance of SB and SB based ration are presented in Table 4.3. NDF represents the cell wall contents portion of a fiber. In treatment T1(C), where only SRL was taken as the source of rumen microorganisms, percent NDF disappearance was 17.62 ±0.38 after 48 hours. But after addition of zoospores of *Neocallimastix* GR1 i.e. T1 (A) to the incubation flask it increased to 28.05 ±0.71

percent, which differed significantly ($P<0.01$) from the control values. In treatment T2(C), percent NDF disappearance was 31.301 ± 0.84 . After addition of zoospores of *Neocallimastix* GR1 i.e. T2 (A) it increased to significant ($P<0.05$) values of 39.00 ± 0.83 percent. However, after addition of zoospores of *Piromyces* WNG12 i.e. T2 (A), the percent NDF disappearance increased to significant ($P<0.01$) levels of 26.05 ± 0.54 as compared to control (19.34 ± 0.66). In treatment T2(C), in which TMR, broth without fungal zoospores and SRL were incubated, the percent NDF disappearance was 33.85 ± 0.49 which increased significantly ($P<0.01$) to 37.44 ± 0.50 percent after addition of zoospores of *Piromyces* WNG12 (T2(B)). The increase in percent NDF disappearance was higher for T1 (A) i.e. sugarcane bagasse (28.05 ± 0.71) and SB based total mixed rations (39.00 ± 0.83) with addition of zoospores of *Neocallimastix* GR1, showing that it was better than *Piromyces* WNG12.

Table 4.3 Percent disappearance of neutral detergent fiber (NDF) of sugarcane bagasse and sugarcane bagasse based ration using zoospores of fungi.

| Treatment | Percent IVNDF | |
|--|---------------------------|------------------------|
| | Incubation Time (48 h) | |
| | <i>Neocallimastix</i> GR1 | <i>Piromyces</i> WNG12 |
| SB + Buffer + SRL + Broth without fungal zoospores | 17.62 ± 0.38 | 19.34 ± 0.66 |
| SB + Buffer + SRL + Broth containing fungal zoospores | $28.05 \pm 0.71^{**}$ | $26.05 \pm 0.54^{**}$ |
| TMR + Buffer + SRL + Broth without fungal zoospores | 31.301 ± 0.84 | 33.85 ± 0.49 |
| TMR + Buffer + SRL + Broth containing fungal zoospores | $39.00 \pm 0.83^*$ | $37.44 \pm 0.50^{**}$ |

**** Significant ($P<0.01$)**

*** Significant ($P<0.05$)**

Through earlier studies it has been observed that administration of elite fungal cultures led to a significant increase in the percent disappearance of NDF contained in RS or WS and TMR based on RS and WS (Manikumar *et al.*, 2004; Thareja *et al.*, 2006; Dayananda *et al.*, 2005).

The present studies have indicated that fungal zoospores are quite comparable in leading to disappearance of NDF contained in SB or SB based TMR under an in vitro system.

4.2.3. Acid detergent fiber (ADF)

ADF represents all the cell wall components of plants without hemicellulose. In treatment T1(C), after 48 hours of incubation the percent ADF disappearance was 23.44 ± 0.70 . After addition of zoospores of *Neocallimastix* GR1, the percent disappearance increased significantly ($P < 0.01$) to 31.18 ± 1.26 (Table 4.4). In treatment T2(C), percent ADF disappearance was 34.44 ± 1.01 in control where zoospores were not administered, but after addition of zoospores of *Neocallimastix* GR1 i.e. T2(A) the percent disappearance of ADF increased significantly ($P < 0.01$) to 47.90 ± 0.90 . Addition of zoospores of *Piromyces* WNG12 i.e. T2 (A), percent ADF disappearance also increased significantly ($P < 0.01$) to 29.27 ± 0.78 as compared to control (22.47 ± 0.61). In treatment T2(C), in which TMR, broth without fungal zoospores and SRL were inoculated, the percent ADF disappearance was 32.07 ± 0.61 and addition of zoospores of *Piromyces* WNG12 i.e. T2(B) led to a significant ($P < 0.01$) increase in disappearance of ADF (42.29 ± 0.83). The increase in percent ADF disappearance was higher for sugarcane bagasse (31.18 ± 1.26) after addition of zoospores of *Neocallimastix* GR1, as compared to *Piromyces* WNG12 (29.27 ± 0.78), and similarly percent ADF disappearance of TMR by addition of *Neocallimastix* GR1 zoospores was higher (47.90 ± 0.90) than that obtained by adding zoospores of *Piromyces* WNG12 (42.29 ± 0.83). The results showed that *Neocallimastix* GR1 was a better fungus than *Piromyces* WNG12 (Debanujit *et al.*, 2006).

The disappearance of ADF correlates positively to the degradation and utilization of highly complex polysaccharides present in dry roughages. Thus through present study, it was shown that by the incorporation of fungal zoospores, complex polysaccharides

present in SB could be utilized to release energy which can be further used by the animals for increasing their productive performance (Dayanand *et al.*, 2005; Thareja *et al.*, 2006).

Table 4.4 Percent disappearance of acid detergent fiber (ADF) of sugarcane bagasse and sugarcane bagasse based ration using zoospores of fungi.

| Treatment | Percent IVADF | |
|--|---------------------------|------------------------|
| | Incubation Time (48 h) | |
| | <i>Neocallimastix</i> GR1 | <i>Piromyces</i> WNG12 |
| SB + Buffer + SRL + Broth without fungal zoospores | 23.44±0.70 | 22.47 ±0.61 |
| SB + Buffer + SRL + Broth containing fungal zoospores | 31.18 ±1.26** | 29.27 ±0.78** |
| TMR + Buffer + SRL + Broth without fungal zoospores | 34.44 ±1.01 | 32.07 ±0.61 |
| TMR + Buffer + SRL + Broth containing fungal zoospores | 47.90 ±0.90** | 42.29 ±0.83** |

** Significant (P<0.01)

4.2.4. Total volatile fatty acid (TVFA)

The results of total VFA's (mM/ 100ml) under *in vitro* system have been presented in Table 4.5 and figure 4.2. The TVFA's (mM/ 100ml) were found to be less in control groups i.e. T1(C) and T2(C) than zoospores administered treatment groups. In treatment T1(C), TVFA content was 8.91 ±0.21. After addition of zoospores of *Neocallimastix* GR1 the TVFA content increased significantly (P<0.05) to 10.57 ±0.28 mM/100ml at 48 h. of incubation. In treatment T2(C), TVFA content was 11.36 ±0.23 (mM/ 100ml). Addition of zoospores of *Neocallimastix* GR1 i.e. T2 (A) led to significant (P<0.05) increase (13.10±0.31 mM/ 100ml). However, after addition of zoospores of

Piromyces WNG12 i.e. T1(A), TVFA increased to 9.90 ± 0.08 ($P < 0.01$) as compared to control (8.34 ± 0.17). In treatment T2(C), in which TMR, broth without fungal zoospores and SRL were incubated. The TVFA content was 10.50 ± 0.15 . After addition of zoospores of *Piromyces* WNG12, the TVFA content increased significantly ($P < 0.01$) to 12.14 ± 0.15 m/ 100ml. The administrations of fungal cultures have been shown to increase the TVFA production under an in vitro system of degradation of urea ammonia treated wheat straw (Dayananda *et al.*, 2005). Addition of fungal isolates (*Orpinomyces* sp.) in Korean sheep fed on orchard grass based TMR enhanced the nutrient digestibility and TVFA production (Lee *et al.*, 2000). Under the present investigations, the fungal zoospores which might have grown to mature fungus led to a significant increase in fibrous degrading hydrolytic enzymes which help to break the lignocellulosic bonds of complex polysaccharide contained in SB and SB based TMR and thereby led to release of energy in the form of TVFA.

Table 4.5 Effect of addition of fungal zoospores on TVFA (mM/100ml) production under *in vitro* evaluation of different substrates

| Treatment | TVFA | |
|--|---------------------------|------------------------|
| | Incubation Time (48 h) | |
| | <i>Neocallimastix</i> GR1 | <i>Piromyces</i> WNG12 |
| SB + Buffer + SRL + Broth without fungal zoospores | 8.91 ± 0.21 | 8.34 ± 0.17 |
| SB + Buffer + SRL + Broth containing fungal zoospores | $10.57 \pm 0.28^*$ | $9.90 \pm 0.08^{**}$ |
| TMR + Buffer + SRL + Broth without fungal zoospores | 11.36 ± 0.23 | 10.50 ± 0.15 |
| TMR + Buffer + SRL + Broth containing fungal zoospores | $13.10 \pm 0.31^*$ | $12.14 \pm 0.15^{**}$ |

**** Significant ($P < 0.01$), * Significant ($P < 0.05$)**

The anaerobic fungi play an active role in the plant fibre degradation by producing a wide variety of potential hydrolytic enzymes in the rumen.

In treatment T1(A), the addition of zoospores of *Neocallimastix GRI* showed significantly higher ($P<0.05$) level of CMCCase (17.95 ± 0.61 vs 13.71 ± 0.41 mIU/ ml), FPase (12.58 ± 0.75 vs 9.09 ± 0.21 mIU/ ml) and xylanase (29.28 ± 0.94 vs 23.37 ± 0.62 mIU/ml) as compared to that of control. However, in treatment T1(B), the addition of zoospores of *Neocallimastix GRI* showed highest ($P<0.05$) level of CMCCase (23.15 ± 0.38 vs 15.71 ± 0.84 mIU/ ml), FPase (16.85 ± 0.91 vs 16.85 ± 0.91 mIU/ ml) and xylanase (32.29 ± 1.08 vs 25.45 ± 0.32 mIU/ ml) as compared to that of control. The results are shown in Table 4.6.

Table 4.6 Effect of addition of fungal zoospores of *Neocallimastix GR1* on activity of hydrolytic enzymes ($\mu\text{mol/ ml}$)

| Treatment | Hydrolytic Enzyme Activity (48 h) | | |
|--|-----------------------------------|--------------------|--------------------|
| | <i>Neocallimastix GRI</i> | | |
| | CMCase | FPase | Xylanase |
| SB + Buffer + SRL + Broth without fungal zoospores | 13.71 ± 0.41 | 9.09 ± 0.21 | 23.37 ± 0.62 |
| SB + Buffer + SRL + Broth containing fungal zoospores | $17.95 \pm 0.61^*$ | $12.58 \pm 0.75^*$ | $29.28 \pm 0.94^*$ |
| TMR + Buffer + SRL + Broth without fungal zoospores | 15.71 ± 0.84 | 10.73 ± 0.71 | 25.45 ± 0.32 |
| TMR + Buffer + SRL + Broth containing fungal zoospores | $23.15 \pm 0.38^*$ | $16.85 \pm 0.91^*$ | $32.29 \pm 1.08^*$ |

* Significant ($P<0.05$)

In treatment T2(A), the addition of zoospores of *Piromyces* WNG12 showed significantly higher ($P < 0.05$) level of CMCase (17.61 ± 0.41 vs 15.70 ± 0.95 mIU/ ml), FPase (10.91 ± 0.24 vs 7.09 ± 0.36 mIU/ ml) and xylanase (27.85 ± 0.29 vs 21.85 ± 0.42 mIU/ ml) as compared to that of control. However, in treatment T2(B), the addition of zoospores of *Piromyces* WNG12 showed significantly higher ($P < 0.05$) level of CMCase (21.01 ± 0.12 vs 18.36 ± 0.08 mIU/ ml), FPase (13.45 ± 0.80 vs 8.46 ± 0.90 mIU/ ml) and xylanase (30.80 ± 0.35 vs 24.84 ± 0.95 mIU/ ml) as compared to that of control (Table 4.6 and Figure 4.2).

The enzymes tested in the present experiment are the major enzymes responsible for the degradation of lignocellulosic feed. Therefore the increased activity of fibrolytic enzymes resulted in a better in vitro feed degradability. The findings of these results indicate that the addition of fungal zoospores of *Neocallimastix* GR1 and *Piromyces* WNG12 accelerated the rate of fermentation, digestibility of lignocellulosic feeds and the synthesis of enzymes involved in fibre degradation.

4.2.6. Average number of zoospores

In treatment T1(A), after the addition of zoospores of *Neocallimastix* GR1 (2×10^3 zoospores/ ml), number of zoospores increase significantly (3.00×10^3 zoospores/ml) as compare to that of control (1.04×10^3 zoospores/ ml). However, in treatment T2(A), after the addition of zoospores of *Neocallimastix* GR1 (2×10^3 zoospores/ml), number of zoospores increase significantly (3.13×10^3 zoospores/ ml) as compare to that of control (1.08×10^3 zoospores/ ml). In treatment T2(A), after the addition of zoospores of *Piromyces* WNG12 (2×10^3 zoospores/ ml), number of zoospores increase significantly (2.96×10^3 zoospores/ ml) as compare to that of control (1.08×10^3 zoospores/ ml). However, in treatment T2(A), after the addition of zoospores of *Piromyces* WNG12 (2×10^3 zoospores/ ml), number of zoospores increase significantly (3.26×10^3 zoospores/ ml) as compare to that of control (1.10×10^3 zoospores/ ml).

Fig. 4.1 Hydrolytic enzyme activity of Neocallimastix GR1 and piromyces WNG 12

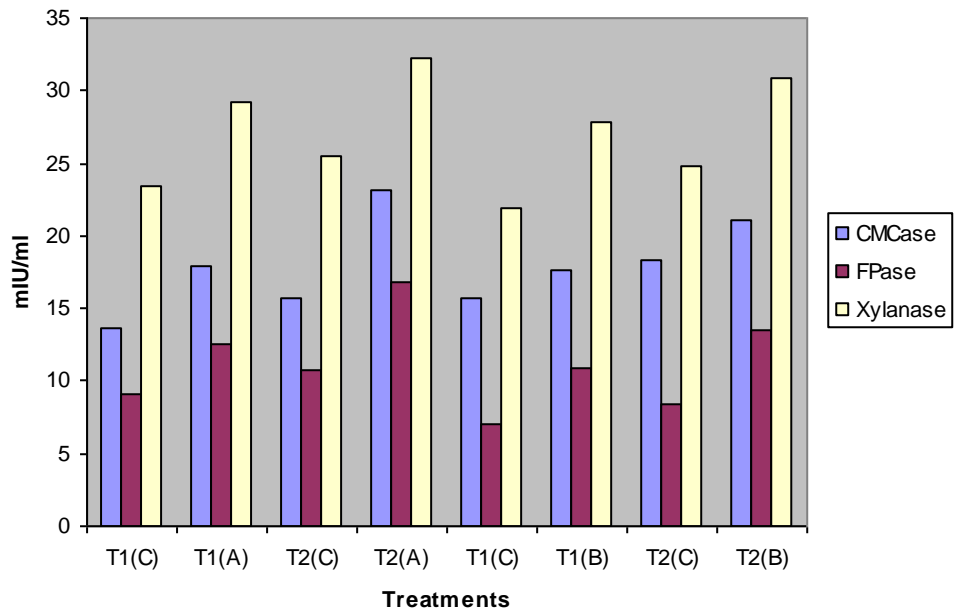


Fig 4.2 Effect of addition of fungal zoospores on TVFA (mM/100ml) production

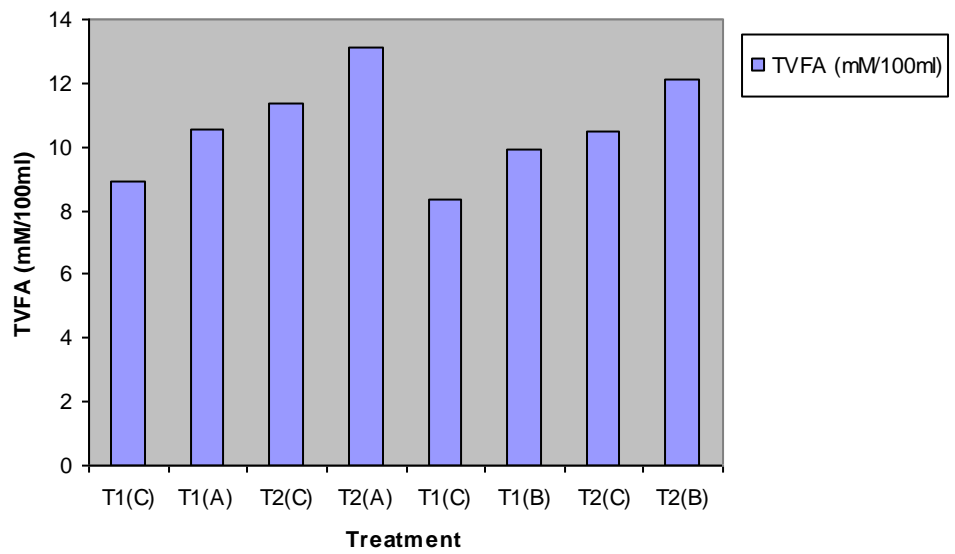


Table 4.7 Effect of addition of fungal zoospores of *Piromyces* WNG12 on activity of hydrolytic enzymes ($\mu\text{mol}/\text{ml}$)

| Treatment | Hydrolytic Enzyme Activity (48 h) | | |
|--|-----------------------------------|-------------------|--------------------|
| | <i>Piromyces</i> WNG12 | | |
| | CMCase | FPase | xylanase |
| SB + Buffer + SRL + Broth without fungal zoospores | 15.70 \pm 0.95 | 7.09 \pm 0.36 | 21.85 \pm 0.42 |
| SB + Buffer + SRL + Broth containing fungal zoospores | 17.61 \pm 0.41* | 10.91 \pm 0.24* | 27.85 \pm 0.29** |
| TMR + Buffer + SRL + Broth without fungal zoospores | 18.36 \pm 0.08 | 8.46 \pm 0.90 | 24.84 \pm 0.95 |
| TMR + Buffer + SRL + Broth containing fungal zoospores | 21.01 \pm 0.12** | 13.45 \pm 0.80* | 30.80 \pm 0.35* |

** Significant (P<0.01), * Significant (P<0.05)

Table 4.8 Average number of zoospores/ ml of fluid after 48 h of incubation

| Treatment | Average number of zoospores/ ml of fluid | |
|--|--|-------------------------------------|
| | Incubation Time (48 h) | |
| | <i>Neocallimastix</i> GR1 | <i>Piromyces</i> WNG12 |
| SB + Buffer + SRL + Broth without fungal zoospores | 1.04 X 10 ³ \pm 0.08 | 1.08 X 10 ³ \pm 0.05 |
| SB + Buffer + SRL + Broth containing fungal zoospores | 3.00 X 10 ³ \pm 0.17** | 2.96 X 10 ³ \pm 0.02** |
| TMR + Buffer + SRL + Broth without fungal zoospores | 1.08 X 10 ³ \pm 0.07 | 1.10 X 10 ³ \pm 0.03 |
| TMR + Buffer + SRL + Broth containing fungal zoospores | 3.13 X 10 ³ \pm 0.06** | 3.26 X 10 ³ \pm 0.09** |

** Significant (P<0.01)

5. SUMMARY & CONCLUSION

In the recent past, inadequate availability and escalating prices of cereal straws alarmed the farmers to look for alternate sources of roughages, therefore in sugarcane growing areas farmers have started feeding to their livestock sugar cane bagasse (SB), a by product of sugar industry, as a source of roughage. But, SB being highly lignified lacks palatability and efficient digestibility. Rumen fungi have shown to play a very important role in fibre degradation by breaking the lignocellulosic bonds through fungal enzymes and letting the cellulose and hemi cellulose exposed and be attacked by rumen bacteria for further break down and release of utilizable energy in the form of volatile fatty acids. There are many reports showing an improvement in the efficiency of nutrient utilization of straw based diets within the ruminant system by administering inoculums of elite ruminal fungi.

The chemical composition of SB is almost similar to WS , therefore digestibility of SB based rations could also be improved by administration of fungi. However, live fungal isolates contained in large volumes of media lack feasibility of administration in animals on day to day basis. Thus, there was a need to transform the fungi to zoospores by augmentation of zoosporogenesis in order to administer the fungi along with the diet of ruminants so that these zoospores on getting a suitable substrate and environment in the rumen will grow into fungus and will be able to degrade and digest the highly fibrous plant tissues. Secondly, availability and low cost of SB demands its use as animal feed. Therefore, it was desirable to evaluate the dry roughage based rations by replacing wheat straw with SB. Thus, the present study was conducted to enhance the zoosporogenesis of elite fungi and to see the effect of administration of those zoospores on the *in vitro* digestibility of sugarcane bagasse and sugarcane bagasse based ration.

Three anaerobic ruminal fungal isolates viz. *Neocallimastix* sp. GR1 (Thareja *et al.*, 2006), *Orpinomyces* sp. C-14 (Manikumar *et al.*, 2004) and *Piromyces* sp. WNG-12 (Tripathi *et al.*, 2007) were obtained from Fungal Biotechnology Laboratory, Dairy

Microbiology Division, NDRI, and were revived using Hungate's roll-tube technique as described by Joblin (1981). These three cultures were tested for identical morphology based on their zoospores flagellation, rhizomycelium and shape of sporangium to the parent cultured characteristics. Attempt was made to promote the zoosporogenesis, so as to get more number of zoospores/ zoosporangium by using media deficient in essential nutrients. The deficient media was prepared by changing quantity of cellobiose (100 mg/100ml) and by incorporating complex substrates (i.e. SB @100, 200 and 400 mg/100ml) in media instead of cellobiose for the growth of anaerobic fungal culture. The cultures were finally selected based on their efficiency to produce zoospores in response to different nutrients in media.

To promote the zoosporogenesis fungal cultures (*Neocallimastix* sp. GR-1, *Orpinomyces* sp. C-14 and *Piromyces* sp. WNG-12) were inoculated into deficient media and normal media. After 96 h of incubation, normal media contained more number of zoospores (2×10^3 zoospores/ ml) as compared to that of deficient media (0.8×10^3 to 1.3×10^3 zoospores/ ml). The normal media was used for further growth of fungal zoospores in bulk with incubation time of 96 hours. Fungal cultures i.e. *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12 (2×10^3 zoospores/ ml) produced more number of zoospores as compared to that of *Orpinomyces* sp. C-14 (0.7×10^3 zoospores/ ml). Therefore, two fungal cultures i.e. *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12 were used for further *in vitro* digestibility studies of SB and SB based ration.

In vitro digestibility of SB and SB based TMR (SB: WS: Conc. mixture, 20: 30: 50) was carried out by Tilley and Terry (1963) method and analyzed for IVDMD, disappearance of ADF and NDF, TVFA concentration, activity of hydrolytic enzymes and average number of zoospores.

IVDMD of sugarcane bagasse increased ($P < 0.01$) to 27.98 ± 0.38 Vs 22.48 ± 0.54 and 26.51 ± 2.92 Vs 22.82 ± 0.39 after addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12 respectively. DM digestibility of total mixed ration also increased ($P < 0.01$) to 52.38 ± 0.54 Vs 47.99 ± 0.53 and 52.84 ± 0.61 Vs 49.61 ± 0.63 after addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12 respectively. The NDF disappearance in sugarcane bagasse

Piromyces sp. WNG-12 respectively. The NDF disappearance in sugarcane bagasse increased ($P < 0.01$) to 28.05 ± 0.71 Vs 17.62 ± 0.38 and 26.05 ± 0.54 Vs 19.34 ± 0.66 by the addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12 respectively. The NDF disappearance in total mixed ration increased significantly ($P < 0.01$) to 39.00 ± 0.83 Vs 31.301 ± 0.84 and 37.44 ± 0.50 Vs 33.85 ± 0.49 by the addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp.

The percent ADF disappearance of sugarcane bagasse was 23.44 ± 0.70 and 22.47 ± 0.61 without addition of fungal zoospores but after the addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12, it increased ($P < 0.01$) to 31.18 ± 1.26 and 29.27 ± 0.78 respectively. In case of total mixed ration, the ADF disappearance increased ($P < 0.01$) to 47.90 ± 0.90 Vs 34.44 ± 1.01 and 42.29 ± 0.83 Vs 32.07 ± 0.61 by the addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp.

The data on the biochemical parameters of the *in vitro* digestibility studies of sugarcane bagasse showed that the total volatile fatty acids (mM/100 ml) were found to be 8.91 ± 0.21 and 8.91 ± 0.21 without addition of fungal zoospores but after the addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12, the TVFA increased ($P < 0.05$) to 10.57 ± 0.28 and 9.90 ± 0.08 respectively. The values for total volatile fatty acids (mM/100 ml) were found to be 11.36 ± 0.23 and 10.50 ± 0.15 without addition of fungal zoospores where the SB based TMR were used as a substrate, but after the addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12, the TVFA increased significantly ($P < 0.05$) to 13.10 ± 0.31 and 12.14 ± 0.15 respectively.

The anaerobic fungi play an active role in the plant fibre degradation by producing a wide range of potential hydrolytic enzymes in the rumen. The hydrolytic enzyme activity of CMCase, FPase and xylanase increased significantly ($P < 0.01$) after the addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12 in the *in vitro* system using sugarcane bagasse or sugarcane bagasse based ration as substrates. Addition of zoospores of *Neocallimastix* sp. GR-1 in the *in vitro* digestibility studies of sugarcane bagasse showed increased ($P < 0.01$) in the activity of hydrolytic enzymes i.e. CMCase, FPase and xylanase to 17.95 ± 0.61 Vs 13.71 ± 0.41 , 12.58 ± 0.75 Vs 9.09 ± 0.21 and 29.28 ± 0.94 Vs 23.37 ± 0.62 respectively. However, addition of

zoospores of *Piromyces* sp. WNG-12 showed increased ($P < 0.01$) in the activity of hydrolytic enzymes i.e. CMCCase, FPase and xylanase to 17.61 ± 0.41 Vs 15.70 ± 0.95 , 10.91 ± 0.24 Vs 7.09 ± 0.36 and 27.85 ± 0.2 Vs 21.85 ± 0.42 respectively. The average number of zoospores/ml of supernatant increased significantly ($P < 0.01$) after the addition of fungal zoospores of *Neocallimastix* sp. GR-1 and *Piromyces* sp. WNG-12 as compared to that of control when sugarcane bagasse and sugarcane bagasse based ration were used as substrates.

Conclusions

From the results of the present study it can be concluded that:

- ❖ The zoosporogenesis was maximum after 96 h of incubation of fungal cultures (*Neocallimastix* sp. GR-1, *Piromyces* sp. WNG-12 and *Orpinomyces* sp. C14) using media with normal composition.
- ❖ *Neocallimastix GR1* and *Piromyces WNG12* produced more number of zoospores as compared to that of *Orpinomyces C14*.
- ❖ Administration of zoospores of *Neocallimastix GR1* and *Piromyces WNG12*, increased significantly, the *in vitro* digestibility of DM, disappearance of NDF and ADF of sugarcane bagasse and sugarcane bagasse based rations
- ❖ Administration of zoospores of *Neocallimastix GR1* and *Piromyces WNG12*, increased TVFA production and activity of hydrolytic enzymes (CMCase, FPase and Xylanase) significantly under *in vitro* studies of SB and SB based ration

Thus, the zoospores of elite rumen fungi, *Neocallimastix GR1* and *Piromyces WNG12* are quite promising to be used as probiotics in ruminants to improve the nutritive value of SB and SB based ration. However, the zoospores of *Neocallimastix GR1* showed better performance than zoospores of *Piromyces WNG12*.

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