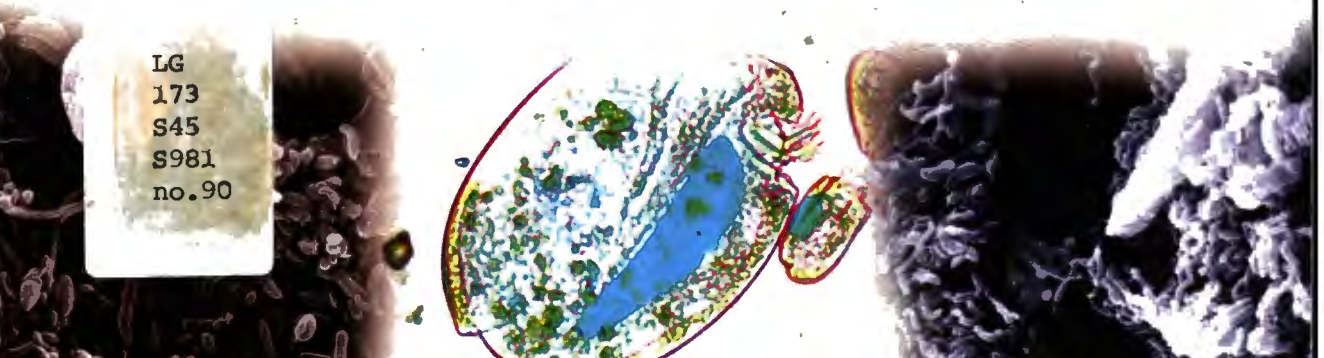
UPM Inaugural Lecture Series

RUMEN MICROBES AND SOME OF THEIR BIOTECHNOLOGICAL APPLICATIONS

Prof. Dr. Norhani Abdullah



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Tel : 603-8946 6003
Fax : 603-8948 7273
e-mail: cco@admin.upm.edu.my

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INAUGURAL LECTURE

PROF. DR. NORHANI ABDULLAH

Rumen Microbes and Some of Their Biotechnological Applications

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UNIVERSITI PUTRA MALAYSIA

NORHANI ABDULLAH

Professor Dr. Norhani Abdullah was born in Kuala Pilah, Negri Sembilan in 1951. She completed her secondary schooling at College Tunku Kurshiah, Seremban. She graduated in 1976 with a B.Sc. (Hons.) degree majoring in Biochemistry from the University of Malaya, and in the same year, began her academic career as a tutor in the Department of Biochemistry and Microbiology, Universiti Putra Malaysia (UPM) (previously known as Universiti Pertanian Malaysia).

In the following year, she was granted a scholarship by the Australian government under the 'Asian Australian University Co-operation Scheme (AAUCS)' to do her MS degree at the Department of Biochemistry and Nutrition, University of New England, Australia and returned to UPM as a lecturer in 1980. After about three years of teaching in UPM, she did her doctoral degree in animal biochemistry at the Department of Animal Science, UPM. She conducted a comparative study between cattle and swamp buffaloes on various aspects of feed utilisation and digestion, rumen microbes and their activities and urea recycling.

She was involved in a number of coordinated research programmes, including 'The Use of Nuclear Techniques to Improve Domestic Buffalo Production in Asia – Phase II Programme' (1984-1987) under the auspices of the Food and Agriculture Organisation (FAO) of the United Nations and the International Atomic Energy Agency (IAEA); 'Evaluation of Different Buffalo Genotypes' organised by the Australian Centre for International Agricultural Research (ACIAR); 'Isolation, enumeration and identification of rumen microorganisms in Malaysian livestock' under the Japan-Malaysian collaborative research organized by the Japanese Society for the Promotion of Science (JSPS); 'Isolation and characterisation of cellulolytic rumen bacteria' funded by FAO and the 'Development, standardisation and validation of nuclear based technologies for measuring microbial protein supply in ruminant livestock for improving productivity' also organised by the FAO/IAEA. She was also involved in a collaborative research on the microbiology and physiology of mouse deer organized by the Japan International Research Centre for Agricultural Sciences (JIRCAS).

Professor Norhani has been awarded numerous study/travel grants including the FAO/IAEA to attend the Interregional Training Course on the Use of Isotope-Aided Techniques in Ruminant Nutrition at the Seibersdorf Laboratory, Vienna, Austria; the Japanese Society for the Promotion of Science (JSPS)/VCC Scientific Exchange Programme, to spent a couple of weeks at the Tokyo University of Agriculture and the National Institute of Animal Health, Tsukuba, Japan; the British Council to stay for a month at the at the Hannah Research Institute, Ayr, Scotland and by JIRCAS to visit the National Institute of Animal Health, Tsukuba. Most of these stays involved short studies on techniques to isolate and characterize rumen microbes.

From her involvement in research on rumen microbiology and nutritional biochemistry, she has authored or co-authored more than 70 publications in local and international journals. Research products from her group have been recognized in the form of awards in research competitions organised by UPM and other organisations within the country.

Her other duties beside teaching and research include supervising post-graduate students at MS and PhD levels and conducting workshops or training for microbiologists from universities and local industries. She is a member of a number of committees within the Department and the Faculty and has been the Honorable Secretary of the Malaysian Society for Microbiology and an Executive Committee Member of the Malaysian Society of Animal Production for two terms. Just recently, she was appointed the Head of Department for the Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, UPM.

RUMEN MICROBES AND SOME OF THEIR BIOTECHNOLOGICAL APPLICATIONS

ABSTRACT

The rumen is a unique ecosystem, well suited to maintain a dense population of microbes. Its anaerobic and reduced conditions are favourable for an intensive microbial degradation of feedstuffs consumed by the host. The rumen bacteria, protozoa and fungi produce the enzymes to hydrolyse the various nutrients present in the feed. The cellulases and hemicellulases degrade the complex plant cell wall polysaccharides and the products are assimilated and fermented by the microbes for their own energy requirement. The end-products of fermentation, mainly the volatile fatty acids and microbial protein are made available to the host. The massive amount of genetic materials and gene products present in the rumen offers a source of unlimited materials for the development of biosensors, bioassays and enzymes for biotechnological applications.

INTRODUCTION

Microorganisms are present in the gut of all animals and their contribution to the host digestive systems depend on the diet and the construction of the gut. For herbivorous animals, the microorganisms present are of central importance to the digestive processes, particularly in the utilization of plant cell wall materials such as cellulose and hemicellulose which cannot be digested by the normal intestinal enzymes. The ruminants have developed modified digestive tracts that can maintain suitable environments for the microbial digestion of the plant materials. On the other hand, monogastrics are animals that have a simple stomach, where their diets consist of feed that can be easily digested in the gastrointestinal tract. The dense microbial population in the rumen offers an unlimited source of genetic materials and gene products for development of various biotechnological applications like bioassays and microbioassays using bacteria or enzymes to measure chemical toxicity in the environment; biosensors for biochemical and clinical analyses and enzyme supplements for livestock production.

THE RUMINANT

Despite the large proportion of plant materials constituted by cellulose and related structural carbohydrates, mammals including herbivores do not synthesise enzymes capable of degrading β-linked polymeric carbohydrates like cellulose and xylans. Ruminants are a class of animals which differ from the monogastric by having a large complex compartmental stomachs and by the process of rumination or cud-chewing. Their fore-stomachs are made up of four compartments, namely, the rumen, reticulum, omasum and abomasum (true stomach). The rumen is the first and the largest compartment of the digestive tract and is highly reduced and anaerobic. Its volume is 4 to 10 litres in sheep and 100 to 300 litres in cattle. It contains a large population of microbes consisting of bacteria, protozoa and fungi, which play an important role in digesting the feed before it is passed to the other parts of the digestive tract. The microbial mix in the rumen is complex and highly dependent on diet. Under normal conditions of feeding, the rumen contents of adult animals contain about 10^{10}-10^{12} bacterial cells/g rumen content (solid and liquid), and 10^5 protozoal cells per ml of rumen fluid (Abdullah *et al.*, 1995). The population density of rumen fungi (fungal zoospores) is difficult to estimate but a range of 10^3-10^5 ml^{-1} had been reported (Joblin, 1981). The microbial populations are maintained reasonably constant in number by being swept out of the rumen with the movement of fluid digesta, and also by the break-down within the rumen. Bacteria are generally believed to constitute most of the microbial biomass in the rumen, although estimates of up to 40% have been recorded for protozoal biomass in some animals. The amount of fungal biomass is thought to contribute less than 8% of the total microbes (Orpin and Joblin, 1997).

RUMEN BACTERIA

The rumen contains a mixed population of bacteria. Although many species are present in the rumen, authentic ruminal bacteria were found to consist of only abut 30 species (Ogimoto and Imai, 1981). The bacteria can be associated with rumen digesta particles and the epithelial wall as well as those that are present in the fluid phase. In general, the conditions and types of microbial population are different between the fluid and solid phase, but they coexist and interact with each other. The primary end-products of one species will be secondarily fermented by another and only the volatile fatty acids (acetic, propionic and butyric acids) are produced in quantity.

The majority of rumen bacteria are able to digest starch, while a number of the predominant bacteria exhibit high cellulase and hemicellulase activity. *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* are able to synthesise very active cellulases which can degrade crystalline cellulose, while *R. albus* and certain strains of *Butyrivibrio fibrisolvens* are cellulolytic towards the more amorphous types of cellulose. These bacteria are also able to degrade hemicellulose. *Prevotella* spp. do not hydrolyse native cellulose, but actively degrade the hemicellulose xylan.

Fibre digestion involves specific adhesion of microorganisms to their particular substrates. Figure 1 is a scanning electron micrograph (SEM) showing the attachment of a mixed population of bacteria on a grass fragment after 24 h incubation in the buffalo rumen. The cellulolytic bacteria degrade cellulosic materials and provide monomers as substrates to other microorganisms which cannot degrade this polymer.

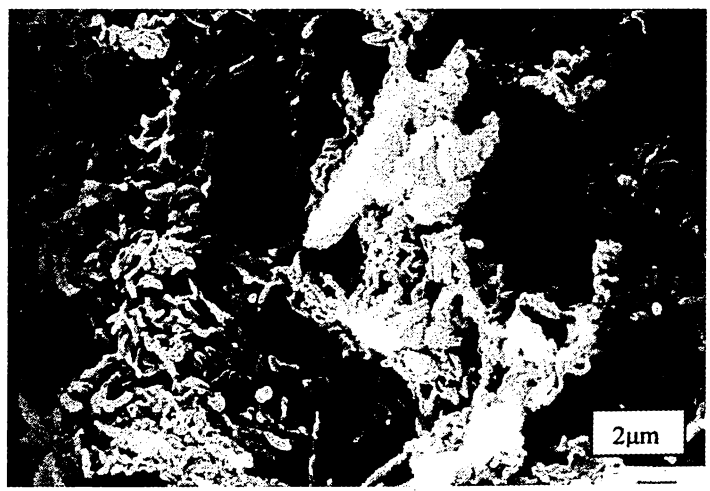


Figure 1: SEM showing the attachment of a mixed population of bacteria on a guinea grass fragment. Note the disorganized tissue surface and digestion pits (empty cavities)

Cellulose digestion requires close proximity between the microbes and the substrates. Bacterial attachment is an important aspect and a prerequisite for fibre degradation. Figure 2 shows the attachment of a pure culture of *R. albus* D3 isolated from a Sika deer on avicel (a crystalline cellulosic material).

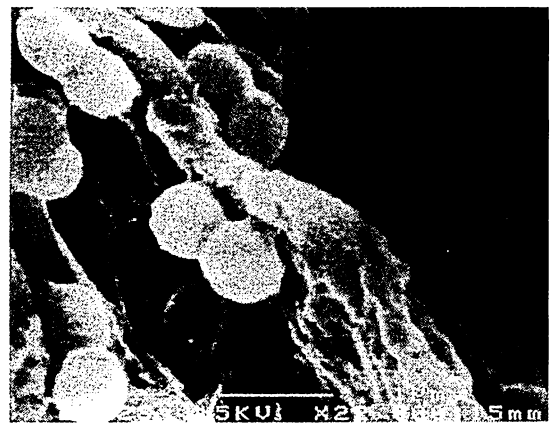


Figure 2: SEM showing the attachment of coccobacillary form of *R. albus* D3 on avicel after 10 min incubation (Sieo *et al.*, 1999).

Ruminococcus albus D3 produces glycocalyx as shown in Figure 3. Glycocalyx has long been recognized as a common structure involved in the attachment of bacteria. Further studies showed the involvement of cellulose binding proteins (CBPs) for attachment of *R. albus* D3 on avicel, where one of the CBPs had xylanase activity (Sieo *et al*, 2000).

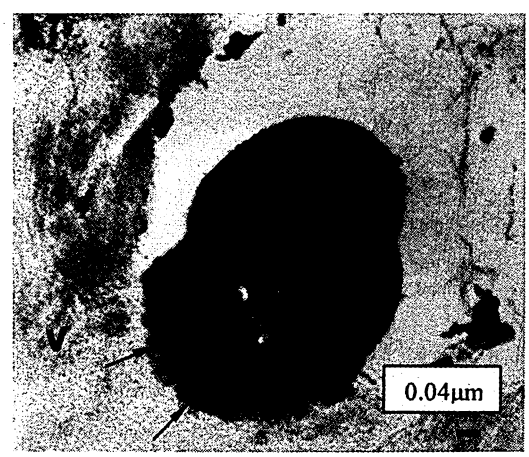


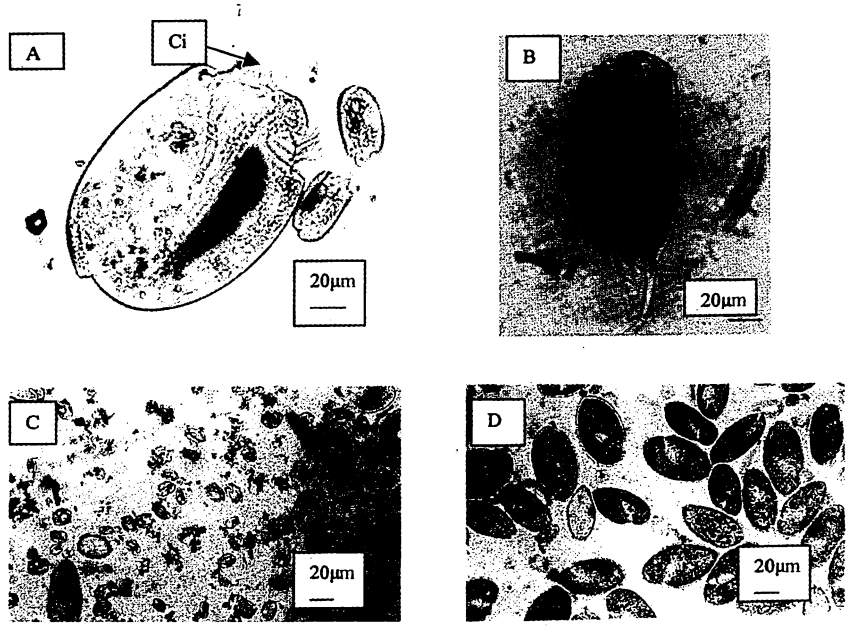
Figure 3: Transmission electron micrograph showing attachment of *R. albus* D3 to avicel after 18 h incubation. Note the diffused glycocalyx (arrows) at the point of contact (Sieo *et al.*, 1999).

RUMEN PROTOZOA

Rumen protozoa are part of the microbial population in the rumen. They are mainly ciliates and the two important groups of ciliates are the holotrichs and the entodiniomorphids. The holotrichs are almost completely covered in cilia, whereas the entodiniomorphids possess cilia at certain parts of the body. The holotrichs use mainly soluble carbohydrates, while the large species of entodiniomorphids ingest and utilize particulate materials. The protozoa to a certain degree are responsible for digestion of plant material either by direct enzymatic action or by breaking up of the tissues for further microbial colonization and digestion.

The metabolic end products formed during carbohydrate utilization by the holotrichs are lactic acid, acetic acid, butyric acid, H_2, CO_2 and storage polysaccharide (Williams and Coleman, 1992). Hydrogen is produced in subcellular organelle called hydrogenosome. Like mitochondria, hydrogenosomes are double-membrane bounded organelles that produce ATP using pyruvate as the primary substrate. Hydrogenosomes are, however, markedly different from mitochondria as they lack DNA, cytochromes and the citric acid cycle. Instead, they contain enzymes typically found in anaerobic bacteria that are capable of producing molecular hydrogen (Bui *et al.*, 1996).

Figure 4 (A-F) shows light micrographs of a few examples of rumen protozoa fixed in methylgreen formalin-saline (MFS) solution observed in the local animals. Figure 4 G shows a light and SEM of *Isotricha jalaludinii* n. sp. found in the rumen of lesser mouse deer, *Tragulus javanicus* in Malaysia (Imai *et al.*, 1995).



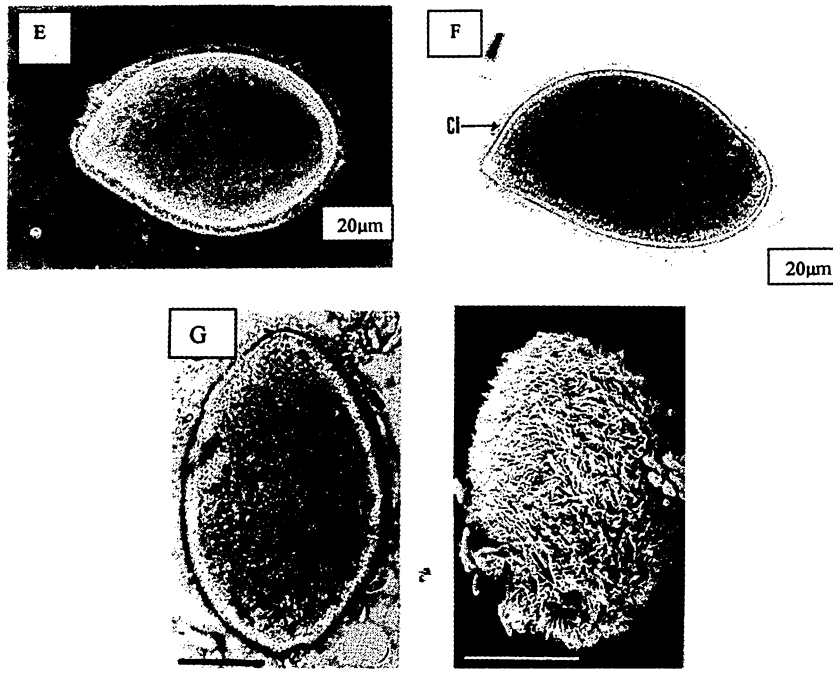


Figure 4: Some examples of rumen protozoa

A,B : Entodiniomorphids
C : Mixed population holotrichs and entodiniomorphids
D : A pure sample of holotrichs
E : *Isotricha prostoma*
F : *Isotricha intestinalis*
G : *Isotricha jalaludinii* (light and SEM micrographs, Bar =30 µm)
Ma : Macronucleus
Ci : Cilia
Ca : Spine
S : Skeletal plate

Generally, the composition of rumen ciliate protozoal population of domestic ruminants is similar, but characteristic variations in the occurrence of some species within host species may occur due to geographical distribution of the hosts as well as the diets. It was reported that the levels of *Metadinium* and *Eudiplodinium* (considered to be fermenters of cellulosic materials) were higher in both the local cattle and swamp buffalo. This indicates that Malaysian cattle and buffalo possess a ciliate protozoal composition favaorable for digestion of cellulosic feed materials (Imai *et al.*, 1995). The total number of ciliates per ml of rumen contents was 12.7×10^4 in swamp buffalo and 11.6×10^5 in Kedah Kelantan cattle. In both animal species, the majority of ciliates were *Entodinium*.

Protozoa engulf bacteria and dietary proteins for their nitrogen requirements and release amino acids and ammonia as waste materials. This predatory behaviour reduces the efficiency of microbial growth and hence the net yield of microbial amino acids available

for intestinal absorption. A number of methods have been employed to defaunate the rumen. Dietary manipulation is probably safer than chemical drenching. It was reported that protozoa were absent in steers fed palm kernel cake (Abdullah and Hutagalung, 1988). Hence palm kernel cake (PKC) could be a natural substrate to reduce the protozoal population in the rumen. This was confirmed in a later study where the number of rumen protozoa decreased in sheep fed PKC. Figure 5 shows the changes in protozoal counts in the rumen of sheep fed PKC. In this study, bentonite (finely divided montmorillonite clay) was added to reduce the detrimental effects of PKC on rumen metabolism and the host. The copper (~ 30 ppm) and zinc (~ 45 ppm) present in PKC may contribute to the defaunation effect of PKC (Abdullah *et al.*, 1995). Sheep fed grass showed diurnal variation in protozoal counts, probably due to the changes in the nutrient composition of the feed.

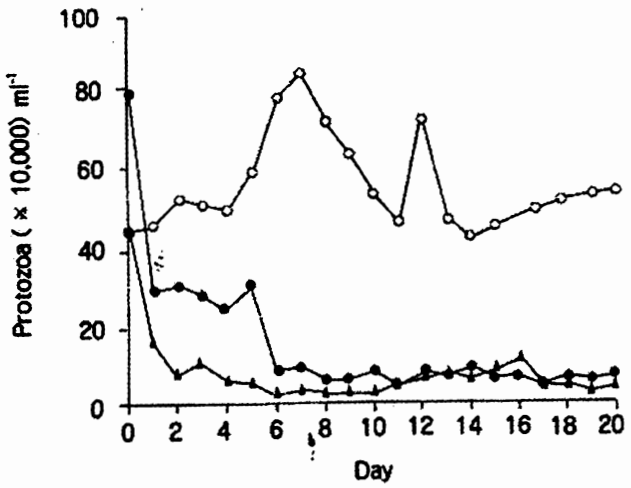


Figure 5: The mean daily protozoal numbers per ml of rumen fluid of sheep fed palm kernel cake (▲) palm kernel cake + bentonite (●) or grass (○). All animals were initially fed with guinea grass

RUMEN FUNGI

Anaerobic fungi were first discovered in the rumen of a sheep by Orpin (1975). The delay in discovering this group of microbe in the rumen is attributed to the common practice among researches that usually work with strained rumen fluid and discarding the solid digesta. Following this discovery, studies on rumen fungi became one of the major interests of rumen microbiologists. The significance and possible role of rumen fungi in fibre digestion was recognized when extensive colonization of fibrous plant materials by the fungi was observed in the rumen of sheep, cattle and buffaloes (Orpin, 1977; Abdullah, 1987; Ho *et al.*, 1996a; Abdullah *et al.*, 1991a,b; Ho and Abdullah, 1999). Figure 6 shows the extensive colonization and tissue degradation by the rumen fungi on straw fragments incubated in the cattle rumen. The fungi occupy a unique niche in the digestive tract of

ruminants and other mammalian herbivores where they participate in primary colonization of plant cell walls. Rumen fungi preferentially colonise thick-walled lignified tissues. Hence diets that are high in fibre content support a higher population of rumen fungi. To date there is no report on the presence of rumen fungi in the lesser mousedeer, probably because of the succulent or concentrate nature of the animal's diet.

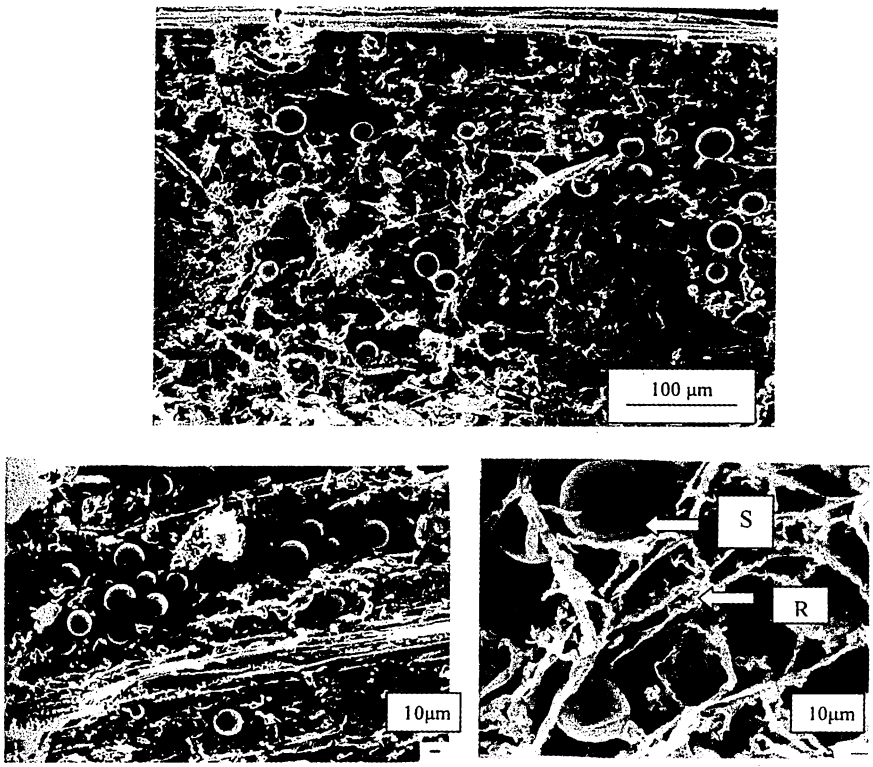


Figure 6: SEM showing fungal colonization of padi straw fragment after 24h incubation in the cattle rumen. Note the disorganised tissue surface which indicate digestion. R: Rhizoids; S: Sporangium

To date, six fungal genera have been established. They were *Neocallimastix*, *Piromyces*, *Caecomyces*, *Orpinomyces*, *Anaeromyces* and *Cyllamyces* (see Ho *et al.*, 2000; Ozkose *et al.*, 2001). The life-cycle (Figure 7) of the fungi consists of a motile, flagellated zoospore stage, that alternates with a vegetative, reproductive stage associated with the digesta fragments. The numbers in Figure 7 represent hours after encystment of a zoospore. Rapid development of an extensive, highly branch of rhizomycellium occurred during the first 6.5h of growth of the thallus. After 21h of encystment, at the base of the zoosporangium, a septum was formed and by 28h, zoosporangia were formed within the sporangium (Trinci *et al.*, 1994). The fungi can be distinguished into two main morphological forms i.e. monocentric and polycentric. In the monocentric species, the thallus usually develops a single sporangium derived from the zoospore cell (Ho *et al.*, 1993), while in the polycentric species, numerous sporangia are produced (Ho *et al.*, 1990).

Figure 8 shows sporangia of polycentric fungi with developing zoospores. The zoospores were eventually liberated through pores formed in the zoosporangial wall (Figure 9). The zoospores can be uni- or multiflagellates depending on the fungal species. Species of *Neocallimastix* produce multiflagellate zoospores (Figure 9A), while species of *Anaeromyces* (*Ruminomyces)* produce uniflagellate zoospores (Ho *et al.*, 1990).

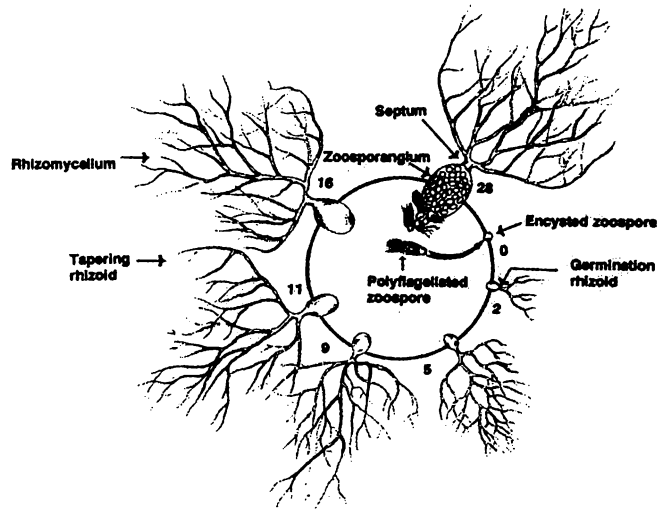


Figure 7: Diagrammatic representation of the life cycle of the monocentric, anaerobic fungus, *Neocallimastix hyrleyensis* originally isolated from the rumen of sheep. The numbers represent hours after encystment of the zoospore (Adapted from Trinci *et al.*, 1994).

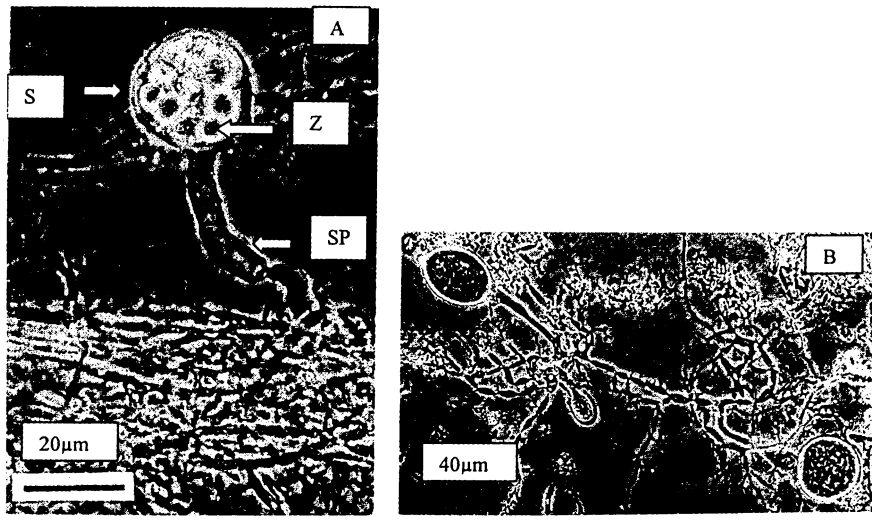


Figure 8: Examples of polycentric anaerobic fungi
A. Orpinomyces joyonii, with developing zoospores (Ho and Barr, 1995)
B. Ruminomyces (Anaeromyces) elegans rhizomycelium with three young sporangia (Ho *et al.*, 1990)
(S: Sporangium, Z: Zoospore, SP: Sporangiophore)

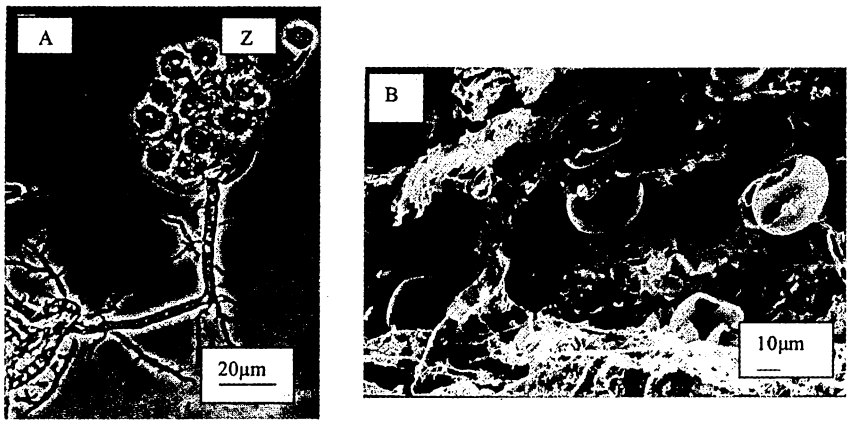


Figure 9: A. A monocentric fungus *Neocallimastix variabilis* showing a multiflagellate zoospore (Z) released from a matured sporangium (Ho *et al.*, 1993)
B. SEM of sporangia (unidentified fungus) with pores through which zoospores were released (grass fragment incubated in the buffalo rumen for 48h)

All strains of rumen fungi so far examined are capable of degrading structural carbohydrates of plant cell walls. Rumen fungi produce cellulases capable of solubilising both the amorphous and crystalline cellulose. High activities of endo-1,4-β-D glucanase (CMCase) have been detected in the growth supernatants of various rumen fungi (Abdullah *et al.*, 1990; Ho *et al.*, 1996b). Rumen fungi are among the few fungi that can degrade crystalline cellulose. Fungi produce a wide range of hydrolytic enzymes which include cellulases, hemicellulases and phenolic esterases (Teunissen *et al.*, 1993; Ho *et al.*, 1996b). Most fungal species produce acetate, formate, lactate, ethanol, carbon dioxide and hydrogen as end products (Borneman *et al.* 1989, Ho *et al.*, 1996b). Hydrogen production is a common feature of all anaerobic rumen fungal fermentation. The formation of hydrogen is localized in microbodies called hydrogenosomes (Yartlett *et al.*, 1986).

Anaerobic rumen fungi may not seem to be important to rumen digestive function since they are absent or occur in very small number when the animal is fed low-fibre diet, but the widespread colonization of fibrous plant materials by the fungi indicates that they play a role in fibre digestion. The cellulases, hemicellulases and phenolic esterases produced by the fungi enable them to invade and degrade structural carbohydrates in lignified plant tissues. In tropical regions where most of the forages are fibrous and poor quality, the development of methods to manipulate the fungi in the rumen could offer a means of improving feed efficiency in ruminants fed high-fibre poor quality feeds.

RUMEN DIGESTION AND FERMENTATION

Cellulose digestion by microorganisms is a comparatively slow process, hence the passage of ingesta needs to be slowed down for maximal degradation. Passage rate of small particles and fluid outflow rate would also influence the microbial population in both the fluid and solid compartments (Abdullah *et al.* 1991c). The ability of the reticulo-rumen to retain large feed particles is an important adaptation for microbial digestion of plant fibre. Only the small feed particles can enter the omasum from the reticulum, while large fragments remain, hence the rumen is never emptied even after a prolonged fast. It is in the reticulo-rumen that most of the microbial activity takes place.

Microbial digestion in the rumen is relatively unrelated to the host's digestive processes in the gut, in which hydrolytic enzymes breakdown more complex compounds of plant cell wall polysaccharides to nutrient molecules that can be used by the microbes for their own metabolic needs. Cellulose is a polymer of glucose linked by β-1,4-glucosidic bonds and the chains form numerous intra- and intermolecular hydrogen bonds to form an insoluble cellulose microfibrils. Microbial hydrolysis of cellulose to glucose involves three major classes of cellulases namely endo-β-glucanase (EC 3.2.1.4) or carboxymethylcellulase (CMCase), which cleave β-1,4-glucosidic links throughout cellulose molecules; exo-β-glucanase or cellobiohydrolase (EC 3.2.1.91) or avicelase which digest cellulose from the non-reducing end, releasing cellobiose and β-glucosidase (EC 3.2.1.21) or cellobiase which hydrolyse cellobiose and low molecular-mass cellodextrins to release glucose (Beguin, 1990).

The digestion and fermentation processes of feed constituents like cellulose, hemicellulose, starches, sugars, proteins and amino acids by the rumen microbes (bacteria, protozoa and fungi) in the rumen result in end products, principally volatile fatty acids (VFAs) i.e., acetic, propionic and butyric acids; the gasses methane and CO_2 and NH_3 and the microbial cells. Succinate, hydrogen, lactate, ethanol and formate, important fermentation products of pure cultures of rumen bacteria, protozoa and fungi occur at very low concentrations in the rumen. The composition of the gas space in the rumen is 65% CO_2 and 35% methane and the gasses leave the rumen during eructation through the mouth and enter the environment. The VFAs are largely absorbed through the walls of the fore-stomach, where they are utilised as a source of energy and glucogenic precursor by the host. The microbial cells are digested in the hind gut, where the microbial protein synthesised in the rumen becomes the source of protein for the host. The amino acid components of the microbial protein which are made available to the host may differ in nature and proportions from the amino acids present in the ingested protein.

Conditions for normal fermentation are maintained in the ruminal fluid by continual adjustment of pH chiefly by saliva inflow and absorption of VFAs into the blood across the rumen wall. However, due to the rapid fermentation processes after the onset of feeding, changes in pH still occur. Figure 10A shows the changes in rumen pH at various

times after the onset of feeding of sheep fed chopped guinea grass and four types of supplements. The supplements tested were two types of energy source (corn flour and paper pulp) and two types of protein (fish meal and soybean meal). At 3 h after feeding, rumen pH of sheep fed soybean meal + corn flour was the lowest. Sheep fed this supplement also showed rapid fermentation activity as indicated by the highest concentration of VFAs in the rumen (Figure 10B) (Jetana *et al.*, 2000).

As can be seen from Figure 10, rumen fermentation pattern is greatly affected by diet. However, the activity of a microbial population adapted to a particular carbohydrate shows a typical fermentation pattern. With less easily hydrolysed carbohydrates or highly fibrous feeds like roughages, acetic acid predominates, but with hexose and easily hydrolysed carbohydrates like molasses, acetic acid still predominates, but the production of propionic and butyric acids increased (Abdullah, 1984; Abdullah *et al.*, 1992). Palm kernel cake with a high protein content showed higher propionate production. The change in molar proportions indicate the shift in microbial population. The molar proportions of individual fatty aids of animals fed different diets are shown in Table 1.

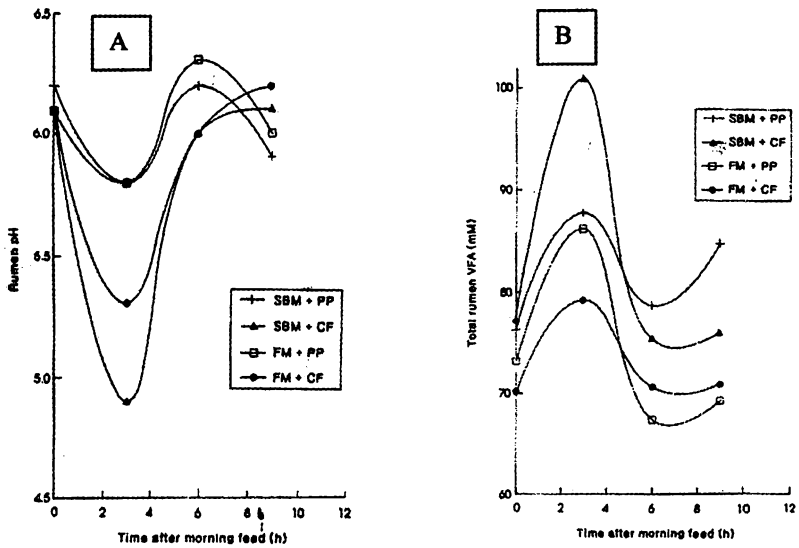


Figure 10 : A. Rumen pH

B. Total VFA

Sheep fed chopped guinea grass *ad libitum* and four different supplements.
SBM + PP = Soybean meal + paper pulp
SBM + CF = Soybean meal + corn flour
FM + PP = Fish meal + paper pulp
FM + CF = Fish meal + corn flour

Table 1: Changes in total rumen VFA and molar percent of acetic, propionic and butyric with different diets

Diets	Total VFA (mM)	Molar %		
		Acetic	Propionic	Butyric
Guinea grass	96.4±1.3	74.5±0.2	17.0±0.2	7.0±0.2
Straw	98.0±2.2	76.6±0.2	17.1±0.1	5.0±0.1
Straw + molasses	81.5±1.2	65.0±0.4	18.2±0.1	13.8±0.3
Palm kernel cake	74.8±5.5	54.4±2.7	26.4±1.3	11.6±1.6
Molasses –based diet	95.1±1.2	41.7±0.8	24.9±1.1	30.8±1.3
Molasses + fish meal	113.9±1.5	43.4±1.3	19.7±0.7	34.6±1.3

The addition of molasses which contain highly fermentable sugars result in high butyrate fermentation. The shift in fermentation to high butyrate production could be due to the increase in protozoal population as they are known to be butyrate producers (Williams and Coleman, 1992). The holotrichs develop in large numbers when soluble carbohydrates are readily available in the diet (Jouany and Ushida, 1990).

The equations below show production of acetate (HAc), propionate (HPro), butyrate (HBu), methane and energy (ATP) from hexose fermentation.

$$\text{Hexose} \rightarrow 2\,\text{pyruvate} + 4\,[H] + 2ATP$$
$$2\text{pyruvate} + 2H_2O \rightarrow 2HAc + 2CO_2 + 2H_2 + 2ATP$$
$$2\text{pyruvate} + 8[H] \rightarrow 2HPro + 2H_2O + 2CO_2 + 2ATP$$
$$2\text{pyruvate} + 4[H] \rightarrow HBu + 2H_2O + 2CO_2 + 2ATP$$
$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O + ATP$$

High cellulolytic activity is associated with high acetate and methane production. Molecular hydrogen produced by the rumen microbes is the major electron donor for methanogenesis by the various species of methanogens. However, to a lesser extent, methane is also synthesized from formate. The excretion of methane from the rumen can represent a loss of up to 15% of the digestible energy depending on the type of diet.

BIOTECHNOLOGICAL APPLICATIONS OF RUMEN MICROBES

Lignocellulolytic microorganisms and their lignocellulolytic enzymes have many potential biotechnological applications ranging from the production of bio-fuel, chemicals, proteins, and to improving textiles, wood-pulping and animal feeds for domesticated herbivores. In the baking industry, xylanases are used for improving desirable texture, loaf volume and shelf life of bread. Hemicellulases have the potential use for pulping and bleaching in the pulp and paper industry to modify the structure of xylan and glucomannan in pulp fibres to enhance chemical delignification (see Howard *et al.*, 2003). Different industries have different needs and consequently require specific enzymes. The detergent,

textile and paper deinking industries prefer cellulases which remove short protruding ends to restore smooth surfaces and which do not hydrolyse intact cellulose fibres.

Ruminal microbes and microbial enzymes offer the possibility of eliminating anti-nutritional factors and toxins in plants, enhancing fiber digestion and nutritive value of feed (Bonneau and Laarveld, 1999). A lot of work on genetic manipulation on rumen microbes has been conducted, in particular genes involve in polysaccharidases production. Over 100 different genes encoding enzymes for fiber digestion have been identified and cloned from ruminal bacteria such as *Butyrivibrio fibrisolvens, Fibrobacter succinogenes, Prevotella ruminicola, Ruminococcus albus* and *Ruminococcus flavefaciens*. At least 30 genes that encode cellulase, xylanases, mannanases, and endoglucanases have been isolated from ruminal fungi. These are of particular interest due to their powerful fibrolytic activity and ability to break down very resistant cell wall polymers (Bowman and Sowell, 2003).

The rumen *Streptococcus bovis* is tolerant to O_2 and gene transfer methods have been developed for this species that make it a candidate as a host for the expression of genes from other organisms. Ekinci *et al.* (2002) were able to use a β-glucanase promoter found in *S. bovis* to express a cellulase gene from the anaerobic rumen fungus *Neocallimastix patriciarum* that is found in very low levels in the rumen, and is important for the degradation of crystalline cellulose. The resulting enzyme product was active against a wide variety of cellulosic substrates. The advantages of using fungal enzymes are their stability to low pH and their extremely high activity level (Ekinci *et al.*, 2002). Some ruminal bacterial species like *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* are found widely in significant numbers in ruminant animals on varied diets. These species can be considered as logical choices to introduce new or enhanced genetic material into the rumen (Selinger *et al.*, 1996).

Protein engineering has been used to increase the catalytic activity and substrate diversity of fibrolytic enzymes from ruminal microbes. This has resulted in enzymes with up to 10 times higher specific activity, changed pH and temperature optima and increased substrate binding activity than the enzymes from which they originated (Selinger *et al.*, 1996).

MICROBIAL ENZYMES AS FEED SUPPLEMENTS

Increasing competition in the livestock industry has forced producers to cut costs by adopting new technologies aimed at increasing production efficiency. One particularly promising technology is feeding enzymes as supplements for animal diets. Supplementation of diets for non-ruminants (e.g., swine and poultry) with fibrolytic enzymes, such as cellulases, xylanases and β-glucanases, increases the feed conversion efficiency and growth rate of the animals. Enzymatic hydrolysis of plant cell wall polymers (e.g., cellulose, xylan, β-glucans) releases glucose and xylose and eliminates the antinutritional effects of β-glucans and arabinoxylans.

Phytase supplementation has been found to increase not only the growth rate of monogastric animals but also the efficiency of phosphate utilization in feeds. This would reduce phosphorous excretion and the chances of environmental pollution.

RUMEN BACTERIA AS A SOURCE OF THE PHYTASE ENZYME

Phytate or phytic acid (*myo*-inositol 1,2,3,4,5,6 hexakisphosphate, IP6) is the main storage form of phosphorus (P) in cereal grains, legumes, pollens and oilseeds which form a major component of animal feed. More than 60% of P in these products are present as phytate-P. Phytate is also considered as an anti-nutritional factor as it can chelate important minerals such as Ca, Zn, Cu, Mn and Fe and binds protein to form insoluble phytate-protein complexes. Theoretically, the P contents of feeds originating from plant materials should be sufficient to meet the requirements of poultry. Unfortunately, phytate-P is poorly utilized by poultry because of the lack of the digestive enzyme, phytase, to hydrolyse phytate into inorganic-P. The limited ability of poultry to utilise phytate-P poses two problems, i.e., the need to supplement inorganic-P in the feed (e.g. dicalcium phosphate) where P is the third most expensive nutrient in poultry production after energy and protein; and the excretion of large amounts of P in manure which pollutes the environment.

Phytase or myo-hexakisphosphate phosphohydrolase hydrolyses myo-inositol hexakisphosphate into inorganic orthophosphate and myo-inositol. The enzyme is widespread in nature, occurring in microorganisms, plants and in some animal tissues. Ruminants are able to utilize phytate-P efficiently as they have phytase-producing bacteria in the rumen. In the process of a large scale screening for phytase-producing bacteria from the rumens, a new phytase-producing bacterial species, *Mitsuokella jalaludinii*, which can hydrolyze phytate in the feed of chickens effectively *in vitro* has been isolated from the rumen of cattle in Malaysia (Lan *et al.*, 2002a). The rod-shaped Gram negative bacterium (Figure 11) hydrolysed sodium phytate rapidly and the phytase production was strongly induced by phytate present in the growth medium.

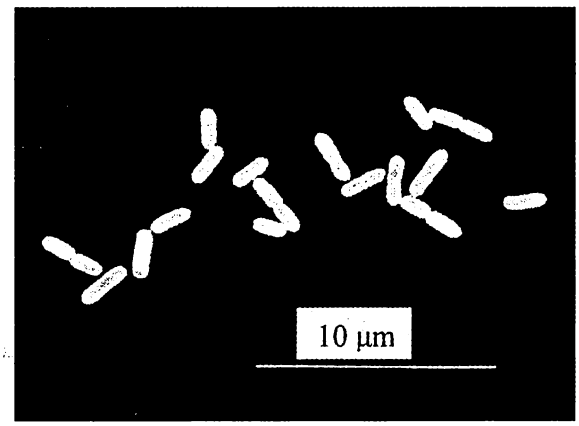


Figure 11: SEM of *Mitsoukella jalaludinii*

Rice bran and soybean milk were found to be the best carbon and nitrogen sources, respectively (Lan *et al.*, 2002b). Under optimum conditions (Lan *et al.*, 2002c), this bacterium produces 12.93 Ug^{-1} culture broth of phytase activity. The production of phytase was comparable to the yield of phytase produced by *Aspergillus ficuum* NRRL 3135 - a wild fungal strain which produces the highest phytase activity among fungi (Wodzinski and Ullah, 1996). The enzyme produced is very active at pH 4.0 – 4.5 and stable up to 60°C.

Feeding trials were conducted to determine the efficacy of supplementation of *M. jalaludinii* culture on the performance and nutrient utilisation in broiler chicken (Lan *et al.* 2002d). A total of 360 one-day old chicks (Avian-43) were fed *ad libitum* with one of the four diets: T1: low available-P (aP) feed+2.0% inactivated *M. jalaludinii;* T2: low-aP feed+2% active *M. jalaludinii* (500U phytase/kg feed); T3: low-aP feed+2% inactivated *M. jalaludinii* +500U Natuphos® phytase/kg feed and T4: normal-aP (0.44% aP) feed+2% inactivated *M. jalaludinii.* The experiment was conducted for 21 days. The growth performance and nutrient utilisation of the chickens were compared to those supplemented with a commercial phytase (Natuphos® phytase). Figure 12 shows the growth performance of broiler chickens and Figure 13 shows the P excretion at 21 days.

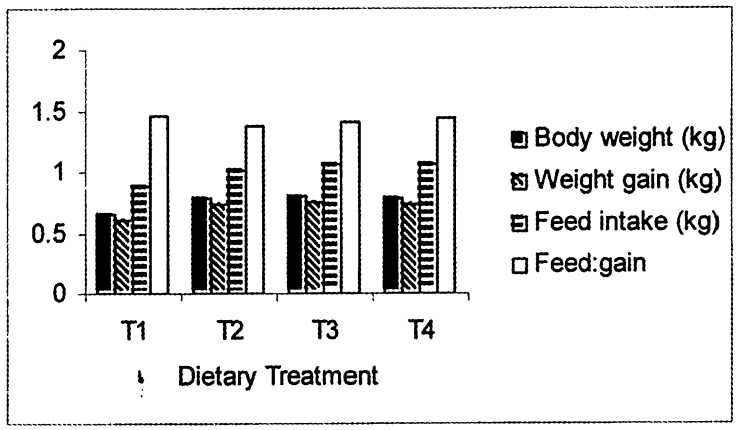


Figure 12: Effects of T1: low available-P (aP) feed+2.0% inactivated *M. jalaludinii;* T2: low-aP feed+2% active *M. jalaludinii* (500U phytase/kg feed); T3: low-aP feed+2% inactivated *M. jalaludinii*+500U Natuphos® phytase/kg feed and T4: normal-aP (0.44% aP) feed+2% inactivated *M. jalaludinii* on growth performance of broiler chickens at 21 days.

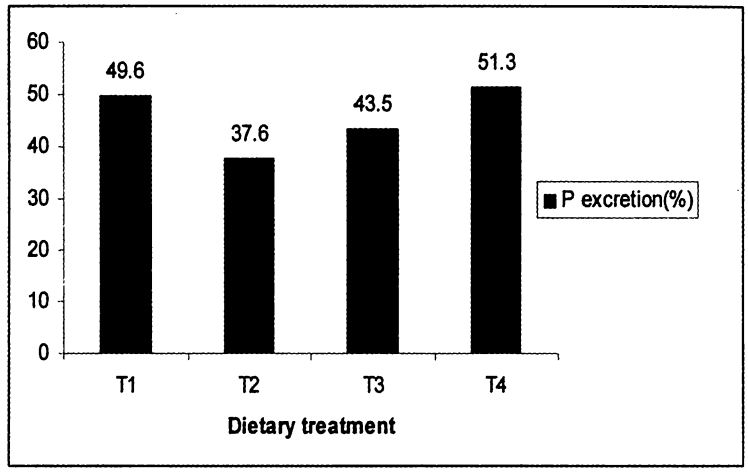


Figure 13: Effects of T1: low available-P (aP) feed+2.0% inactivated *M. jalaludinii*; T2: low-aP feed+2% active *M. jalaludinii* (500U phytase/kg feed); T3: low-aP feed+2% inactivated *M. jalaludinii*+500U Natuphos® phytase/kg feed and T4: normal-aP (0.44% aP) feed+2% inactivated *M. jalaludinii* supplementations on % excretion of phosphorus (P) of broiler chickens at 21 days.

Feeding trials conducted on broiler chickens have shown the ability of freeze-dried culture of *M. Jalaludinii* to enhance P utilisation, hence decreasing the amount of organic-P excreted and reducing the requirement of inorganic-P in the diet. The bacterial preparation also improves the feed intake, feed conversion rate, body weight gain, retention of Cu and Zn, P, Ca, Mn and ash content of the tibia and the concentrations of plasma P and Zn of broiler chickens.

In addition, the apparent metabolisable energy value of feed in broilers fed low available-P diet has been significantly enhanced and digestibilities of N and DM have also been improved. These results indicate the effectiveness of the product in improving the nutritive value of the feed. The activity of the phytase enzyme is comparable to that of the commercial phytase (Natuphos® phytase). However, the presence of other enzymes in the bacterium like amylase and protease further enhances the digestive process of the birds. Hence, *M. jalaludinii* supplementation is more efficient in improving the dietary nutrient utilisation in broiler chickens than Natuphos® phytase.

CLONING OF PHYTASE GENE

Based on the 16s rRNA gene sequence, *M. jalaludinii* is closest to *Selenomonas ruminantium*, where both of them were rumen bacteria producing high phytase activity. To clone the phytase gene from *M. jalaludinii*, degenerate primers were designed based on phytase gene sequence of *S. ruminantium*. PCR amplification was carried out by using different combinations of four pairs of forward and reverse primers. As a result, a few specific bands were obtained from PCR amplifications. Two longest PCR fragments with sizes 689 bp (*pPHY1*) and 700 bp (*pPHY2*) were cloned and sequenced. Sequence alignment of

pPHY 1 and *pPHY 2* were done and it represents a contig which consist of 736bp fragment. Through Blast homology search and clustal alignment, it showed that it is a partial phytase gene.

DNA walking was chosen as an approach to obtain the up and down stream of the phytase gene. DNA walking was performed by using DNA walking SpeedUp™Kit (Seegene) which consists of three steps PCR amplification. PCR amplification were performed by using three different specific inverse primers with annealing control primer. As a result, a 1.1kb fragment and 300bp fragment were amplified. Both of these fragments were cloned and sequenced. Clustal alignment showed that the 1.1kb fragment had high homology to the upper stream of the phytase gene and 300bp fragment to the down stream of the phytase gene. A pair of specific primer was designed based on the upper and down streams sequence obtained from DNA walking. The full length of the phytase gene was successfully isolated from *M. jalaludinii.* The gene is 1047bp in length and consists of 348 amino acids. Through a Blast homology search, the sequence cloned was similar to *S. ruminantium* JY35 myo-inositol hexaphosphate phosphohydrolase precusor (phyA) gene (E value = 0.0) and shared 98% similarities at the DNA nucleotide level with *S. ruminantium* JY35 phyA.

A phytase gene phylogenetic tree (Figure 14) was generated and the phylogenetic analysis shows that there are differences among fungal and bacterial phytases. *Bacillus* spp. phytase genes show high similarity among each other. The analysis also indicates that *M. jalaludinii* and *S. ruminantium* phytase genes are different from the other genes where both of them separated to form a new branch (Phang et al., 2005).

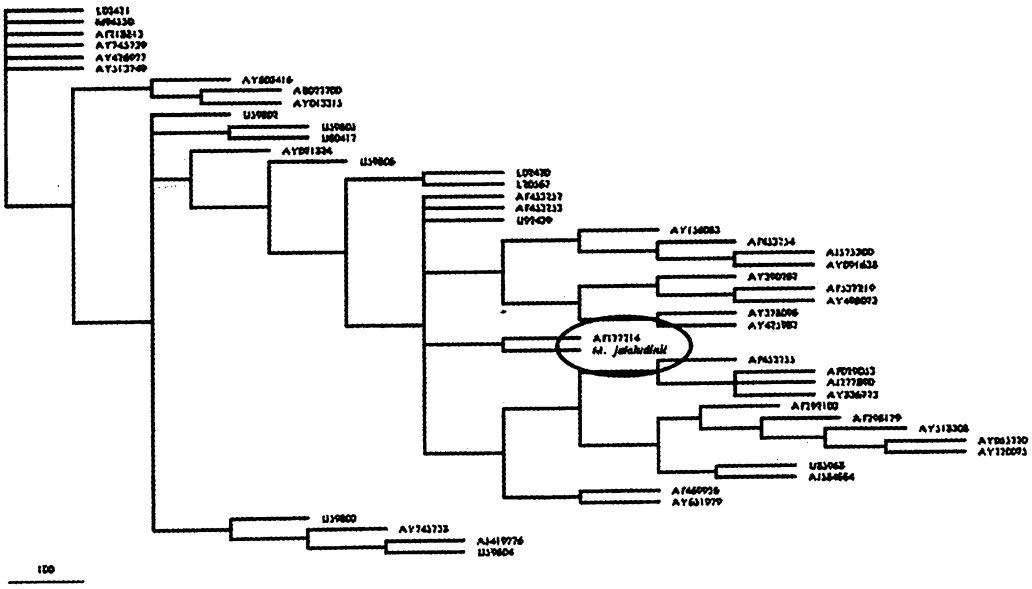


Figure 14 : Phylogenetic relationship of *M. jalaludinii* phytase gene with other microbial phytase genes.

CONCLUSION

The ruminal ecosystem is among the novel enzyme sources currently being explored for the production of industrial enzymes. Understanding the role of enzymes in feed digestion through characterization of the enzymology and genetics involved in digestion of feedstuffs will provide the information required to improve or develop new products for livestock production and other biotechnological applications. The use of microbial enzymes in the various industries has been limited largely because of the cost of development and production of enzymes. Developments in recombinant DNA technology have increased the efficiency of existing microbial production systems and facilitated exploitation of alternative sources of industrial enzymes. Characterization of genes encoding a variety of hydrolytic enzymes, such as cellulases, xylanases, β-glucanases, amylases, pectinases, proteases, phytases, urease and tannases and other microbial enzymes, will promote the development of more effective enzyme supplements and enzyme expression systems. Characteristics of the original source organism need no longer restrict the production of a useful enzyme.

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