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Fungal population and diversity in partially digested cellulose from the abomasum of beef cows

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The fungal population and mycoflora of partially digested cellulose from the abomasum of beef cows were investigated over a period of seven days, using standard methods. The temperature of the partially digested cellulose immediately after collection from the abomasum was 35.9 ±0.58ºC. The fungal populations ranged from $1.6x10^3$ cfu/g to $4.2x10^3$ cfu/g with a mean count of $3.2x10^3$ cfu/g ± $0.51x10^3$ cfu/g. Fungal populations of the partially digested cellulose were generally highest on the first day of sample collection but there was no significant difference among cows on the first day of collection. However, marked reductions ($p \leq 0.05$) in fungal population were noted on the seventh day after collection, possibly due to the influence of external environmental factors. The fungi isolated from partially digested cellulose and their frequency of isolation were *Aspergillus clavatus* (3.2%), *Aspergillus flavipes* (16.1%), *Aspergillus fumigatus* (6.5%), *Aspergillus niger* (12.9%), *Aspergillus sulphureus* (3.2%), *Aspergillus wentii* (6.5%), *Mucor* sp., (12.9%), *Penicillium aurantiovirens* (6.5%), *Penicillium breviscaupactum* (9.7%), *Penicillium raistrickii* (16.1%), and *Rhizopus* sp. (6.5%). These Fungi are important initiators of fermentative breakdown of insoluble plant materials.

Key words: cellulose, cows, fungal populations, plant materials

Introduction

The cow is a ruminant animal that feeds mainly on grasses, which after ingestion are digested, absorbed and utilized (Iwena, 2002) by the animal. Cellulose is the chief component of plant cell walls and is a polysaccharide chemically bonded in a way that most organisms can not digest (Gottfried, 1993). In the plant, cellulose chains are formed in an ordered manner to produce compact aggregates (microfibril) held together by both inter and intra molecular hydrogen bonding. In the plant cell wall, the cellulose is closely

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associated, physically and chemically, with other components especially hemicelluloses and lignin (McDonald *et al*., 1998). Hemicelluloses are alkali soluble cell wall polysaccharides closely associated with cellulose. Lignin is not a carbohydrate but is closely associated with the group of compounds and confers chemical and biological resistance to the cell wall and mechanical strength to the plant (McDonald *et al*., 1998).

Ruminants feeds consist mainly of starch and water in-soluble carbohydrates such as cellulose and hemicelluloses, which cannot be broken down by mammalian digestive enzymes (McDonald *et al*., 1998). Cellulases are enzymes which degrade at least some form of cellulose. Since they are not produced in animals, herbivores must rely on symbiotic cellulolytic microorganisms to digest their dietary cellulose (Singleton and Sainsbury, 2001). Many fungi, bacteria and a few protozoa can degrade cellulose. The cow stomach (rumen) inhabits a large number of microorganisms that digest cellulose contained in its feed (Orpin, 1976; Bauchop, 1980; Iwena, 2002). Bacteria are the principal agents for fermenting plant cell wall carbohydrates but fungi appear to have a close relationship with other microbes in the rumen (Orpin and Letcher, 1979).

The cow's stomach is divided into four compartments (McDonald *et al*., 1998): the rumen, its continuation the reticulum, omasum and abomasum - true stomach (Orskoy, 1983). When a ruminant animal like a cow feeds, it cuts the grass and swallows it with minimal chewing through the oesophagus to the rumen where the grass is stored. By anti-peristaltic movement of the stomach the undigested grass passes from the rumen to the reticulum from where it re-enters the oesophagus (regurgitate) back to the mouth. The food is now chewed properly by using the molar and premolar teeth (chewing the cud) into a semi liquid cud which is-swallowed. This liquid cud now moves into the Omasum from where it passes to the Abomasum. This whole process is called rumination (Ulyatt, 1982). In the abomasums, enzymes are secreted which act on the food. Further digestion and absorption of the food take place progressively along the digestive tract. The digested food is then absorbed into the blood through the villi in the small intestine while the undigested food passes to the large intestine where they are removed through the anus as cow dung or feaces (Iwena, 2002). The resultant faecal matter is rich in minerals. Colour ranges from greenish to blackish. In due course of time, the resulting matter turns yellow due to chemical changes caused by sunlight (Syed, 2004).

Cow dung is used as soil conditioner or fertilizer in many parts of the developing world, especially India where it is known as *gober*. Cattle dung is a potentially enormous energy resource (Dahiya and Vasudevan, 1986). The net effect of digestion in the "rumen" is the conversion of dietary materials to a

mixture of fatty acids (mainly acetic, butyric and propionic acids), gases (primarily CO_2 and CH_4 – which are voided by eructation) and microbial biomass. The successful conversion of manure into biogas depends on manipulating the digestive and respiratory process of microorganisms. When respiration occurs in the absence of air or oxygen, the gas produced is a mixture of methane and carbon dioxide in the ratio of about 2 volumes of methane for every 1 volume of carbon dioxide. After the carbon dioxide is removed, the methane provides a clean and efficient fuel and the residual sludge is a good fertilizer (Fullick, 1994).

A mixed population of microorganisms - bacteria, fungi and protozoa is needed to digest the dung and the process is similar to that which occurs in the guts of ruminants like the cow. Small family biogas producers are currently used in both China and India and the digester produces excellent fertilizer and high quality gas (Herald, 2004). Capturing and utilizing the methane gas has significant benefits to the environment. When the methane is allowed to occur naturally and escape into the atmosphere, it may contribute to the green house gases that alters the intensity of the sun.

There is a lot of literature on biogas production and fertilizer from cow dung. However, there is little information on the associated microorganisms - mostly on bacteria and not on fungi which are important initiators of cellulose degradation or breakdown. In fact, some rumen microorganisms which were once thought to be protozoa are now known to be fungi – e.g., the species of *Neocallimastix frontalis, Piromonas communis and Sphaeromonas communis* (Orpin, 1979; Orpin and Letcher, 1979; TIM, 1997). Our objective was to isolate and identify the fungi in partially digested cellulose with the aim of determining the fungal population and types associated with the degradation of cellulose in the abomasum of cows as well as production of biogas and organic fertilizer.

Materials and methods

Slaughter house and collection of partially digested cellulose

The slaughter house in which the cows were slaughtered and partially digested cellulose (feed material) collected was located along Airport Road in Rumuokoro in Obio/Akpor Local Government Area of Rivers State, Nigeria. The slaughter house was visited at about 6:30 am on the days of sample collection. The partially digested cellulose (feed material) was aseptically collected from the abomasum of freshly killed cows into four sterile 500mL flasks. A mercury thermometer was immediately immersed into each flask containing the partially digested cellulose to determine the temperature. Flasks

were then corked and labeled as samples A, B, C, and D respectively. The samples were then transported immediately to the laboratory for analysis. Sample collection was carried out on three occasions from the abomasums of three cows designated as Cow No. 1, Cow No. 2, and Cow No. 3. A total of twelve samples were collected during the investigation.

Media for isolation of fungi

Potato dextrose agar (PDA) was used for isolation and enumeration of total heterotrophic fungi. The composition of the medium was potato (200 g), distilled water (500 ml), glucose – D (15 g), and agar No. 1 (20 g). Tetracycline was added to prevent bacterial growth and facilitate selective isolation of yeasts and moulds (Walker and Colwell, 1976; Paul and Clark, 1988; Harrigan and McCance, 1990). The medium was cooled to 45^{o}C under aseptic condition, mixed thoroughly and then dispensed into sterile Petri dishes to set.

Cultivation, isolation and enumeration of saprophytic fungi from partially digested cellulose

On arrival in the laboratory, the four samples were treated or analyzed separately as to have a thorough sampling study of the partially degraded cellulose. Saprophytic fungi in partially digested cellulose were estimated by dilution plate count method (IPS, 1990). Sterile physiological saline i.e. 0.85% (w/v) sodium chloride was used as diluent for inoculum preparation.

One gram (1.0 gm) of homogenized sample of partially digested cellulose was aseptically transferred, using a flame-sterilized steel spatula, into a sterile test tube containing 9.0 ml of the diluent. This gave 10^{-1} dilution. Subsequent serial dilution was carried out with the use of sterile 1mL pipette to a second test tube containing 9.0vml of diluent to give a 10^{-2} dilution. Another 1mL was transferred from the 10^{-2} dilution to the next test tube to give 10^{-3} dilution (Cheesebrough, 1984). A zero point one millitre (0.1 ml) aliquot of 10^{-3} dilution of each sample was aseptically removed with a sterile pipette and separately spread plated with flame-sterilized glass spreader on well-dried PDA plates in triplicates. The cultured plates were inverted and incubated at room temperature for 3-5 days. After incubation, the colonies that developed on the PDA plates were counted and recorded as counts of total viable saprophytic fungi. The colonies counted were computed and expressed as colony forming unit (cfu) per gram of partially digested cellulose. The diameter, colour, colonial characteristics of the colonies were also observed and recorded. Discrete colonies were subcultured onto freshly prepared medium for the development

of pure isolates, which were stored on potato dextrose agar slants for subsequent characterization and identification.

Presumptive identification of fungal isolates

Pure Fungal cultures were observed while still on plates and after wet mount in lacto-phenol on slides under the compound microscope. Observed characteristics were recorded and compared with the established identification key (Malloch, 1997).

A small portion of each colony was picked with a sterile needle and teased out in a drop of water on a clean slide over-laid with cover glass. Prepared slide was examined under the microscope starting with a low power objective (x10), then the high power dry objective (x40) for better field view and magnification. This is the wet mount method. The microscopic examination was done by observing cultural characteristics, asexual and sexual reproductive structures like sporangia, conidia head, arthrospores, vegetative mycelium, septate and non septate (Barnett and Hunter, 1972; Malloch, 1997).

On the alternative, slide culture was carried out by using 20 ml of glycerol which was measured into 80 ml of distilled water. This is 20% glycerol. About 1 cm square medium of prepared SDA was cut with a sterile blade onto a clean slide. The four sides of the PDA solid medium were inoculated with culture and covered with cover ship. Slide was transferred onto glass rods in closed moist petridishes containing some layers of blotting paper soaked with 20% glycerol and incubated at room temperature for 3 days. We carried out a microscopic examination of cultural characteristics and asexual and sexual reproductive structures like sporangia, conidial head, arthrospores, vegetative mycelium, septate and coenocyte (Abbey, 1995).

Statistical analysis

The experimental design was complete randomized block with three replicates. Statistical analysis was performed using analysis of variance (ANOVA) on data obtained for the fungal counts according to Gomez and Gomez (1984).

Results and discussion

The average temperatures of the four replicate samples of partially digested cellulose soon after collection from the abomasums of Cow numbers 1, 2, and 3 were 35.0°C, 37.0°C and 35.8°C, respectively. The average temperature of the samples from the three abomasums was 35.93°C ± 0.58°C.

This temperature range is within the mesophillic range. The population of fungi from each sample of partially digested cellulose was determined by calculating the mean of replicate cultures of each sample. The result of the fungal population in partially digested cellulose from the three cows is as shown in Table 1.

Table 1. Fungal population in partially digested cellulose from the abomasum of three cows.

	Incubation period and fungal counts (cfu/g) for cow No. 1			
Sample	**Day 1**	**Day 3**	**Day 7**	**Average**
A	3.9×10^3	1.9×10^3	1.2×10^3	2.3×10^3
B	1.7×10^3	2.9×10^3	8.5×10^3	4.4×10^3
C	4.8×10^3	3.0×10^3	1.5×10^3	3.1×10^3
D	3.9×10^3	1.5×10^3	1.2×10^3	2.2×10^3
Average	3.6×10^3	2.3×10^3	3.1×10^3	3.0×10^3
	Incubation period and fungal counts (cfu/g) for cow No. 2			
Sample	**Day 1**	**Day 3**	**Day7**	**Average**
A	3.2×10^3	3.6×10^3	5.7×10^3	3.9×10^3
B	2.2×10^3	5.6×10^3	9.0×10^2	2.9×10^3
C	8.0×10^3	1.6×10^3	8.0×10^2	3.4×10^3
D	3.4×10^3	2.2×10^3	7.0×10^2	2.1×10^3
Average	4.2×10^3	3.3×10^3	1.9×10^3	3.1×10^3
	Incubation period and fungal counts (cfu/g) for cow No. 3			
Sample	**Day 1**	**Day 3**	**Day 7**	**Average**
A	1.8×10^3	3.4×10^3	-	1.7×10^3
B	2.6×10^3	2.1×10^3	-	1.6×10^3
C	1.7×10^3	-	-	5.7×10^2
D	1.1×10^3	1.0×10^3	-	7.0×10^2
Average	1.7×10^3	1.6×10^3	-	1.1×10^3

The mean of fungal population for Cow No. 1 decreased from 3.6×10^3 cfu/g on day 1 to 3.1×10^3 cfu/g on day 7. The mean population of fungi for Cow No. 2 was 4.2×10^3 cfu/g for day 1, 3.3×10^3cfu/g for day 3, and 1.9×10^3 cfu/g for day 7. The mean population of fungi for Cow No. 3 was 1.7×10^3 for day 1 and 1.6×10^3 for day 3; day 7 showed no growth. The fungal populations of the partially digested cellulose were generally highest on the first day of sample collection and analysis. This was so because the partially digested food provided soluble nutrients for the mycoflora of the abomasum which made use of the readily metabolisable nutrients in the food. The readily metabolisable nutrients decreased with time which also resulted in the decrease in fungal population. Statistical analysis using analysis of variance (F-test) showed no significant difference in the fungal population in partially digested cellulose from the abomasums of the three cows on the first day of collection. However, a substantial ($p \leq 0.05$) increase in the population was noted on the seventh day after collection. This shows that external environmental factors had an effect on

the fungal population in the partially digested cellulose. The abomasum of the cow from which the partially digested food was collected can be regarded as an anaerobic environment and it is therefore expected that anaerobic mycoflora will proliferate. However, the incubation of cultures was carried out under aerobic condition which may have resulted in the reduction in population of fungi with increased incubation period and the non proliferation of fungi on the 7th day in some cases. This shows that some of the fungi in the partially digested cellulose were unable to adapt to aerobic conditions under which the cultures were studied. The inability of fungi to proliferate after several days of incubation affirmed that the fungi isolated in this study were actually associated with the digestion of plant materials in the anaerobic digestive tract of the cows.

The occurrence of fungi isolated from partially digested cellulose from the abomasum of the cows and their frequency (%) of isolation are as shown in Table 2.

The fungi isolated from partially digested cellulose and their frequency of isolation were *Aspergillus clavatus* (3.2%), *A. flavipes* (16.1%), *A. fumigatus* (6.5%), *A. niger* (12.9%), *A. sulphureus* (3.2%), *A. wentii* (6.5%), *Mucor* sp., (12.9%), *Penicillium aurantiovirens* (6.5%), *P. breviscaupactum* (9.7%), *P. raistrickii* (16.1%), and *Rhizopus* sp. (6.5%). Generally, the fungal species isolated belonged to the four genera of *Aspergillus, Mucor, Penicillium,* and *Rhizopus*. These fungi genera are the first invaders to commence digestion of structural plant components (Okigbo, 2005; Okigbo and Obire, 2009). They are also known to be ubiquitous in nature and are characteristically saprophytic.

Table 2. Occurrence of fungal isolates in partially digested cellulose from the abomasum of beef cows and their frequency (%) of isolation.

Fungal isolates	Cow No. 1	Cow No. 2	Cow No. 3	% Frequency
Aspergillus clavatus	-	+	-	3.2
Aspergillus flavipes	+	+	+	16.1
Aspergillus fumigatus	-	+	+	6.5
Aspergillus niger	+	+	-	12.9
Aspergillus sulphureus	-	-	+	3.2
Aspergillus wentii	-	+	-	6.4
Mucor sp	+	+	+	12.9
Penicillium aurantiovirens	+	-	-	6.5
Penicillium brevicompactum	-	+	+	9.7
Penicillium raistrickii	+	+	+	16.1
Rhizopus sp	+	+	+	6.5

\+ = Fungus isolated
\- = Fungus not isolated

The mesophillic range of temperature characterizing the partially digested cellulose in the abomasum will favour the activities of thermophillic fungi

(especially the mucorales - *Rhizopus* and *Mucor* which are then replaced by Hyphomycetes – *Aspergillus* and *Penicillium*). These become predominant at a temperature range of 30 – 50 °C as is common during composting. Fungi are ubiquitous in nature and are found in soil, air and water (Jawetz *et al*., 2002). Species of the genera *Candida, Aspergillus, Penicillium, Rhizopus* and *Mucor* are common (Nester *et al*., 1998). These fungi usually gain entrance into the digestive tract of ruminants through food and water. It is therefore not unusual to find species of these genera in the partially digested cellulose collected from the abomasum of cows and in cow dung (Preston, 1987; Obire *et al*., 2008).

The species of fungi isolated from the dung as reported in Gadre *et al.,* (1986) included *Aspergillus* spp*., Piramonas commonis, Sphaeromonas communis, Rhizopus* spp., *Mucor* sp. and *Penicillium* spp. The saprophytic fungi (yeasts and moulds) isolated from cow dung (Obire *et al*., 2008) were *Alternaria* sp., *Asper*g*illus* sp., *Cephalo*spo*rium* sp., *Cladosporium* sp., *Geotrichum* sp., *Monilia* sp., *Mucor* sp*., Penicillium* sp*., Rhizopus* sp*.,* Spo*rotrichum* sp*., Thamnidum* sp., *Candida* sp., *Rhodotorula* sp. and *Torulopsis* sp.

Health hazards may be associated with improper handling of animal wastes. Their use and disposal are some of the major sanitation problems in many developing countries, including Nigeria. The fungi isolated in this study can further be used to stimulate enhanced degradation of animal wastes, plant material and other decomposable organic materials for the production of biogas in biodigesters and in the production of compost or high quality soil conditioners (fertilizers). The fungal isolates may also be used in the production of enzymes for other industrial applications.

References

Abbey, S. A. (1995). Foundation in Medical Mycology. Port Harcourt. Kenalf Publication p. 25.

Barnett, H. L. and Hunter, B. B. (1972). Illustrated Genera of Fungi Imperfecti. 3rd Edition, Burgess Publishing Company, Minneapolis.

Bauchop, T. (1980) Scanning Election Microscope in the Study of Microbial Digestion of Plant Fragments in the Gut. In: Ellwood, C. B. C, Hedger, J. W, Latham, M. J. and Slater, J. H(eds). Contemporary Microbial Ecology, Academic Press, London.

Cheesebrough, M. (1984). Media Preparation and Culturing of Organisms. Medical Laboratory Manual for Tropical Countries 3: 43-45.

Dahiya, A. K. and Vasudevan, D. (1986). Biogas Plant Slurry as an Alternative of Chemical Fertilizer. Food and Agricultural Organization of the United Nations Rome 9: 67-74.

Fullick, A. (1994). Heinemann Advanced Science Biology. Portsmouth (NY) USA. p. 168-169.

Gadre, R. U., Ronade, D. R. and Godbole, S. H. (1986). A Note on Survival of Salmonellas During Anaerobic Digestion of Cattle Dung. Journal of Applied Bacteriology 60: 93-96.

Gomez, K. A. and Gomez, A. A. (1984). Statistical Procedures for Agricultural Research. 2nd edn. Singapore, John Wiley & Sons Inc. 680 pp.

Gottfried, S. S. (1993). Biology Today. United States of America, Moshy Year Book, Inc City p. 32.

Harrigan, W. F. and McCance, M. E (1990). Laboratory Methods of Food and Diary Microbiology. 8th Edition Academic Press London p. 452.

Herald, M. C. (2004). Cow Waste is Power of the Future. http/ww/montereyheraod.com/mld. Monterey Herald Business/9344785, htm.

Institute of Pollution Studies (1990). Laboratory Manual. Institute of Pollution Studies Rivers State University of Science and Technology, Port Harcourt, Nigeria. p.35.

Iwena, O. A. (2002). Essential Agricultural Science for Senior Secondary Schools. Ikeja, Tonad Publishing, p. 35-36.

Jawetz, E., Muniek, J. L., Adelberg, E. A, Brooks, G. F., Butel, J. S. and Ornstm, L. N. (2002). Medical Microbiology. 22nd Edition Prentice Hall International, in USA p. 592.

Malloch, D. (1997). Moulds Isolation, Cultivation and Identification. Department of Botany University of Toronto, Toronto USA.

McDonald, P., Edwards, R. A., Greenhalgh, J. F. D. and Morgan, C. A. (1998). Animal Nutrition 5th Edition, Pearson Education Limited, UK.

Nester, W.E., Roberts, C.E., Pearsall, N.N., Anderson,D.G. and Nester, M.T. (1998). Microbiology – A Human Perspective. 2nd ed. McGraw-Hill. N.Y

Obire, O., Anyanwu, E.C. and Okigbo, R.N. (2008). Saprophytic and crude oil degradingfungifrom cow dung and poultry droppings as bioremediating agents. Journal ofAgricultural Technology (Thailand) 4(2): 81-89.

Okigbo, R.N. (2005). Biological control of postharvest fungal rot of yams (*Dioscorea* spp.)with *Bacillus subtilis*. Mycopathologia (Netherland) 159: 307 – 314.

Okigbo, R.N. and Obire, O. (2009). Mycoflora and production of wine from fruits of soursop (*Annona muricata* L.*)* International Journal of Wine Research **(**Auckland**,** New Zealand) 1: 1-9.

Orpin, C.G. (1976). Studies on the Rumen Flagellate (Neocallimastix frontalis. Journal of General Microbiology 81: 249-269.

Orpin, C.G. (1979). The Rumen Flagellate Piromonas communis, its Life History and in Version of Plant Material in the Human. Journal of General Microbiology 99: 107-117.

Orpin, C.G. and Letcher, A.J. (1979). Utilization of Cellulose, Starch, Xylan and other Hemicelluloses for Growth of Rumen Phycomycetes, NeocaIlimastix frontalis. Current Microbiology 3: 121-124.

Orskoy, E.R. (1983). Possible Nutritional Constraints in Meeting Energy and Amino Acid Requirement of the highly Productive Ruminant. In: Ruckebugh, Y; and Thivend, P. (eds.). Digestive Physiology and Metabolism in Ruminants. M. T. P. Press, Lancaster p. 309-324.

Paul, E.A. and Clark, F.E. (1988). Soil Microbiology and Biochemistry. Academic Press Incorporated, New York.

Preston, T. R. (1987). Matching Ruminant Product Systems with Available Resources in the Tropics and Subtropic. International Colour Production, Stand Thorpe, Queensland; Australia 4: 380.

Singleton, P. and Sainsbury, D. (2001) (eds). Cellulases. In: Dictionary of Microbiology and Molocular Biology. 3rd Edn. John Wiley & Sons, New York. pp. 140 - 141.

TIM (1997). New developments in rumen microflora. Trends in Microbiology 5: 483 – 488.

Syed, M. H. (2004). Grameen's Programme for Research on Poverty Alleviation and Biodigester Experiment in Grameen. The Grammen Trust, Murphur Two, Dhaka-1216, Bangladesh p. 1-4.

Ulyatt, M. J. (1982). Plant Fibre and Regulation of Digestion in the Ruminant. In: Fibre in Human and Animal Nutrition. Royal Society of New Zealand, Bulletin 20: 103-107.
Walker, J. D. and Colwell, R. R. (1976). Enumeration of petroleum-degrading microorganisms. Applied and Environmental Microbiology 31: 198- 207.

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