

IN VITRO CELLULASE PRODUCTION FROM FIVE HERBIVORE MICROBIAL ECOSYSTEMS AND CONSORTIA*

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Abstract

As the most abundant biomass in nature, cellulose is the main chemical component in herbivore forages. The energy locked in these complex polymers can only be released by cellulolytic enzymes. Therefore, research aiming to increase the expression of cellulolytic enzymes or browsing uncultured microbial ecosystem in search of potential fibrolytic enzymes is imperative. The main objectives were to: (a) investigate the variation of cellulase enzymes in cow (CW), horse (H), miniature horse (mH), wildebeest (WB) and zebra (ZB); and (b) identify their presence and activeness in microbial consortia, N1 (H+WB), N2 (H+ZB), N3 (WB+ZB) and N4 (H+WB+ZB). Fresh faecal or rumen inocula were cultured in the laboratory on maize stover and lucerne (1:1) with salivary buffer for 72 h at 38°C. Crude proteins (CPZ) were precipitated from both fresh and cultured inocula using 60% ammonium sulfate for enzyme assays and zymography. Endocellulases and their activity were identified on 1% (m/v) carboxymethyl cellulose (CMC) zymograms stained with Congo red. All CPZ extracts were active as reducing sugars were produced after incubation with crystalline cellulose, CMC and xylan. The number and types of proteins with endocellulase activity varied ($P < 0.05$) among and within the different animal species CW (15), H (14), mH (14), WB (13) and ZB (13). Microbial consortia were active with relatively higher number of endocellulases, N1 (17), N3 (14), N4 (14), and N2 (13). Cellulase enzymes vary among and within herbivore species grazing on the same or different fields. Therefore, identifying specific enzymes and microbes with higher fibrolytic potentials from different ecosystems for transinoculation could play a vital role in improving forage digestibility in ruminants.

Key words: fibrolytic enzymes, rumen, faecal inoculum

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Cellulose is a plant polysaccharide composed of β -D-glucopyranosyl units joined together by β -1, 4-glycosidic bonds. It is the major component of plant cell walls and most abundant plant polysaccharide. The breakdown of cellulose to glucose units occurs in three basic steps involving three major cellulase enzymes, namely exocellulase, endocellulase and cellobiase (Percival Zhang et al., 2006); (1) endocellulase randomly hydrolyzes β -1,4 glycosidic linkages in the amorphous regions of the cellulose chains, (2) exocellulase hydrolyzes β -1,4 glucocidyl linkages at either the non-reducing or reducing ends of cellulose forming cellobiose and (3) β -cellobiase hydrolyzes cellobiose into two glucose molecules. These glucose molecules are fermented by the anaerobic microbes for energy generation (ATP) yielding short chain fatty acids (SCFA) as by-products which are the main metabolic substrates for energy production in ruminants.

Cellulases and related enzymes are commonly used in many industrial applications including food, brewery, paper, animal feed and agricultural research (Beauchemin et al., 2003). Animal feed will be the main focus as more than 600 million tonnes of feed worth more than 50 billion US dollars are produced annually (Bhat, 2000). Cellulases and xylanases have been shown to have a wide range of feasible applications in both monogastric and ruminant animals (Yang and Xie, 2010). In monogastric animals, cellulases are used either to eliminate anti-nutritional factors present in grains or vegetables, degrade certain cereal components in order to improve the nutritional value of feed or to supplement animals' own digestive enzymes whenever these enzymes are insufficient, especially in the post-weaning period in piglets (Bhat, 2000). In ruminants, it has been used to improve feed digestibility, milk yield, body weight gains and animal performance (Arriola et al., 2011). Although many studies on enzyme supplementation have shown substantial improvements in feed digestibility and animal performance (Bala et al., 2009; Titi and Tabbaa, 2004), others reported negative effects or none at all (O'Connor-Robison et al., 2007; Peters et al., 2010).

Enzyme sources can be either pure or crude (but richer in the application enzyme). Pure cellulase enzymes are expressed by specific microorganisms using molecular biology techniques in the laboratory while crude cellulase enzyme mixtures are concentrated from *in vitro* incubators. Although cellulase expressions by microbes or genetically modified microbes have been the main sources of enzymes, attempts have also been made to clone cellulase and xylanase genes in order to produce transgenic animals (Hall et al., 1993). These animals would secrete the required enzymes into the gastrointestinal tract to facilitate its feed digestion efficiency. Research of this sort is not very common though it has the potential of unveiling more information on cellulase functions. Despite many reports describing the isolation and characterization of cellulases and xylanase from different environmental genomic libraries, the biotechnological potential of novel cellulases from uncultured microbial community is far from being fully explored. Therefore, exploring a variety of ecosystems to identify the different types (size) of cellulase enzymes present, their fibrolytic potential as well as their combined effect might be beneficial in ruminant nutrition.

The main objectives were: (a) to identify the types of cellulase enzymes available in the following individual microbial ecosystems (IS): cow (CW), horse (H), miniature horse (mH), wildebeest (WB) and zebra (ZB); and (b) identify their pres-

ence and activeness in consortia (to investigate combined effect), N1 (H+WB), N2 (H+ZB), N3 (WB+ZB) and N4 (H+WB+ZB).

Material and methods

Samples (faeces) were collected randomly in winter from four animals: horses (H) and miniature horses (mH) (Ukulinga Research farm, University of KwaZulu Natal), blue wildebeests (WB) and plains zebras (ZB) with no preference to sex. *Equus quagga* (ZB) and *Connochaetes taurinus* (WB) were available at the Tala Game Reserve, uMbumbulu. The ZB and WB were grazing on a dry land in an open field where *Pennisetum clandestinum* (Kikuyu grass) was dominant among other dry grasses. Rumen fluid was collected from the cows (CW) alone. Although the CW, mH and H were grazing on a dry land in an open field where Kikuyu grass was dominant, they were supplemented with veld hay while in pens.

Faeces were collected within 2 min of defecation from mH, H, ZB and WB before transferring into an airtight insulated flask (38°C) which had been flushed with CO₂. While in the laboratory, 300 g of faeces was mixed with 300 ml of warm incubation buffer (made by titrating 2 l of warmed solution A (solution A, NaHCO₃ (19.60 g), Na₂HPO₄ (7.40 g), KCl (1.14 g), NaCl (0.94 g), MgCl₂·6H₂O (0.26 g) and (NH₄)₂SO₄ (5.8 g), in 2 l distilled water) with 2 ml of Solution B (53 g CaCl₂·2H₂O in 1 l distilled water) and flushed with CO₂ (Tilley and Terry, 1963). The mixture was stirred thoroughly before squeezing through four layers of cheese cloth (pre-warmed and flushed with CO₂) to make faecal fluid (FF). For the cow, rumen fluid (200 ml) was collected through a fistula, strained through cheese cloth (pre-warmed and flushed with CO₂) to make rumen fluid (RF). All inocula were prepared in the shortest possible time in order to minimize the exposure of anaerobic microbes to oxygen. For each animal species, two samples were collected and pulled together to represent that species before sampling.

For rumen or faecal fluid culturing, 198 ml of each filtrate (CW, mH, H, WB or ZB) was pipetted into 402 ml of incubation buffer (flushed with CO₂ and placed in an incubator to equilibrate to 39°C for 1 h) containing 6 g of 1:1 mixture of lucerne (LS) and maize stover (MS) milled through a 1-mm sieve. For microbial consortia, N1 (H+WB), N2 (H+ZB), N3 (WB+ZB) and N4 (H+WB+ZB) were mixed in equal proportions to investigate microbial synergism. The culture samples were flushed with CO₂ and incubated for 72 h at 38.5°C in airtight Duran® bottles (Duran Group, Germany) (4 L). After 72 h, cultured RF or FF was used for extraction of crude protein (CPZ) for enzyme assays and zymography. After culturing, CW, mH, H, WB and ZB became CW1, mH1, H1, WB1 and ZB1, respectively.

Crude protein was extracted from cultured filtrate using 60% (NH₄)₂SO₄, dialyzed, concentrated using polyethylene glycol 20000 (Fluka, USA, product no 81298) and concentration determined by Bradford assay. Enzyme assays were carried out to confirm the activeness of each CPZ prior to zymography. Exocellulase was assayed by pipetting 0.5 ml of 1% (m/v) crystalline cellulose (product no 11363,

Sigma-Aldrich®, Germany) with the reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0) into 0.5 ml of crude protein solution obtained from rumen fluid or faeces and incubated for 72 h at 39°C. The enzyme reaction was stopped by boiling at 100°C. The reaction mixture for endocellulase assays, contained 0.5 ml of 0.5% (m/v) carboxymethyl cellulose (CMC) from Fluka Biochemica (product no 419273), in the reaction buffer at pH 5.5 and 0.5 ml of CPZ. The enzyme reaction was stopped by boiling at 100°C after incubating at 39°C for 2 h. Samples were centrifuged at 6000xg at RT for 5 min, then 400 µl of the reaction mixture was analysed for reducing sugars. Xylanase activity was assayed by pipetting 0.6 ml of 0.1% (m/v) xylan (Sigma, USA, product no X4252) solution in the reaction buffer (pH 5.0) into 0.4 ml of CPZ solution and incubating at 39°C for 1 h. The reaction was stopped with 100 µl of 200 mM NaHCO_3 . All stopped reactions were centrifuged (6000xg, 5 min at 4°C) and 400 µl samples analysed for reducing sugars using 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959), using a spectrophotometer and absorbance read at 540 nm. Reducing sugars in unknown reaction samples were determined on glucose or xylose standard curves as described by Colombatto and Beauchemin (2003). Each enzyme assay was replicated three times with four pseudo repeats. Enzyme specific activity was defined as µg of reducing sugar (glucose or xylose)/mg crude protein.

Both reducing and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was used in this study to monitor the presence of proteins in the CPZ samples. Crude protein extracts (10 µg/5 µl) were separated on a 10% running gel. The gels were stained with Coomassie blue-250 or by silver staining (when the proteins were too small to be stained by Coomassie blue-250) for the detection of protein bands on the gel (Blum et al., 1987). Crude protein extracts were run alongside a molecular weight marker (150, 100, 75, 50, 35, 25 and 15 kDa) (Sigma) for estimating unknown molecular weights of protein bands. Each CPZ ecosystem extracted at three different times of collection was run on gels more than three times to establish the presence of protein bands.

For zymography assays, CPZ were first separated on a 10% non-reducing SDS-PAGE gels which had been polymerized with 1% (m/v) CMC substrate. After separation, re-naturation of proteins was carried out by soaking the gels in two changes of 50 ml of 2.5% (v/v) Triton X-100 (to remove SDS) over 3 h at room temperature (Dodia et al., 2008; Heussen and Dowdle, 1980). Following this, the gels were incubated in the reaction buffer pH 5.0 at 33°C for 48 h. After incubation these gels were stained with 0.2% (w/v) Congo red to identify protein bands with cellulase activity as previously described by Beguin (1983). After staining the gels were rinsed with 5% acetic acid to change the background to blue black for easy photographing. Crude protein extract samples were run alongside a molecular weight marker which was cut off after re-naturation and stained with Coomassie blue-250. These markers were used to plot a standard curve (distances migrated versus molecular weights) to estimate the molecular weight of the unknown active cellulase bands.

In vitro enzyme specific activities estimates derived from CW, mH, H, WB, ZB, N1, N2, N3 and N4 were subjected to the analysis of variance (ANOVA) using the general linear model of SAS (2002):

$$Y_{ij} = \mu + E_i + e_{ij}$$

where:

Y – is the individual observation,

μ – is the overall mean,

E_i – is the effect of the inoculum source and

e_{ij} – is the random variation.

Results

Exocellulase, endocellulase and xylanase specific activities were higher ($P < 0.001$) in the *in vitro* cultured microbial ecosystems than in the fresh microbial ecosystems (Table 1). For exocellulase enzymes, N1, N2 and N4 had the highest specific activities followed by H1 with intermediate activity. Endocellulase activity differed ($P < 0.001$) among the microbial ecosystems with systems N4, H1, ZB1 and N1 recording the highest specific activities. Systems N3, N2, WB1, mH1 and CW were intermediate in their endocellulase specific activities but lower for the fresh microbial ecosystems.

Table 1. Xylanase, endocellulase, and exocellulase specific activities of crude protein extracts

Inoculum source	Xylanase (μg xylose/mg)	Endocellulase (μg glucose/mg)	Exocellulase (μg glucose/mg)
CW	38.0 e \pm 2.34	2.9 f \pm 0.01	1.8 g \pm 0.02
mH	30.0 f \pm 2.14	5.9 e \pm 0.05	4.5 g \pm 0.07
H	46.3 e \pm 3.11	6.4 e \pm 1.00	5.4 g \pm 0.20
WB	8.0 g \pm 2.01	3.7 e f \pm 0.10	3.2 g \pm 0.06
ZB	43.5 e \pm 4.10	5.9 e \pm 0.14	4.4 g \pm 0.30
CW1	83.2 c \pm 1.21	78.6 c \pm 2.11	68.2 f \pm 3.00
mH1	75.4 c \pm 1.66	83.3 bc \pm 3.01	94.9 de \pm 3.08
H1	98.3 b \pm 1.90	92.7 b \pm 2.31	141.8 b \pm 9.03
WB1	57.3 e \pm 5.76	72.6 d \pm 0.45	90.5 e \pm 2.67
ZB1	88.8 bc \pm 1.53	89.2 b \pm 2.11	107.1 c \pm 0.04
N1	81.4 c \pm 1.17	87.8 b \pm 4.57	163.1 a \pm 6.67
N2	104.5 b \pm 2.62	77.7 d \pm 2.65	162.6 a \pm 2.73
N3	68.6 d \pm 2.85	80.5 bc \pm 0.53	98.1 d \pm 2.76
N4	183.1 a \pm 4.74	108.5 a \pm 4.11	160.9 a \pm 9.33
P-value	0.001	0.001	0.001

\pm SE = standard error, CW, mH, H, WB, ZB = fresh CPZ extracts, CW1, mH1, H1, WB1, ZB1 = cultured.

System N4 recorded the highest ($P < 0.05$) xylanase activity while N2, H1 and ZB1 were intermediate. Xylanase specific activities were much lower in the fresh systems than in the rest of the cultured systems.

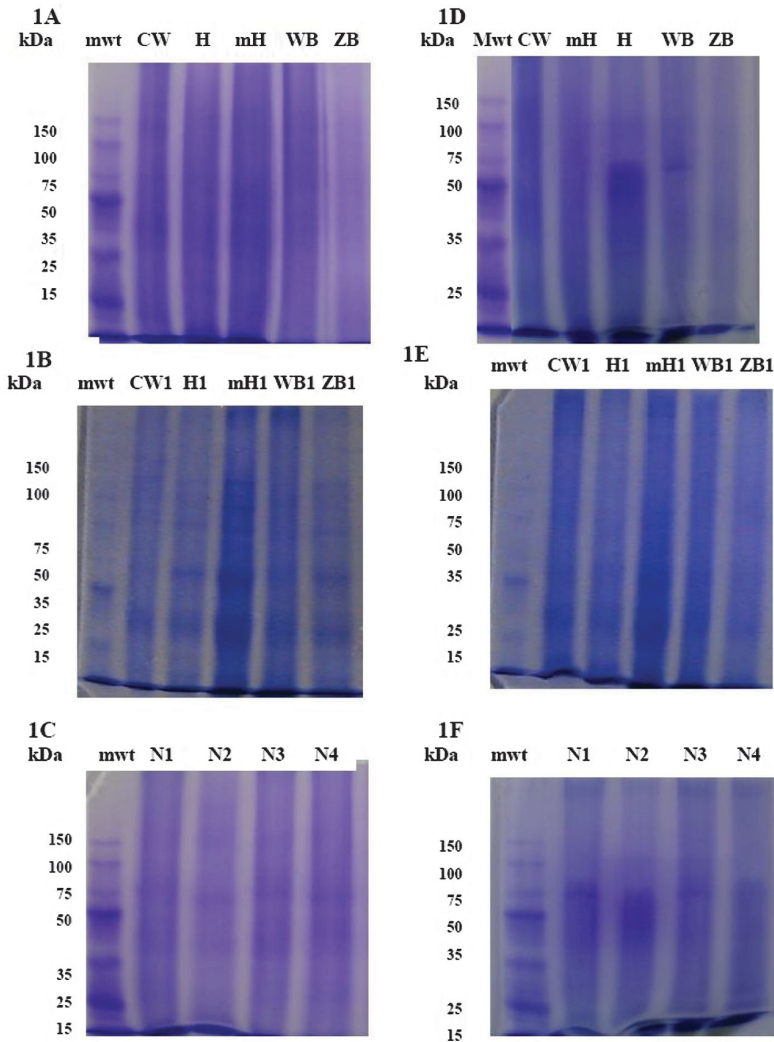


Figure 1. Coomassie stained Laemmli reducing and non-reducing 10% SDS-PAGE monitoring the presence of proteins in crude protein extracts from different microbial ecosystems. 1 A, B and C are reducing SDS-PAGE while D, E and F are non-reducing SDS-PAGE, mwt=molecular weight markers (150, 100, 75, 50, 35, 25 and 15 kDa), fresh crude protein extract were from cow (CW), horse (H), miniature horse (mH), wildebeest (WB) and zebra (ZB) while cultured crude protein extracts from CW1, H1, mH1, WB1, ZB1, N1 (H+WB), N2 (H+ZB), N3 (WB+ZB) and N4 (H+WB+ZB)

SDS-PAGE demonstrated that all the CPZ extracts from the different microbial ecosystems contained proteins of diverse molecular weights. These CPZ contain numerous protein bands ranging from 1 to 250 kDa (Figure 1 A, B, C, D, E and F). The thickness of protein bands from fresh CPZ was less than those observed after incubation. This was demonstrated by the prominent protein bands observed in fresh CPZ (Figure 1 A and D) compared to cultured CPZ (Figure 1 B, C, E and F). Specific

protein abundance was variable in the different microbial cultures, Figure 1 C (30, 48, 50 and 91 kDa), Figure 1 B (30, 40, 50, 70, 75 and 80 kDa), Figure 1 E (30 and 38 kDa) and Figure 1 F (75 and 85 kDa). All nine CPZ extracted from *in vitro* microbial ecosystems exhibited endocellulase activities as demonstrated by the clear zones observed on zymograms (Figure 2). One major active band (216 kDa) was observed in all the CPZ from fresh systems (Figure 2 A). Besides protein 216 kDa, the protein 180 kDa also exhibited a higher endocellulase activity in WB and ZB. The active band 216 kDa was common to all the cultured systems. The enzyme of 191 kDa was present in all the combined systems (Figure 2 C) including H1 (Figure 2 B). There were many other active bands on the gels that were too small to be captured by the photographic equipment available but were read and reported in Table 2. The total number of active protein bands varied among the nine microbial ecosystems. Cultured microbial ecosystems CPZ had more active cellulases than fresh CPZ. In fresh CPZ, the number of active endocellulases was 10, 7, 6, 5 and 5 for ZB, CW, WB, mH and H, respectively. In the cultured systems, the highest number of endocellulases was found in N1 (17), followed by N3, N4, and mH1 with 14, N2 and ZB1 having the least numbers.

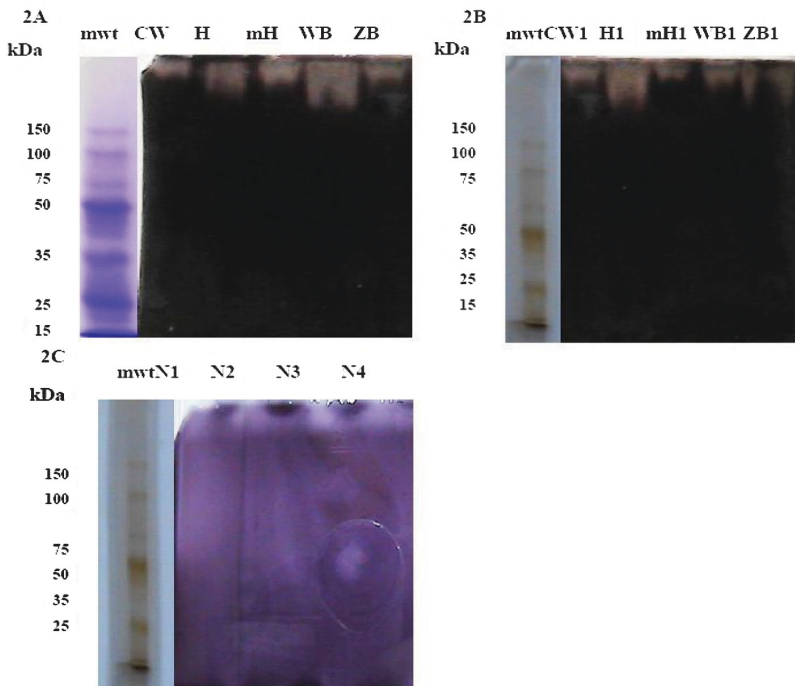


Figure 2. Zymograms of cellulase activities from crude protein extracts of nine *in vitro* microbial ecosystems on Laemmli non-reducing polyacrylamide gel electrophoresis. Endocellulases activities detected on non-reducing polyacrylamide gels containing 1% CMC (2 A, B and C). The wells were loaded with crude protein samples (10 µg/5 µl) extracted from both fresh (CW, H, mH, WB and ZB) and cultured (CW1, H1, mH1, WB1, ZB1, N1, N2, N3 and N4) microbial ecosystems and incubated for 48 h at 38°C. Gels were stained with Congo red, rinsed with 5% acetic acid, mwt were stained with Coomassie-blue-250 or silver stain

Table 2. Apparent molecular masses of cellulase enzymes from crude protein extracts from five herbivores

Enzyme source	Approximate molecular weight of enzymes (kDa)									
	216	175	124	119	114	74	23			
CW	216	175	124	119	114	74	23			
mH	216	165	124	119	48					
H	216	175	150	135	48					
WB	216	124	74	48	48	38				
ZB	216	180	165	150	99	74	58	48	18	3
CWI	216	185	180	175	150	145	124	114	99	84
mHI	216	185	175	165	155	150	145	135	124	104
HI	216	191	175	165	150	145	124	109	104	99
WBI	216	206	195	185	175	160	150	145	135	124
ZBI	216	165	155	150	135	124	114	99	74	64
N1	216	195	191	175	170	156	150	135	124	74
N2	216	191	170	150	124	114	99	74	53	48
N3	216	191	185	175	150	135	114	109	74	48
N4	216	201	191	175	165	160	150	135	124	99

CW, mH, H, WB, ZB = fresh enzyme extracts, CW1, mH1, H1, WBI, ZBI = cultured enzyme extracts.

Discussion

This study was designed to investigate the presence and variation of cellulases in 5 *in vitro* herbivore microbial systems and their activeness in a consortium. All CPZ extracts were active as demonstrated by their exocellulase, endocellulase and xylanase activities when incubated with their specific substrates. Crude protein extract separation on both reducing and non-reducing SDS polyacrylamide gels was successful after staining with Coomassie blue G-250 but did not form prominent bands that could be captured by the camera at our disposal. The lack of prominent bands on the gels was due to low individual protein quantity since CPZ extracts contain numerous proteins and only a limited amount can be loaded on the gels at a time (10 µg). Using a more sensitive method (Silver stain) to make these bands more visible failed as the whole gel turned black due to numerous protein bands (Oakley et al., 1980). However, the protein bands were more prominent for cultured CPZ than fresh. This was probably due to the absence of endogenous protein contamination (from the rumen or hindgut) in cultured systems than in fresh CPZ. Secondly, specific cellulase enzyme concentrations might have been enriched in cultured CPZ during culturing on maize stover and lucerne feed as the population of specific microbes increased. The lower number of endocellulases in fresh CPZ was probably due to smaller quantities of specific enzymes than their absence. Therefore, clear band formation on the gels might have been limited by smaller quantities of specific proteins on gels given the time of incubation. These results showed that where cellulase enzyme purification methods are limited, cultured CPZ extracts for enzyme assays and identification would be preferred to fresh CPZ.

The number and type of cellulases vary within and among herbivore species grazing on the same field. Seven of the protein bands with endocellulase activities were common in CW, H and mH grazing on the same field while the remaining 13 cellulases were either present in one or two of the three animals. Five of the proteins (216, 150, 124, 99 and 74 kDa) with endocellulase activities from herbivores in the wild were also found in the domesticated herbivores. The active protein bands 206, 195 and 58 kDa were unique for herbivores in the wild. Though feed type does influence type of microbes and enzyme expression, its effect in this study was considered as minimal since all animals were being fed or grazing high fibre diets. Secondly, culturing with the same diet attempted to zero diet effect to enable enzyme potential to be the main dependent variable. Metagenomic studies of bovine rumen microflora by Ferrer et al. (2005) revealed nine active endo- β -1,4-glucanases less than observed in this study. This implies that crude method screening could be vital prior to enzyme purification and expression.

Clear bands on zymograms were evidence of CMC digestion as previously established by other researchers (Kim et al., 2009). Apart from N2, the total number of active proteins in N1, N3 and N4 were higher than those of its individual systems. This was associated to an increase in population of specific cellulase enzymes during culturing. The total number of active proteins ranked the systems as N1>N3>N4>N2. Although system N1 had the highest number of endocellulase (17), it was formed from individual systems with a combined number of 20 endocellulases. The differ-

ent types of cellulase numbers observed in this study goes a long way to confirm the diversity of enzyme and microbes in the same or different microflora (Duan and Feng, 2010). Nine enzymes from the individual systems H1 and WB1 were absent in N1. There is likelihood that the expression of these missing enzymes might have been inhibited (proteolysis) by other microbes in the systems during recolonization of the different ecological niches (Morgavi et al., 2001). It is also possible that these enzymes might still be there but in smaller quantities (not detected on the gels) if not expressed by the dominant microbes in the niche.

The combined microbial ecosystems N1, N2, N3, and N4 were found with 5, 4, 5 and 2 new enzymes, respectively, which were not visible on the individual zymograms. These new enzymes may be coming from microbes which might have proliferated to become dominant in the new system conditions that was probably lacking in the previous individual ecological system. It is also possible to suggest that these enzymes (from variable microbial population) might have been there but in smaller quantities unable to form clear bands on the zymograms given the incubation time.

The molecular weights of proteins with CMCase activities in this study were browsed online (UniProtKB/Swiss-Prot, 2013) to verify their presence on protein databases. Almost all the bands were matched with cellulases of the same or similar molecular weights with protein names as cellulases or endoglucanase or endocellulase. These matching proteins were mainly from ruminant or hindgut microbes. Some of the active bands (3 and 6 kDa) were not found online from rumen or hindgut microbes but were available from other sources (Bronnenmeier et al., 1997; Clarke et al., 1997). Therefore, more research is required to establish these bands as novel. The molecular weight of cellulases isolated from rumen or hindgut microbes ranges from 9 to 160 kDa online. The bands with higher molecular weights were suggested to be cellulosomes (complexes of cellulolytic enzymes). This was confirmed after browsing online and identifying cellulosomes with similar molecular weight (Najmudin et al., 2006). However, some cellulosomes were found with molecular weights lower than 150 kDa (Gal et al., 1997). With the results obtained from zymography, it is possible to conclude that all the proteins that formed clear bands on the zymogram had endocellulase activities but it cannot be concluded that each clear band was a CMCase or cellulosome enzyme.

In conclusion, this study shows that all seven *in vitro* microbial ecosystems were active as demonstrated by their cellulase activities. Cellulases activities on zymograms are better studied with cultured than fresh inocula as culturing increases specific protein concentration. Cellulase expressions vary within and among animal species grazing on the same field. Endocellulases were higher in CW than mH and H grazing on the same field. Wildebeest and ZB varied with the types of endocellulases expressed but not in numbers. Transinoculation may be possible as indicated by the activeness of microbial consortia. The combined systems were ranked according to the number of cellulases they retained as N1>N3>N4>N2. Although four active bands (206, 216, 6 and 3 kDa) seem to be novel, more research is still required for its purification and characterization which could be used as feed additives.

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