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Morphological Characterization Of Rumen Protozoa Isolated From Sweet Sorghum Hydrolysis Using Carabao (*Bubalus bubalis* Linn) Rumen Fluid

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Abstract

This study was conducted to characterize the morphology of rumen protozoa isolated in the batch type hydrolysis of sweet sorghum using 1% fresh carabao rumen fluid with duration of 0, 3, 6, 9, 12 and 15 days of the novel process hydrolysis. Hydrolysis was conducted using 5% chopped sweet sorghum bagasse. Nitrogen content was augmented with 0.35 g urea per 750 ml effective volume with low speed agitation of fermentation bottle at least 20 minutes twice daily and incubated at room temperature. Characterization was limited to microscopic evaluation of the morphological features of protozoa that was stained with methylene blue formalin saline solution. Protozoon population of 2.61 x 10 4 cfu/ml at initial period had significantly lowered down at the 15 days hydrolysis to 0.62 x 104 cfu/ml. Evaluation of morphology for the types of protozoa at different durations showed the predominance of species types with different orientations of caudal spines, shapes of the macromolecules, size of the body, and presence of adoral ciliary zones were features of species types from genus Eodinium Entodinium of the family Ophryoscolecidae. Evaluation of the hydrolysis condition showed that duration had significant effect on the pH ($P>0.05$). Sweet sorghum hydrolysis has initial pH 7 that significantly declined to pH 5 at 15 days duration. The population of the protozoa in the rumen fluid hydrolysis was significantly affected by duration of the hydrolysis ($P>0.05$). Entodinium were isolated at all durations of the acidic hydrolysis of sweet sorghum bagasse using carabao rumen fluid. In conclusion, morphological characteristics of the rumen protozoan shows how diverse are the composition of rumen protozoa in the carabao rumen fluid hydrolysis. The presence of the rumen protozoa is a justification of the novel carabao rumen fluid hydrolysis conversion of lignocelluloses in sweet sorghum bagasse into soluble carbohydrates for bioethanol production. The information is vital for animal feed utilization and bioethanol production optimization.

Key Words: Rumen protozoa, Philippine Carabao, Hydrolysis, Bioethanol, Bagasse, Feeds

Introduction

Many researches had emerged on rumen protozoa using taxonomical, morphological, physiological and nutritional characterizations of the rumen microbe (Thomkin,1960 Abou et al,1960;

Dehority et al, 1978; Coleman, 1979; Dehority,1993). Hungate et al, 1971 have reported on the role of protozoa as source of nitrogen and amino acids in the ration of its host animal. Furthermore, Hungate et al 1971 indicated that rumen protozoa divide not more than once a day, a their reproduction process known as asexual binary fission wherein parent protozoon divides to a number of daughter protozoon. Accordingly, the weight of protozoa was estimated as 2% of the weight of the rumen contents suggesting high concentration of the protozoa in the rumen. Protozoa functions in the rumen were also disclosed about ciliated protozoan behaviour of ingestion of rumen bacteria during digestion of cellulose and ingestion of starch granules (Dehority, 1993: Coleman, 1980).

Rumen protozoa are anaerobic fermentative microorganisms and found to have important role in rumen metabolism by elimination of carbohydrates from bacterial attack (Ushida, 2011). Predation behaviour of rumen protozoa according to Coleman (1979) is another significant role of protozoon that many microbiologists became interested to study species control of fermentation. Rumen protozoa were unknown species component of the rumen hydrolysis of lignocelluloses feedstock such as rice straws (Abenes and Florendo, 2009). The efficiency of carbohydrate conversion was assumed due to the synergy activities with large number of protozoan and other cellulolytic rumen microbes that had adapted in the hydrolysis (Florendo et al., 2017; 2018). Optimization would require in-depth knowledge of the rumen protists because of their large genera and enumeration second to rumen bacteria in the carabao rumen fluid hydrolysis. Information on the population of the types of rumen protozoa during hydrolysis are vital in justifying the process hydrolysis as pre-treatment intended for bioethanol production and use of carabao rumen microbes as direct source of cellulolytic enzymes. This study had aimed to isolate, characterize the rumen protozoa in the hydrolysis of sweet sorghum using carabao (Bubalus bubalis Linn) rumen fluid.

Bioethanol as industrial product utilizing fermentation are rapidly coming up. Among the exploratory research was the use of microorganisms from animal rumen, like the carabao rumen fluid microorganisms that was discovered to hydrolyze carbohydrate into bio-ethanol from crop residues. Since carabao rumen fluid microorganism process is a mix fermentation, in-dept study of the biological pre-treatment of lignocellulose feedstock for bioethanol production, particularly protozoan are significant contributors to the efficiency of carbohydrates conversion in the carabao rumen and the novel carabao hydrolysis (Hungate, 1993; Florendo et al., 2022). The aim of this research is to determine the types of species of rumen protozoa in the sweet sorghum carabao rumen fluid hydrolysis as pre-treatment of sweet sorghum lignocellulose intended for bioethanol production and determine the nature of rumen protozoon as affected by duration of the carabao rumen fluid hydrolysis of sweet sorghum.

Materials and Methods

Preparation of Biomass

Sweet sorghum was collected at the forage production area of the Small Ruminant Center, Central Luzon State University. After the canes were harvested, the sweet juice was removed by manually pounding the stalk using a hammer, bagasse were air dried for three days. Sun cured stalks (1cm) were shredded using a laboratory blender, washed and drained the fluid and dried. For the determination of moisture content, the dried bagasse were dried in an oven maintained at 105º C for 1 hour. Sub-sample of bagasse was autoclaved at 1210C, 15 psi for 15 minutes.

Rumen collection and preparation

Using a thermo flask, one liter of rumen fluid with digesta was collected from a rumen cannulated carabao (Bubalus bubalis L.) owned by Philippine Carabao Center (PCC) National Genepool. In the PCC at CLSU Nutrition laboratory, the digesta and fluid were squeezed using a cotton cloth and the rumen fluid was bubbled with CO2 gas for 5 minutes prior to inoculation. Process Flow for the collection and preparation and sampling of rumen fluid for protozoa counting.

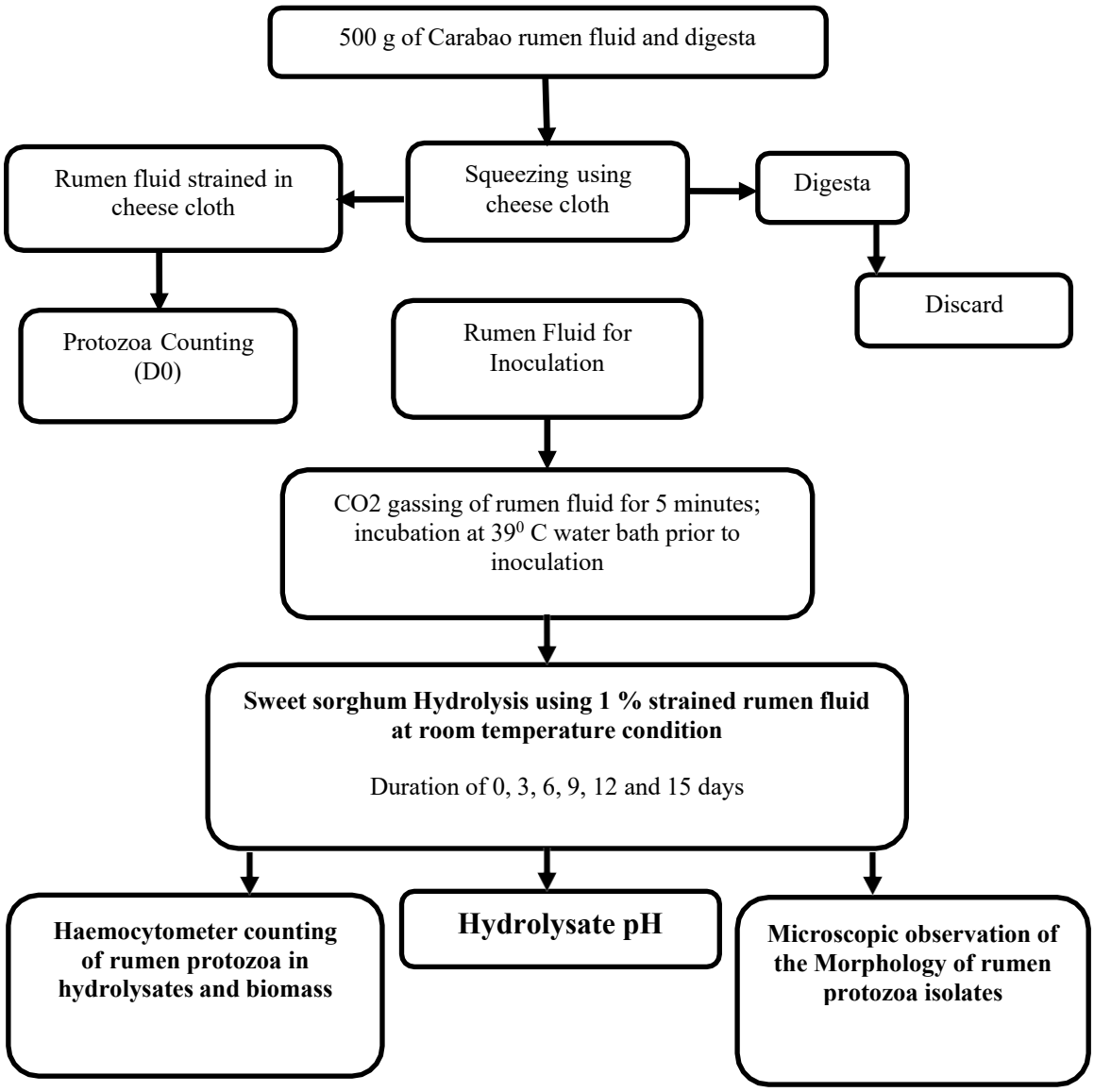

Figure1. Collection and preparation and sampling of rumen fluid for protozoa counting and morphological characterization

Feedstock Hydrolysis

Sterilized shredded sweet sorghum of 5g each replicates were sterilized at 1210C, added 700 ml warm water at (37º C) and 0.39g urea granules into a CO2 pre-gassed 1L capacity bottle. The mixture was agitated at low speed in order to dissolve the granules and prevent lumping of biomass. The final mixture was gassed with CO2 gas for 5 minutes and adjusted the pH to 7 by using NaOH solution. The bottles were sealed with perforated wood cork and balloon as gas trap, stored at room temperature. Every day at 8 to 9 AM and 4 to 5 PM, the fermenters were subjected to low speed agitation of 10 minutes. The durations of sweet sorghum hydrolysis were D0, D3, D6, D9 and D15 days. Subsampling of hydrolysates were done at the end of each duration in 3 fermentation bottles, each bottle serving as replicate of each treatment duration.

Measurement Hydrolysis pH

Ten (10) ml of hydrolytes from fermenter was taken using a sterilized 10 ml pipette. The sample was placed in 100 ml beaker and cooled at Chiller until the hydrolysate attained a temperature of 20º C. After cooling, the pH was measured by mean of a portable pH meter. Data on pH value was taken at day 0, 3, 6, 9, 12 and 15 days duration of sweet sorghum hydrolysis.

Isolation of Carabao Rumen Fluid Hydrolysis Protozoal Fraction

Fresh 500g rumen fluid and digesta was collected via a rumen cannula from a carabao (Bubalus bubalis L). Digesta and rumen fluid was squeezed with cheese cloth to separate rumen fluid into thermoflask. The thermoflask filled with rumen digesta was immediately travelled to the PCC at CLSU Nutrition laboratory for further filtration and carbon dioxide gassing. From the pre-gassed rumen fluid, sub-sample of 7 ml of the strained carabao rumen was mixed with 0.9% saline solution. The mixture was centrifuged for 5 minutes using a Babcock centrifuge, washed the filtrate that contain the protozoa and then re- suspended 7 ml of saline solution. A new Bauer haemocytometer was used in the counting of the protozoa at hydrolysis day 0.

For haemocytometer cell counting, sub-sample of 7 ml ruminal fluid was mixed with 7 ml of 0.9% saline to come up with 14 ml solution. The solution was filtered using a Whatman filter paper 541 to separate the protozoa and re-suspended in 1 ml of mixed Methylene Blue Formalin Saline Solution (MFS). One ml filtrate MFS solution was used in the 5x5 grid of Neubauer Haemocytometer. Results of cell count per square were converted into number of protozoa per ml of rumen fluid following guideline of haemocytometer. Subsampling and protozoa population counts were analyzed at duration day 0, 3, 6, 9, 12 and 15 days. Back up sample were stored at 40C until the completion of protozoa cell counting.

Morphological Characterization of Isolated Rumen Protozoa

The rumen protozoon was characterized by using mixed Methylene Blue Formalin Saline solution (MFS). The composition of mixed methylene blue formalin saline solution was derived from Dehority (1993). For identification of the collected protozoon from the sweet sorghum hydrolysis, the following guidelines were followed;

1. MFS solution was shaken to break the pelleted protozoa and facilitate the protozoon absorption of the MFS stain. Protozoon cell stained with MFS clearly differentiate specie dark blue macronucleus.
2. Shape of macronucleus, skeletal plate, caudal spines or location of the outside cilia and organelle used for movement or food ingestion can be observed using the low power magnification.
3. Dehority (1993) classification of protozoa in terms of morphological features was primary reference in the identification of the rumen protozoa isolated in the carabao rumen fluid hydrolysis. Figure2 -2a shows the identification of the protozoon isolated from carabao rumen fluid hydrolysis of sweet sorghum.

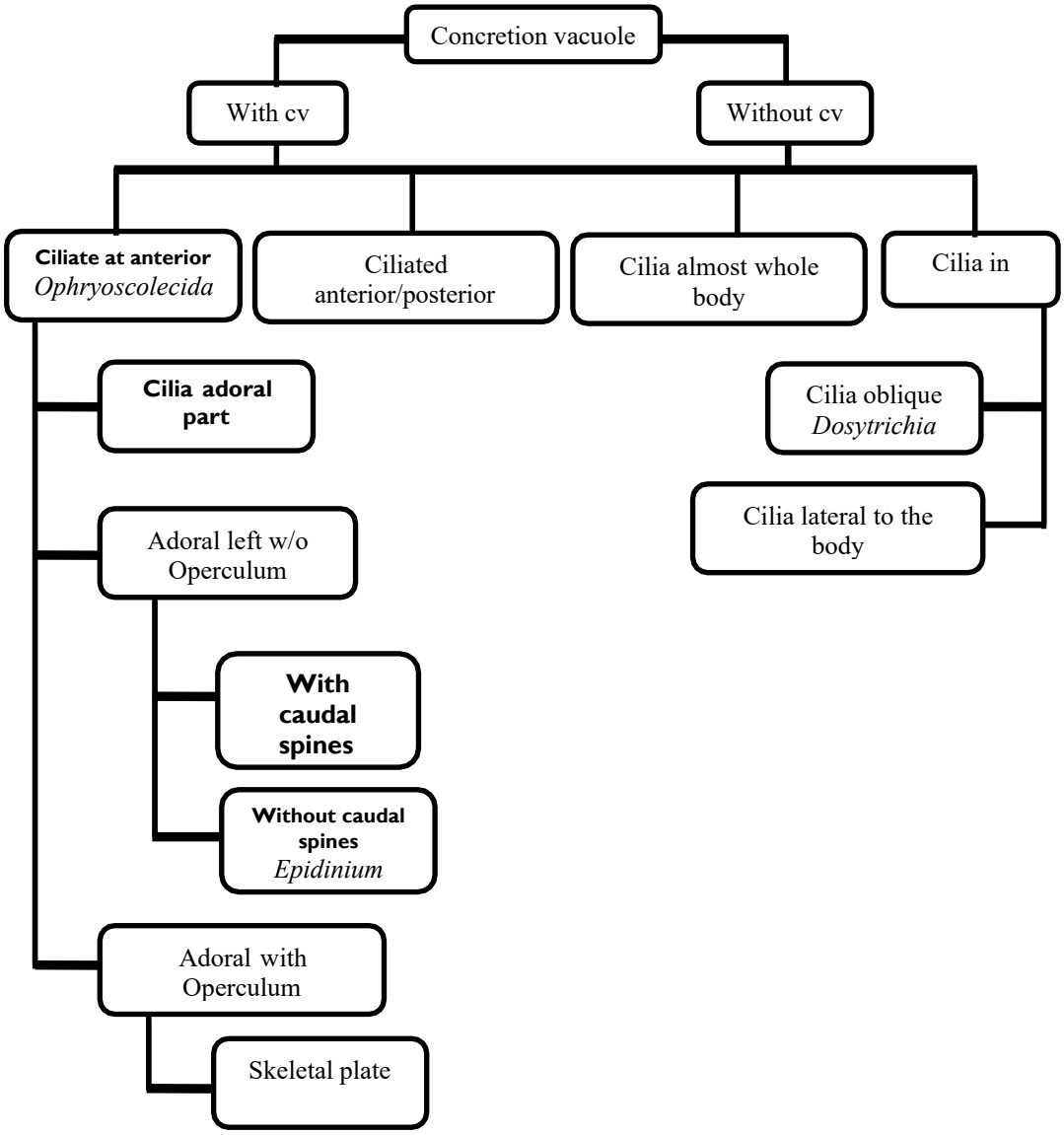

Figure 2. Morphological classification of rumen protozoon with caudal spined rumen protozoon and ciliates (Dehority classification guide, 1993).

4. A digital camera resolution was set at 800 x 360, the following characteristics of MFS stained protozoon and differentiation among species at genus level;
    a. Shape – spherical, ovoid, ellipsoidal, elongate or asymmetrical.
    b. Location of ciliary zones – entire body surface, almost entire body surface, anterior andposterior body surface or anterior body surface only.
    c. Concretion Vacuole – present or absent.
    d. Operculum – present or absent.
    e. Skeletal plate – present or absent.
    f. Number of Skeletal plates – one, two, three, four or five.
    g. Shape of skeletal plates – broad or slender.
    h. Number of contractile vacuole – one, two, three, four, five or more.

i. Shape of macronucleus – spherical, ellipsoidal, rod-shaped or more complicated.
j. Location of macronucleus – anterior, middle or posterior part of macronucleus.
k. Number of caudal spines – zero, one, two, three, four, five or more.

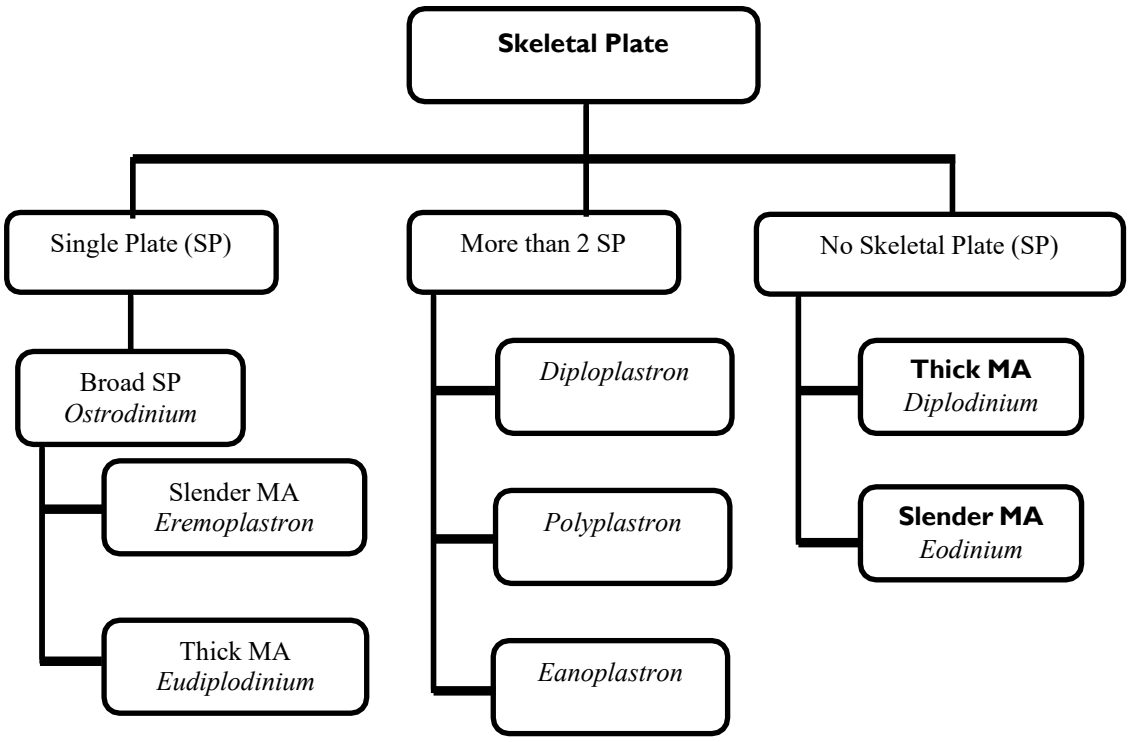

Figure 2a. Morphological Classification of Rumen protozoa isolated from carabao rumen fluid hydrolysis of sweet sorghum bagasse (Dehority, 1993)

Procedure in determining size of the rumen protozoa

Using a compound microscope with a camera that is attached to a laptop program with size measurement in microscale length set at 100um with deviation of 10um, the protozoon cell size and morphological characteristics were viewed at low power magnification and digital camera set at 680 x 380 resolution.

Statistical Analysis

Data on protozoan population count, pH at different duration was analysed using analysis of variance in Complete Randomized Design (CRD), Statistical analysis (SAS software 9.4.) was performed. Comparison of significant means was done using Duncan Multiple Ranges Test at 5% level of significance.

Results and Discussion

Physical characteristics of the hydrolysis

The biomass particles of 10 cm of sweet sorghum were reduced to fine particles while the transparent color of hydrolysate had turned into cloudy yellow green, indicating microbes growth, and activity that has dissociated biomass green pigments due with increasing duration of hydrolysis. Enlargement of the balloon on the top of the fermenter implied that fermentation gas was released

when agitated, indicating the active rumen fermentation in the hydrolysis of sweet sorghum bagasse using carabao rumen fluid. Observations over periodic sampling at 3, 6, 9, 12 and 15 days showed that physical composition such as pH of the hydrolysis had changed with increasing duration. Hydrolysis pH of the hydrolysates are presented in Table 1.

Table 1. The pH value of sweet sorghum using carabao rumen fluid at different duration in days.

	pH value					
TREATMENT	D0	D3	D6	D9	D12	D15
R1	7	6.4	5.9	5.8	5.6	4.9
R2	7	6.3	5.9	5.8	5.6	5
R3	7	6.4	6	5.8	5.7	5.1
MEAN	7a	6.4b	5.9c	5.8d	5.6e	5f

Means with same letter are not significantly different at 5% level of significance

The statistically evaluated durations of hydrolysis had significant effect on the pH of the hydrolysis ($P<0.05$). Average initial pH of 7.0 at day 0 had ensured that the starting condition was within pH suitable for cellulose degrading rumen fluid microbes that was inoculated into the hydrolysis at 1% of the effective volume. The decrease in pH of hydrolysates at increasing duration had changed the physical conditions of the hydrolysis. Evaluation of the pH at different durations showed duration of 3 days with pH 6.4 was still within the pH threshold for cellulolytic rumen microbes. Succeeding durations showed that hydrolysis pH drifted to pH of 5.9 down to pH 5.0 at 15 days, which implied acidic sweet sorghum hydrolysis. The decrease in pH of sweet sorghum hydrolysis was similar with the findings of Florendo et al., 2022; Efrele et al indicating acid effect on rumen microbes.

Hydrolysis Population Size of Rumen Protozoa

The population of rumen protozoa in the sweet sorghum hydrolysis using carabao rumen fluid at different durations are presented in Table 2. Statistical analysis showed duration had significant effect on the population of rumen protozoa in the hydrolysis of sweet sorghum using carabao rumen fluid ($P<0.05$). Mean comparison showed hydrolysis D0 with the highest population of 2.61(x104 cfu/ml). Duration of hydrolysis D3 showed mean population count of 1.71(104 cfu/ml) was significantly higher than populations of protozoa at durations 6, 9, 12 and 15 days. Likewise, the populations that declined at subsequent durations D6, 9, 12 and 15 had significant differences.

Table 2. Population of rumen protozoa in the sweet sorghum hydrolysis using carabao rumen fluid at different duration in days.

	Protozoa Cell Count(x 10^4 cfu/ml)					
TREATMENT	D0	D3	D6	D9	D12	D15
R1	2.55	1.60	1.40	0.85	0.8	0.60
R2	2.50	1.80	1.40	1.10	0.85	0.55
R3	2.80	1.75	1.25	0.75	0.75	0.70
MEAN	2.61 a	1.71 b	1.35 c	0.90 d	0.80 d	0.61 e

Means with same letter are not significant at 5% level of DMRT

Results showed that the duration of hydrolysis had negative impact on the population of rumen protozoa. The decreasing population was attributed to the low pH non-tolerant species in the hydrolysis of the sweet sorghum. Result of the study on the decreasing population in the acidic hydrolysis was in agreement with Franzolin and Dehority (1996a), that a low rumen pH are involved in the survival of rumen ciliates, that it was the length of time with a low pH that had affected the generation of rumen

ciliates in the rumen fluid hydrolysis. Decline in the protozoa population in the study of Owens et al. (1998) suggested that on high concentrate diets, the prevalence of protozoa in the rumen typically declines, probably due to the lack of a fibrous floating mat in the rumen where the ciliate remain attached in order to multiply.

In the present study, the decline in population count from 2.61(x104) to 0.61(x104) was indicator of the protozoa population with unable to grow at low tolerance to acidic hydrolysis. Also, count and identified protozoon species of Edodinium and Entodinium at long durations indicates interaction with rumen bacteria and fungi in hydrolysing the carbohydrates for their energy utilization at prolong duration.

Dehority (1993) had indicated species of protozoon of the family Ophryoscolocedae genus Polyplastron multivesiculatum and Eudiplodinium that survived starvation by engulfing hydrolysed cellulose, indicating enhancement of carbohydrates degradation by protozoa of the hydrolysis. Changes in the population of protozoa with increasing durations was agreeable with Nagaraja & Titgemeyer (2007) that indicated reduction in the number of the rumen ciliate population was a good indicator of acute and sub-acute acidosis attributed to the accumulation of lactic acid or volatile fatty acids.

Morphological Characteristics of Isolated Rumen Protozoa in the Hydrolysis

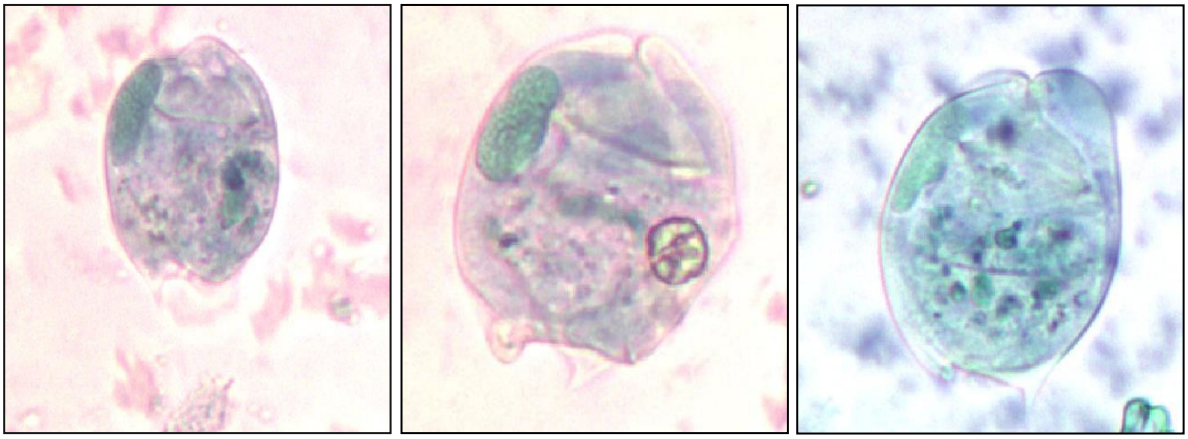

Figure 3. Morphological Characteristics of Carabao Rumen Protozoa. Different types of protozoa from genus *Entodimiun caudatum* taken at day 0 of the sweet sorghum hydrolysis using carabao rumen fluid. The protozoa were stained with mix Methylene Blue Formalin Saline Solution (MFS). Photos taken at low power magnification, using a compound microscope with digital camera with resolution 680 x 360

Figure 3 shows the characteristics of 3 isolated species of genus *Entodinium caudatum,* species with caudal spines from the sweet sorghum hydrolysis using 1% carabao rumen fluid. The bodies of the protozoa were oval shaped, with rod-shaped macronucleus (ma) with micronucleus (not visible) near anterior part. Caudal spines isolated protozoon; a) consisted of two short caudal spine, protruded from the body like claws with pointed tips, (b) round thick lobe on the right caudal spine (RCS) and protruded spike with pointed end left caudal spine (LCS) and in species type c; the caudal spine that showed shorter but larger base and right spine with longer sharp tip. These isolated of rumen protozoa that grew in the hydrolysis of sweet sorghum have body length that ranged 21 to 22 micrometers and width 18 to 21 micrometers. Except that the isolated *Entodinium* were larger in body size compared with other isolates, the protozoa have similar morphological characteristics with *Entodinium babalum* (Imai, 1989 by Dehority, 1993). These types of protozoa were prominent at day 0 and recurred at hydrolysis with durations of 3, 6, 9, 12 and at long duration of 15 days. Because the hydrolysis was acidic beyond duration of 3 days, species of *Entodinium* showed their resilience to acidic condition of the sweet sorghum bagasse using 1% carabao rumen fluid. Species of *Entodinium* was isolated

in sugarcane bagasse, corn stover and rice straw hydrolysed by carabao rumen fluid (Florendo *et al*, 2022) William and Coleman, 1992 indicated had the nature of predation by protozoan species *Diplodinium, Eodinium* and *Polyplastron.*

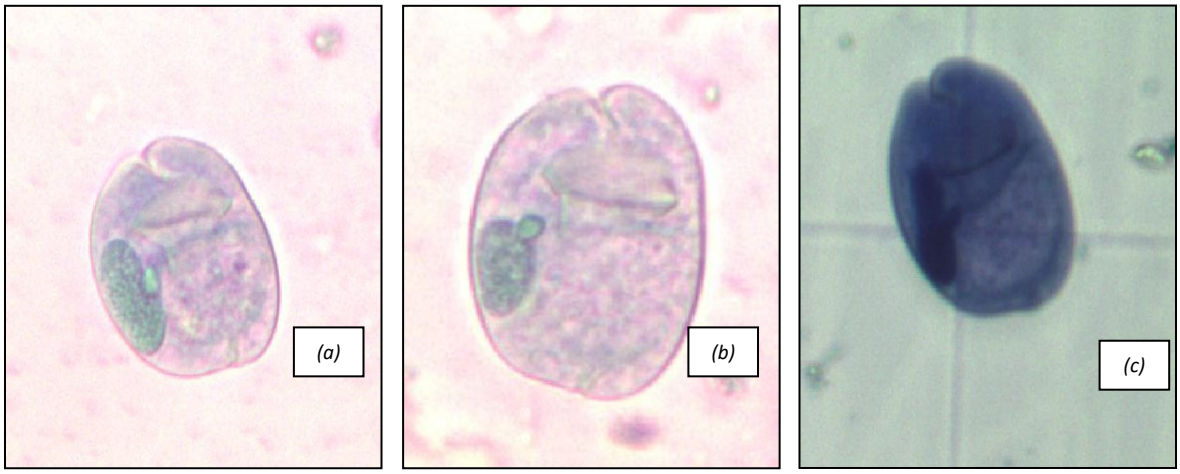

Figure 4. Morphological Characteristics of isolated Protozoa. Different types of protozoa from genus *Entodimiun* without caudal spines taken at day 0 of the sweet sorghum hydrolysis using carabao rumen fluid. The protozoa was stained with mix Methylene Blue Formalin Saline Solution (MFS), photos taken at low power magnification, using a compound microscope with digital camera with resolution 680 x 360.

Figure 4 indicates the characteristics of species of the genus *Eodinium* without caudal spines isolated at sweet sorghum hydrolysis using carabao rumen fluid. The morphological characteristics of the 3 isolated protozoa isolated were ovoidal bodies with anterior position of the mouth for filtering nutrients through their cilia showed the folded adoral lips as 2 thick grooves. Food vacuoles were plenty above the cytoproct of the protozoon between right and left posterior lobes but they have no caudal spines. Specie rod-shaped large size macronucleus were adhering on the right side posterior of the body with small size micronucleus position varies from anterior, middle and posterior of macronucleus. Length of the isolated protozoa of body ranged from 22 micromillimeters and the width ranged from 18 micromillimeters. The morphological features of the isolates from the carabao rumen fluid hydrolysis of sweet sorghum using carabao rumen fluid have characteristics similar to *Eodinium borax* as shown in the manual classification for protozoa by Dehority, (1993).

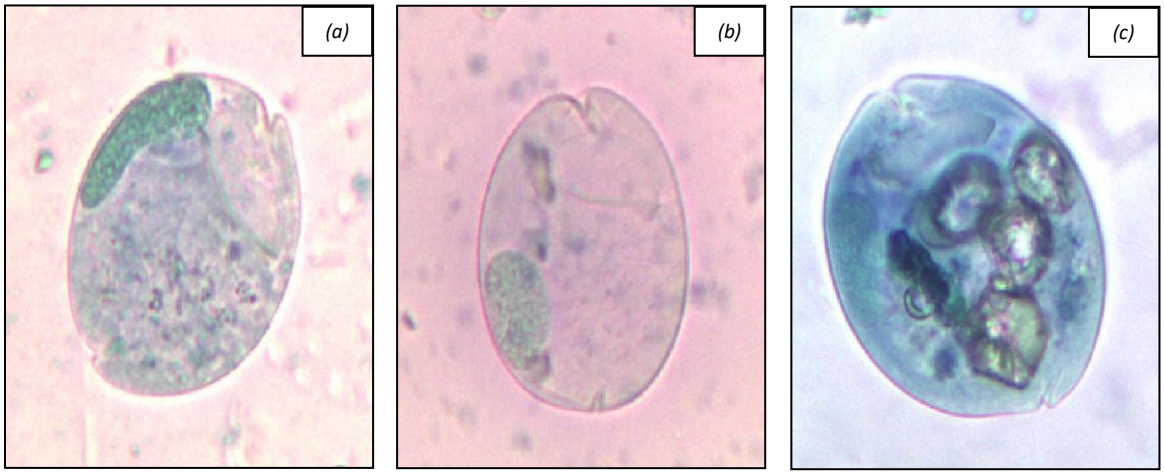

Figure 5. Morphological characterization of isolated Rumen protozoa. Different types of protozoa from genus *Entodinium* without spines taken at day 0, 3, 6, 9, 12 of the sweet sorghum hydrolysis

using carabao rumen fluid. The protozoa was stained with mix Methylene Blue Formalin Saline Solution (MFS), photos taken at low power magnification, using a compound microscope with digital camera with resolution 680 x 360

Figure 5 shows that the bodies of the isolated protozoa *Entodinium* were oval with adoral lips folded into two thick grooves at anterior parts, their macronucleus were elliptical and large with micronucleus located at anterior. Posterior part were rounded with a cytoproct, the anal area for the removal of food wastes were prominent and looked like opening with thin grooves, Cytoproct in the isolated protozoa was located on the left posterior while it was central of posterior in the species protozoon b and protozoon c. Concretion vacuoles were prominent in some of the isolate(c), size of the length of the protozoon body ranges from 20 to 26 micromillemeter and width from 23 to 24 micromillimeters. The isolated species were the biggest species types in the hydrolysis. The above characteristics of the isolated ciliates from the hydrolysis resembled the species from genus *Entodinium* species without spines as described (Dehority, 1993). Morphological characteristics of the isolated rumen protozoa in the hydrolysis of sweet sorghum using 1% carabao rumen fluid have similar morphology with genus *Entodinium* sp of the family *Ophryoscolecidae.* Specifically, the characteristics of the isolated species such as ovoid to ellipsoidal, presence of caudal spines and different shapes and orientation of the spines, and the location of cytoproct were variations in the isolated species of rumen protozoa that originated from a carabao with rumen cannula. Furthermore, the *Entodinium* species were found at durations 3, 6,9,12 and 15 days of the hydrolysis of sweet sorghum as result of their adaptability and resilience to survive at low pH hydrolysates. Among the isolates, *Entodinium* had adapted long period of hydrolysis, indicating high resilience to the condition of the sweet sorghum hydrolysis

Conclusion and Recommendation

The morphological features of isolated rumen protozoa in the carabao rumen fluid hydrolysis indicates the presence of protozoan species from family Ophryosclocedeae, among the characterized, Eodinium and Entodinium has many strains. The rumen protozoan has resilience, meaning resistance to and capability to survive the low conditions of sweet sorghum hydrolysis using carabao rumen fluid. The physical changes in the sweet sorghum hydrolysis like pH are indications of rumen protozoa active participation in the degradation of sweet sorghum lignocellulose, and can improve microbial conversion of lignocelluloses with the nature of synergy with other adapted microbes such as bacteria and fungi that adapted to the carabao rumen fluid hydrolysis. Information are justification of the novel process of carabao rumen hydrolysis for the bagasse of sweet sorghum as alternative feedstocks for bioethanol production. Recommendation is further study of the rumen protozoa and their nature of cellulolytic enzyme characteristics that can be used in the selection of rumen protozoa with high conversion efficiency for lignocellulose feedstock intended for feed utilization and cellulose ethanol production.

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