Volume VI, No. 1: 23 - 48 (2022)
ISSN 2507-9638

THE CLSU INTERNATIONAL JOURNAL OF SCIENCE & TECHNOLOGY
www.clsu-ijst.org

Microbial Identification and Population Successions in the Novel Carabao Hydrolysis Pretreatment of Agriculture Crops Lignocelluloses Intended for Cellulose Ethanol Production

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Submitted: October 4, 2021. Accepted: February 16, 2022. Published online: April 13, 2022.

Abstract

This study was conducted with the objective to elucidate physical, chemical and microbial changes in carabao rumen fluid hydrolysis of bagasses of sweet sorghum and sugarcane, corn stover and rice straw. Identification of microbial species, composition of populations and succession behavior at durations of 3, 6, and 9days of the carabao novel process were determined using API20A kit for anaerobes, spectrophotometry and compound microscopy. Efficiency of microbial conversion of carbohydrates into soluble sugars, pH changes in hydrolysates were determined at various durations. Hydrolysis initial pH 6.98 was reduced at durations of 3 days, 6 days and 9 days while significant pH variations were feedstock related, lowest in sugarcane(pH 4.91) sweet sorghum(pH 5.46), corn stover(pH 5.72) and near neutral in rice straw(pH 6.56). Carabao rumen fluid hydrolysis conversion efficiency was significantly improved in biomass with moderate amount of soluble extractives ($p<0.05$). Highest carbohydrates conversion efficiency was sweet sorghum (66.49%), corn stover (52.43%), sugarcane (52.12%), and rice straw (39.28%). Duration of 6 days had improved carbohydrates conversion efficiency average of 55.02%. Morphology and physiochemical characterizations of strains within bacterial groups *Clostridium, Bacteroidetes, Streptococcus, Actinomyces, Bifidobacterium, Lactobacillus* and *Staphylococcus*, rumen fungi species *Ruminomyces, Orpinomyces* and *Neocallimastix* and various protozoa of the family O*phryoscolocedae* and *Isotrichia* revealed diversity of the noval carabao. Changes in microbial composition, growth, and succession behavior of bacteria, fungi and protozoa were implications of synergy that includes complementation and resilience in the carabao rumen fluid hydrolysis. This is the first study on the microbial community of the novel carabao intended for cellulose ethanol production. Information generated will be of great help in the selection of microbes that can convert lignocellulose wastes into soluble sugars with higher efficiency for the upscaling of the hydrolysis of agriculture wastes as alternative feedstock for cellulose ethanol production.

Key Words: Carabao, anaerobes, rumen bacteria, rumen fungi, rumen protozoa, pretreatment process

Introduction

By virtue of carbohydrates that can be converted into biofuel, agriculture crop residues and lignocellulosic feedstock as alternative to grain starch and sugars play significant role in the energy economy towards mitigation of the globally experienced oil crisis. The cellulose ethanol that came from the lignocellulose portions of food crops non-competitive with human requirements was known with quality that could reduce accumulations of carbon dioxide emissions (CO_2) in the air. In comparison with grain ethanol, cellulose ethanol reduced carbon dioxide emissions by 64% against 23% using E85 corn grain ethanol (http.ceres.net/biofuel-Advantage.html). Conversion of agricultural crops residues into alternative energy can generate additional means of livelihood with increased income, cost reduction in biofuel, clean air in the environment and ultimately, the better socio - economic reputations as stipulated in the energy bill of the Philippine Biofuels Act of 2006.

Cellulose ethanol production makes use of solubilized carbohydrates of agriculture crop residues. Mosier, (2005) indicated that in order to extract the cellulose from lignocelluloses, the biomass must require the action of acid or alkaline solution to dissociate the crystalline forms of cellulose including hemicelluloses from lignocelluloses complex. The dissociated carbohydrates will be further solubilized in succeeding enzymatic hydrolysis to produce soluble sugars prior to yeast fermentation process for cellulosic alcohol. However, pre-treatment use concentrated acids or dilute acids hydrolysis have conversion efficiency that ranges from 50% to 60 % overall process efficiency while the assumed calculated efficiency using both cellulose and hemicellulose was 95% (Badger, 2002). However, these previous pretreatments for cellulosic bioethanol are unlikely to reach commercial operation because the acid process was not cost effective, slow process and current problems on disposal of chemical effluents. Advancement in pretreatment, advancement in biotechnology and combinations of process technology are suggested to improve overall process efficiency (Hamelinck *et al*, 2005; Chen et al, 2007). Alkali treatment such as done by Mateo *et al*, (2017), where in 1.5% NaOH treated rice straw enhanced enzymatic hydrolysis that resulted in 77.8% conversion efficiency is a potential pretreatment of cellulosic biomass.

Until recently and with the advent of biotechnology, biological conversion of lignocelluloses has provided immediate alternative to enzymatic hydrolysis prior to ethanol fermentation (Winter, 2008). In Japan, wood construction wastes and its conversion to cellulose ethanol production was made possible by the use of microbial co-cultures of *Klebsiella oxytoca* and *Escherichia coli.* In Idaho, corn stover was hydrolyzed into ethanol by co-cultures *of Trichorderma reseei* and *Saccharomyces*. Another co-culture that consisted of *Thermoanaeobacterium saccharolyticum* and *Chrysosporium lucknowense* was developed specifically for corn stover fermentation by Abengoa Company (Winters, 2008). Increasing use of biological methods in degrading the lignocellulose in feedstock is becoming advantageous because of the least cost of ethanol production and the availability of safer disposal of effluents. Carabao rumen fluid hydrolysis is a biological pretreatment opportunity that uses rumen anaerobic microbes direct enzyme hydrolysis conversion of agriculture fibrous crop residues and grasses into soluble sugars for cellulose ethanol production (Florendo *et al.,* 2009). In addition, volatile fatty acids like acetate, valerate and and gas methane and carbon dioxide are fermentation co –products that maybe derived for valuable uses (Florendo *et al*., 2018).

This study aimed to (1) to determine the composition of bacteria, fungi and protozoa using morphological and physiochemical characterizations of isolates from the hydrolysis of crop residues like sugar cane, sweet sorghum, corn stover and rice straw; (2) to determine the effect of feedstock carabao rumen fluid hydrolysis on physical and chemical changes like hydrolysates pH and carbohydrates conversion and (3) to determine succession behavior and relate effect on composition of the carabao rumen fluid microbial community to the physical and chemical characteristics of hydrolysates at various duration of hydrolysis.

PROJECT CONCEPTUAL FRAMEWORK

Figure 1. The Carabao Rumen Fermentation Model

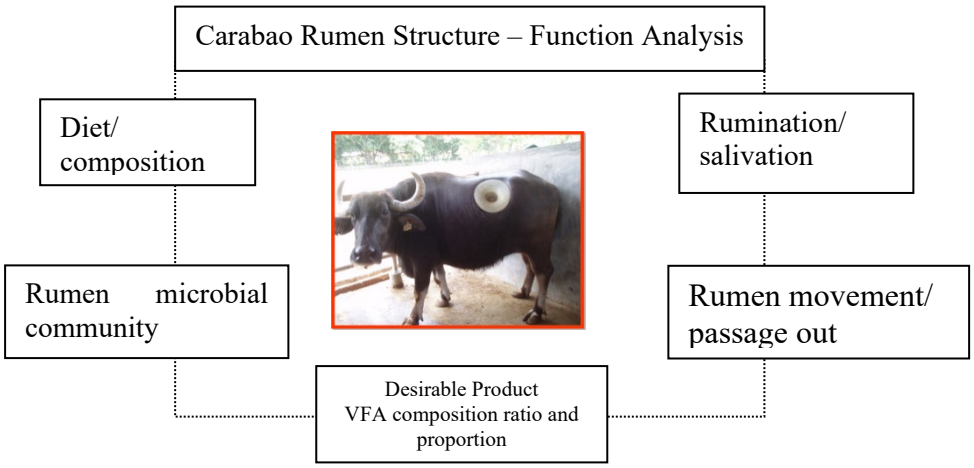
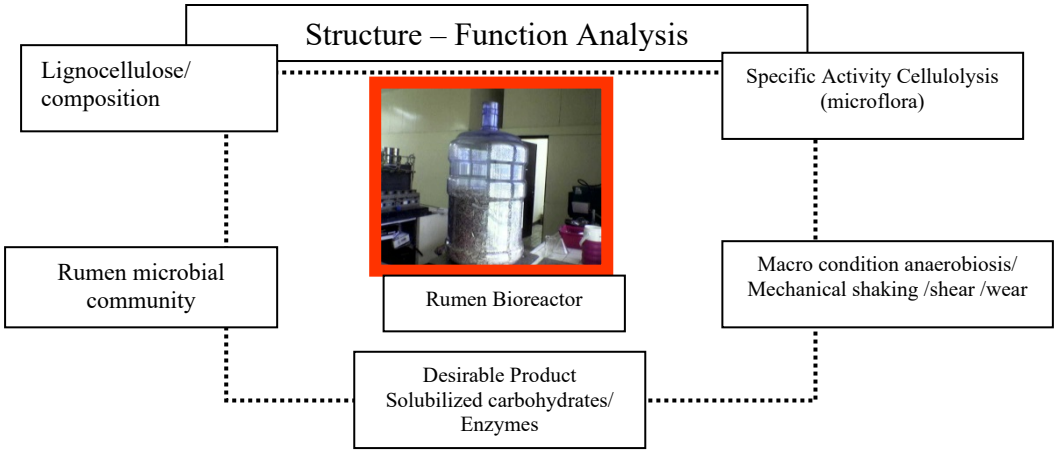


Figure 2. The Batch Type Carabao Rumen Hydrolysis Model Paradigm for Cellulose Ethanol Production



The paradigm batch type carabao rumen fluid hydrolysis model for cellulose ethanol production was designed after the structure – function relationship concept, the rumen fermentation of carabao as model (Figure 1) is transferred in anaerobic container (Figure 2) to effect conversion of lignocellulose into soluble sugars. The hydrolysis was alternative to chemical pretreatment by using microbial direct enzymatic process. Carabao rumen hydrolysis of rice straw with urea and molasses reached an 84.5% efficiency conversion of carbohydrates cellulose and hemicellulose into soluble sugars (Abenes and Florendo 2009). The process of carabao novel hydrolysis was outlined as follows;

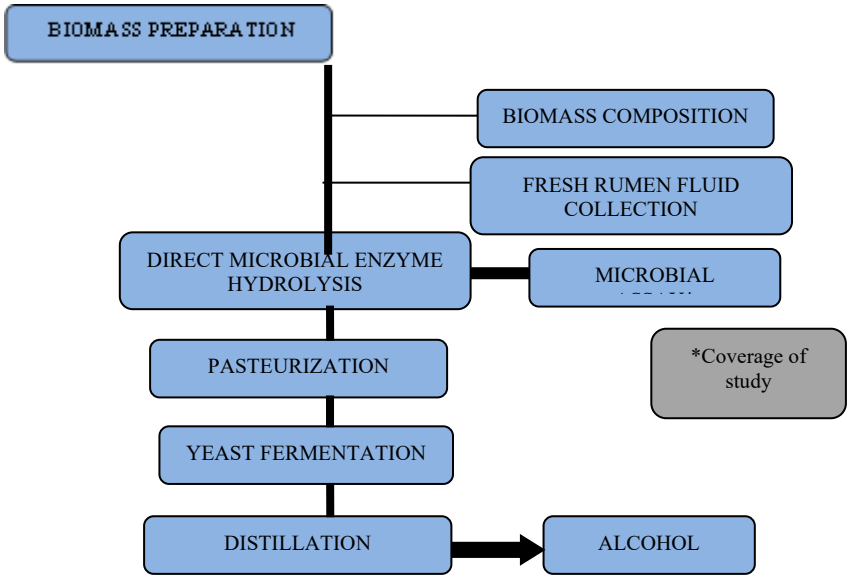


Figure 3. Process of cellulose ethanol production with carabao rumen fluid hydrolysis

Materials and Methods

Biomass Preparation

Bagasses of sugar cane, sweet sorghum and corn stover and rice straw were cleaned, sundried for 3 days, chopped to 10 mm and stored in autoclavable polypropylene plastic. Sweet sorghum syrup was extracted using a juice extraction machine, then sundried, chopped to 10 mm size, and weighed prior to storage. Biomass was steam sterilized at 121^{0}C for 30 minutes. Laboratory samples' moisture content, total solid, reducing sugar, soluble extractives, holocellulose and hemicellulose were analyzed following the analysis protocol for alternative feedstock (Sluiter *et al*., 2008; TAPPI methods 1992-1993).

Procedure of Rumen Cannulation in Carabao

A male buffalo crossbreed (*Bubalus bubalis L.*) was fitted with rumen cannula following the one- stage operation technique (Grant *et al*., 1990; Ocampo *et al.* personal communication). Pre-operative preparation was done, wherein feed was withheld for 24 hours and water for 12 hours. The buffalo was brought to the chute, bathed with the left para-lumbar region for aseptic operation. After cleaning, the animal was positioned on the right side so that operation is carried out in recumbent position. With supervision from the Veterinarian, the male buffalo was given intravenous 2% Xylazine HCl at the rate of 2 mg per kg of body weight coupled with paraventral nerve block using Lidocaine. The left paraventral fossa was anaesthetized by using nerve block, inverted "L" using lidocaine. Prior to incision, the circumference of the cap of the cannula was used as guide for the circular incision on the skin, followed by circular incision in the underlying external abdominal muscles, ligated blood vessels when needed. After removal of the circular pieces of muscles, internal abdominal transverse muscles and peritoneum were bluntly separated, retracted and created an opening to expose the rumen wall. The rumen wall was grasped using towel forceps and clamps for traction and exteriorized the rumen wall. Using #0 catgut the rumen wall muscles and skin were sutured together following a continuous suture pattern. After sutured, the rumen wall was incised, removed the circular piece of rumen wall, wiped the blood and cleaned the sutured area with

70% alcohol. A rubber cannula (Diamond, USA) with inner and outer flanges and the inner tube with circular flange wider than the width of the outer flange was inserted into the freshly opened circular incision of the rumen sutured to the skin. Antibiotic was given for 5 days while iodine solution was applied in the sutured skin until healed. After surgery the buffalo with rumen cannula was allowed to recover under confinement with feeding system that consisted of Napier soilage, urea-molasses treated rice straws, 0.5% body weight of concentrate mixture with 14% crude protein. Adequate clean water was available at all time.

Carabao Rumen Fluid Collection

Collection of fresh rumen digesta and fluid was done via the rumen-cannulated carabao. The cannula was opened by removing the cap and the hand was inserted to manually get digesta from the dorsal and ventral portion of the rumen. Collected rumen digesta was squeezed in double layer of cheese cloth to extract the fluid, loaded in thermoflask and immediately gassed with CO_2 in the laboratory. Carabao rumen fluid was kept in a 37^0C controlled water bath and bubbled with CO_2 gas to prevent the aerobic condition. All bottles, containers, and pipettes used in the collection of fresh rumen fluid were gassed with CO_2 to ensure anaerobiosis before refilling. This process was a modification of Lee *et al* 2000.

Conditions for the Carabao Rumen Fluid Hydrolysis

A 6 L capacity plastic container served as fermentor. Each of the biomasses, sweet sorghum, sugarcane bagasse, corn stover and rice straw with calibrated weight of 0.225 kg was loaded in individual fermentor from each crop residue. Then warm water pre-gassed with CO_2 was loaded in the container, Dissolved urea solution at 2% of the loaded biomass was filtered using 0.25 mm filter membrane (Sartorius). Fresh carabao rumen fluid was filtered and added at 1% of 4.5 L water, which is equivalent to 45 ml. The rumen fluid was bubbled with CO_2 gas for 5 minutes before it was loaded into the fermentor. Hydrolysis pH was adjusted to pH 7 by using 0.1N NaoH solution. In Batch 2 and 3 with glass fermentation vials, the components were adjusted to effective volume of 100 ml (Abenes and Florendo, 2009)

Operation of Carabao Rumen Fluid Hydrolysis

After loading the materials into the plastic vessel, it was sealed with cork with gas release and inflatable balloon. Duration of hydrolysis was 3 days (d3), 6 days (d6), 9 days (d9), 12 (d12) and 15 days (d15 days). A chest freezer partially filled with water maintained at 37^0C to 39^0C was used as incubator. All fermentation vessels incubated inside were pulled out of the incubator and agitated at low speed shaker for 10 minutes. Enlargement of the balloon indicates that gasses were produced and rumen fermentation was active in every plastic bottle. The next batches (B2 and B3) were done using 100 ml volume capacity vials. The amount of same biomasses urea, molasses and fresh carabao rumen fluid were adjusted to the volume of fermentation. Vials were gassed with CO_2 and capped with rubber butyl cap and crimped aluminum cap. The vials were incubated in incubator with temperature of 39^0C. Duration of the hydrolysis in vial was 3 days, 6 days and 9 days.

Sampling of Hydrolysate from Fermenter

Sample hydrolysate was obtained from the three batches of hydrolysis. At each duration period 3d, 6d and 9d, 12d and 15d, 100 mL hydrolysate was collected by destructive sampling method. A wide tip pipettor was used to aspirate 50 mL into pre-gassed centrifuge tube for temperature and pH reading using a thermocouple with pH meter while optical density was evaluated on UV vis spectrophotometer(grated spectrophotometer). For the reducing sugar

analysis, sub-sample hydrolysate was stored at 4^0C until measured using the procedure of Dinitrosalicylic acid assay for reducing sugars (DNS) assay (Miller and Wollins, 1974). For microbial analysis, sample hydrolysate was stored at -20^0C freezer until needed.

Isolation of Microbial Fraction from the Fermentor

Isolation of rumen fluid hydrolysate for microbial fraction was done following the procedure described by Lee *et al.* (2000). Samples of the frozen hydrolysates(N=20) were thawed at room temperature, blended for 1 minute and transferred into CO_2 pre-gassed separatory funnel to allow precipitation for one hour. Then, the liquid portion was collected and centrifuged at 12,000 rpm for 5 minutes. Supernate was re-centrifuged at 4^0C for 30 minutes at low speed, the pelletized microbial cells are collected and washed with 10 ml of 1% TGBroth. The washed pelleted cells represented the liquid-based microbial fractions of rumen bacteria and rumen fungi while the solid portion was used in the isolation of rumen protozoa fraction. All microbial fractions were stored at -20^0C for purifications in growth enhancement using mineral enhancement liquid medium.

For growth enhancement of the isolated microbial fractions, the original formula of Miller and Wolin (1973) for the cultivation of obligate anaerobes in serum bottle was modified to contain the mineral enhancement liquid medium composition presented in Table 1.

Ingredients	Percentage in 1Liter
Cellulose	0.50%
Yeast Extract	0.20%
Mineral Solution 1	4.00%
Mineral Solution 2	4.00%
Resazurin(0.1%)	1.00%
Na_2CO_3 (8%)	5.00%
Cystein HCl	0.05%
Thioglycollate Liquid medium	84.25%
Homogenized carabao rumen fluid	1.00%

Antibiotic*

- Anti-bacteria, Anti-fungus and Anti-protozoon

Composition of Mineral Solution 1	Percentage % in 1L
KH_2PO_4	0.60%
$(NH4)_2 SO4$	0.60%
NaCl	1.20%
$(MgSO_4.)_2SO_4$	0.24%

After mixing all ingredients, the prepared liquid medium was boiled for 10 minutes, and then bubbled with CO_2 gas during the cooling process (Holdeman *et al*., 1997). While under continuous gassing, 5 ml Na_2CO_3 and 50 mg cysteine HCl was added. The reduced solution was indicated by change in blue solution into yellow color. The color change was due to the thioglycollate liquid medium as alternative to distilled water in the original formulation of Lee *et al*. (2000). Once the color turned yellow, cooled 50 ml solution was transferred into a pre-gassed 125 ml serum vials, flasked with CO_2 for 5 minutes before sealed with rubber butyl cap and aluminum cap. Vials were steam sterilized at 121^0C for 30 minutes. Vials of sterilized liquid medium were stored at 4^0C for the antibiotic treatment.

Antibiotic Treatment of the Mineral Liquid Enhancement Medium

Prior to antibiotic treatment of the mineral enhancement liquid medium, 0.5 g of steam sterilized 6 mm disc filter paper (Whatman #1) was aseptically added into each bottle of mineral enhancement medium (MEM) liquid medium. For bacterial MEM, Ketoced and Metronidazole were added at 40*ug* per mL of the liquid medium. For fungi MEM, Amoxicillin and Flagyl were added at 40*ug* per mL of the 100 mL MEM. In the MEM of protozoal fraction, 40*ug* each of Ketoced and Amoxicillin were added per mL of liquid culture medium. This is a modification of Lee *et al*, (2000) combinations of antibacterial, antiprotozoal and anti-fungal treatments.

Inoculation of Microbial Fraction

For bacterial cultivation, sample of 0.1 ml bacterial fraction was added to vial of the bacterial mineral enhancement liquid medium (MEM). Similar procedure was done in the inoculation of fungi and protozoan in the medium. All enhancement growth medium vials were corrected for 100 ml volume using the liquid medium, then pre-gassed with CO_2 for 1 minute. Using filter sterilized 4N NaOH, the pH of the solution was adjusted to 7. The microbial MEM vials were sealed with rubber butyl cap and crimped aluminum cap. The duration of incubation at room temperature was 14 days. After incubation, the microbial fractions cultures of bacteria, fungi and protozoa were stored at 4^0C until isolation and identification of species.

Isolation and Characterization of Rumen Bacteria

MEM content of bacterial fractions MEM was homogenized at 2,000 rpm for 1 minute. The homogenized fraction was precipitated for one hour, and then the supernate was separated from the solid portion using a separatory funnel. One ml of homogenate was evaluated for optical density at A_{600} using a UV-vis spectrophotometer. For bacterial morphological evaluation, dilution ($10^{7}$to 10^{12}) was pour plated in Petri plates with Thioglycollate Fluid Medium added with 15% Agar (TGFMA). TGFMA plates were placed inverted in anaerobic genbags (Biomerieux, USA), anaerobic rectangular jar provided with CO_2 sachet and anaerobic indicator stick. The genbags were incubated at 37^0C for 48 hours. After 48 hour, bacterial cell forming unit was isolated and assayed for cell shape and arrangement, gram reaction, spore formation and catalase. The isolates with distinctive characteristics were re-inoculated in petri plates with TGFMA and TGBroth culture vial. Morphological characteristics of each isolated carabao rumen hydrolysate bacterium were observed using compound microscope with built in digital camera and wide screen images in laptop.

API20A kit for anaerobes (Biomerieux, USA) was used as confirmatory test for each isolated rumen anaerobic bacteria. Cultures of the 52 isolated bacteria in TGFMA and TGBroth were incubated for 24 hours at 37^oC. After incubation, swab of each fresh culture was aseptically inoculated in API 20A liquid culture medium. The culture suspension turbidity was compared with McFarland Standard No. 4 standard before inoculation. Comparison with McFarland standard No.4 indicated that API culture had equivalent cell count of 1.2 (10^9) per ml. The optical density of each bacterium API fresh culture was measured at A_{600} using spectrophotometer (grated spectrophotometer, Shanghai No.3 Analytical Inst. Factory). Following API 20A kit manufacturer instruction, 0.25 mL of API 20A liquid culture was aseptically inoculated into a strip that contains 20 tubules of different fermentation substrates. The filled API strip was placed in humidified tray, incubated in genbag with CO_2 sachet and indicator stick. API20A trays were incubated at 37^0C. Evaluation of the API tubules for positive and negative reactions was done at 24 hours and confirmed at 48 hours of incubation. Re-

evaluation of isolated species reaction to gram staining, spore formation test, and bacterial shape was evaluated in culture grown in both Nutrient Agar (NA) and TGFMA.

Physio-chemical characteristics and morphology of the 52 rumen bacteria isolates were evaluated and then compared with taxonomy of known anaerobes (API20A,Biomerieux.USA) and the classification guide for ruminal bacteria (Dehority,1993). The procedure of API 20A identification of anaerobes was conducted in identifying bacterium isolated at durations of 3day, 6days and 9 days of sugar cane bagasse, sweet sorghum, rice straw and corn stover hydrolyzed by carabao rumen fluid . Bacterial growth in biomass were based on the number of isolated cultures at incubation period of 3, 6 and 9 days, 12 days and 15 days. Population count of isolated bacteria based on genera and strains at duration of 3.6 and 9 days were identified by means of the API20A taxonomy for anaerobes.

Isolation and Characterization of Rumen Fungi

Dilution was prepared from the MEM liquid culture of rumen fungi fractions from sugar cane bagasse, sweet sorghum, rice straw and corn stover hydrolysis. The culture medium was homogenized at 2,000 rpm for 1 minute. Sample of one ml of dilution (10^{-12}) was pour plated in Potato Dextrose Aga PDA vial then incubated at 37^0C for 5 days. Also, a fraction of homogenized filter paper was stubbed in PDA vial and incubated for 5 days. Cultures were observed for colonial features of rumen fungi like color, margin, elevation and colony indentation and sub-surface fungal mycelia growth, presence of spores, cysts and structure of hyphae using a compound microscope with camera that is connected to a laptop. The morphology of anaerobic rumen fungi isolates was compared with database described by Ho and *et al*. (2000) and classification of rumen fungi (Dehority, 1993). Population count of the carabao rumen fluid hydrolysis rumen fungi was based on the presence or absence of the morphological features at different incubation period.

Isolation and Characterization of Rumen Protozoa

Culture of protozoa in MEM liquid medium was filtered in Whatman filter paper #5. The filtered protozoan bodies and degraded filter paper residues were collected, rinsed with distilled water until clear of the culture medium. The bodies of protozoa and filter paper discs were suspended in 50 mL Methylene blue formaldehyde saline (MFS solution) which was prepared with 1:1 mixing ratio of sample and MFS solution. Body structures such as skeletal plates, vacuoles, and spikes of isolated rumen protozoan were observed in microscope. Morphology of each rumen protozoa isolated at 3,6and 9 days was compared to the structure catalog for rumen protozoa (Dehority, 1993).

Statistical analysis

Feedstock and hydrolysis duration effect on pH, carbohydrates conversion efficiency and microbial population count have been analyzed following analysis of variance on factorial experiment in Complete Randomized Design (Sirichai Statistics version 6). Mean comparison was done using Duncan Multiple Range Test (DMRT) at 5% level of significance.

Results and Discussion

Composition of Agriculture Crop Residues as Biofuel Feedstock

Table 1 showed the composition of sweet sorghum, sugarcane, corn stover and rice straw as alternative feedstock for cellulose ethanol production.

Table 1. Chemical composition of alternative feedstock

COMPONENT	[1] Sweet Sorghum Hay	[2] Sweet Sorghum Bagasse	Sugar Cane Bagasse	Rice straw	Corn Stover
Total Solid.%	93.12	93.41	94.31	93.42	99.15
Soluble Extractives,%	20.36 *±1.25*	4.12 *±1.17*	12.82 *±1.30*	1.73 *±0.73*	9.00 *±0.94*
Holocellulose,%	36.27 *±1.90*	42.12 *±0.33*	47.61 *±0.40*	55.53 *±0.57*	43.52 *±1.41*
Hemi-cellulose,%	22.26 *±0.22*	29.30 *±0.32*	30.03 *±0.30*	31.54 *±0.06*	22.93 *±0.21*

[1,2] Feedstock with Batch 1 and 2 Hydrolysis

The crop residues analyzed had soluble extractives, that may vary from lipids, soluble carbohydrates, fat soluble vitamins and waxes soluble in mix benzene - ethanol. Sweet sorghum hay content of soluble extractives was higher than compared with sorghum bagasse, corn stover and rice straw. Soluble extractive in the sample feedstock could be associated with plant maturity. Holocellulose represents hemicellulose and celluloses content, typical components of lignocelluloses wastes. All samples analyzed had high contents of holocelluloses and hemicelluloses. Laboratory results on sweet sorghum and sugarcane were comparable with the composition analysis done by Kim and Day, 2011. Anwar *et al.* 2014 had indicated the advantages of agriculture wastes for being renewable, inexpensive, abundant natural resource which can potentially lower down the cost of large scale energy and cost effective bio based energy production like cellulosic ethanol.

Physico-chemical Changes in the Carabao Rumen Fluid Hydrolysis

Hydrolysate pH of the four crop residues hydrolyzed for 3 days, 6 days and 9 days is presented in Table 2. Hydrolysate pH was significantly affected by feedstock ($p<0.05$). In comparison, rice straw pH 6.56 was slightly acidic than sugarcane (pH 4.91), sweet sorghum (pH5.46) and corn stover (pH5.72). The hydrolysis near neutral pH 6.9 had decreased to pH 5.64, 5.76 and 5. 59 at 3 days, 6 days and 9 days period of hydrolysis. Statistical evaluation showed duration had significant effect on pH particularly between day 0, and durations 3, 6 and 9 days ($p<05$). Hydrolysis pH had decreased over time, indicating the all feedstock hydrolysis turned into acidic conditions. Mean difference existed between periods of 3 days, 6 days and 9 days was not significant.

Table 2. Feedstock and Duration Effect on Hydrolysis pH

Hydrolysis (days)	Sugar cane bagasse	Sweet sorghum bagasse	Rice Straw	Corn Stover	Feedstock Mean
0	6.98	6.98	6.95	6.97	6.98 *a*
3	4.63	5.55	6.62	5.76	5.64 *b*
6	5.11	5.86	6.48	5.59	5.76 *b*
9	5.00	5.48	6.58	5.81	5.59 *b*
Mean	4.91 *d*	5.46 *c*	6.56 *a*	5.72 *b*	

Variations in italic letters (*a-d*) indicate significant difference (p=0.05).

Rumen fermentation produced acidic end products volatile fatty acids acetate, propionate, butyrate and other higher molecular weight like lactic acid (Van Hotert, 1993). Erfle *et al,* 1987 had indicated fermentation pH from 7 to 5 in artificial rumen could vary from decreased volatile fatty acids production, low methane production, low ammonia concentration, decrease protease and deaminase enzyme activity, low free amino acids, lactate acid production increase, and increase in lactate producing bacteria and decrease in proteolytic microorganisms. In anaerobic digestor, anaerobes fermented biomasses into volatile fatty acids were used as indicator of inefficiency of the fermentation process in bioreactor (Ahring *et al,* 1995).

Carabao Rumen Fluid Hydrolysis Carbohydrates Conversion Efficiency

Carbohydrate conversions efficiencies of the four agriculture crop residues are presented in Table 3. Bagasse of sweet sorghum was highly hydrolyzed into soluble sugars from 3 days, 6 days and after 9 days incubation period with average efficiency of 66.49%. Corn stover was hydrolyzed with average 52.43% of three sampling periods. Sugar cane bagasse was converted into soluble sugars with average efficiency of 52.12% while rice straw was converted at average of 39.28% for the same durations of incubation. Statistically evaluated, type of crop residues had significant impact on conversion efficiency ($P<0.05$). Comparison of means showed that hydrolysis of sweet sorghum bagasse had the highest efficiency, followed by sugarcane bagasse, corn stover and rice straw. The study showed that rumen microbial carbohydrates conversion efficiency was improved in feedstock with moderate content of soluble extractive. Results likewise showed that that among the feedstocks, rice straw carbohydrates conversion was lowest due to full maturity.

Table 3. Carbohydrates Conversion Efficiency of Various Feedstock Using Carabao Rumen Fluid Hydrolysis

Hydrolysis (days)	Sugar cane bagasse	Sweet sorghum bagasse	Rice Straw	Corn Stover	Mean
3	48.83	65.30	37.95	50.71	50.70
6	55.07	70.12	40.45	54.43	55.02
9	52.50	64.02	39.44	51.77	51.93
Mean	52.12 *b*	66.49 *a*	39.28 *c*	52.43 *b*	

Variations in column italic letters (*a-d*) indicates significance (P =0.05).

Durations of hydrolysis at 3, 6 and 9 days had indicated carbohydrates efficiency increased from 50.70% to 55.02% at 6 days and decreased to 52.93% at 9 days hydrolysis. Statistical evaluation showed no significant difference in the efficiency between durations 3 days, 6 days and 9 days. Fluctuations in the carbohydrates conversion efficiency were contributed to microbial interactions that provided species acquire nutrients for their energy metabolism, increase colonization, inhibited population and resilience in the acidic condition process on cellulolytic activity. Russel and Domobrowski 1980 indicated an acid effect that produced various microbial responses like altered rumen microbial population, inhibited enzyme production that slowdown of cellulolytic activity.

Growth and Characteristics of the Rumen Bacteria

a) Rumen Bacterial Fraction

Bacterial growth pattern indicated that crop residues of sugarcane, sweet sorghum and rice straw with growth variations are presented in Figure 3. Mass optical density of the bacterial fractions had fluctuations, indicating increased growth and decreased growth while sweet sorghum had exhibited initial low pattern of growth and then increasing mass optical density at later period of the hydrolysis. Evaluation of the individual specie of bacteria from bacterial fraction showed the interaction of genera and type of strains. Changes in bacterial fraction acid hydrolysates effect on the integrity of a bacterial cell (Russell and Dambrowski, 1980. Presented in Figure 4 are bacterial strains in bacterial fractions isolated hydrolysis duration at 3 days, 6 days and 9 days.

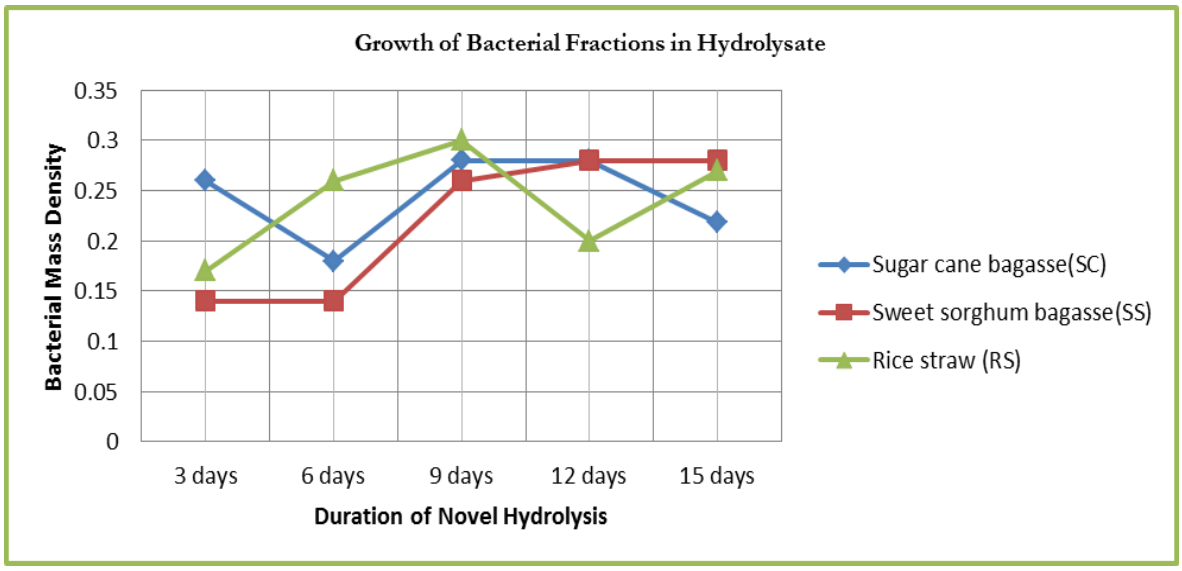


Figure 3. Growth Pattern of bacterial fractions in various feedstock hydrolysis using carabao rumen fluid hydrolysis.

Figure 4. Effect of individual bacterial strain on bacterial fractions of various feedstocks hydrolysis.

Identification of the Bacteria in the Novel Carabao Rumen Bacteria

Genus Clostridium

Twenty four strains of genus *Clostridia* were isolated in four feedstocks sugarcane, sweet sorghum, rice straw and corn stover hydrolysis. Physiochemical and morphological characteristics are presented in Table 4 to Table 7. This group being the only spore forming, Gram-positive, consisted of aerotolerant and obligates anaerobes. Differentiated by physiochemical properties, the strains of Clostridia of the rumen fluid hydrolysis had four strains with indole, two strains with urease enzyme and all isolates have broad sugars acid fermentation substrates. Dehority (1993) had rumen classified *Clostridium, C. clostridiiforme* and *C. cellobiosparum,* have specifications similarities with our isolates. Other isolated spore forming have physiochemical features similarities with *Clostridia beinjerinke, C. bifermentans, C. clostridioforme* and *C. sordelli.* In our study, group *Clostridia* was the most number of isolates, particularly from day 6 to day 9 period of incubation, indicating the dominance of Clostridia in the novel carabao hydrolysis. Nathani *et al*, 2016) had cited the Clostridia with multi-functions like high cellulolytic activity, high producers of volatile fatty acids in ruminant while some species have been used in human because bacteriolytic anti-cancer property. Weimer *et al.* (2015) had indicated that rumen Clostridia that belonging to phyla Firmicute was one of the dominant bacteria in the rumen.

Genus Bacteroidetes

The group of carabao rumen bacteria of genus *Bacteroidetes* was characterized as Gram-positive, non-spore forming and rod shaped, some strains were obligate anaerobe because of the absence of catalase and strains were aerotolerant because of the presence of enzyme catalase. Physiochemical characteristics are presented in Table 4 to Table 7. Nine strains isolated *Bacteroidetes* have broad sugar acid fermentation substrates, and four of the isolates are indole positive and two isolates with urease enzyme. Isolated obligate anaerobes *Bacteroides* have similarities with B*. uniformis* and *B. stercoris, classified* under carabao rumen fluid hydrolysis (API20A system for anaerobes). The aerotolerant strains had similarities with *B. ruminocola subsp. brevis* and *B. ruminicola ruminis* based on the classification guides for rumen bacteria (*Dehority, 1993*). Many strains of *Bacteroidetes* persisted at day 3 and day 6 days but non-occurrence at 9 days may be associated with Bacteroides weak resistance to the low pH environment of the hydrolysis. Russell and Dambrownski,(1980) indicated that cell of bacteria intra-neutral cell is maintained at near neutral pH gradient but when exposed to low pH environment,it decreases the intracellular pH to a point where enzymes that are sensitive to low pH are affected, the exchange of nutrient like protein was inhibited and growth of bacteria eventually slow down. In terms of strain population, *Bacteroidetes* was the second dominant bacteria in the carabao rumen fluid hydrolysis.

Genus Actinomyces

Carabao rumen novel process had isolates of bacteria of the genus *Actinomyces,* a non-spore forming, Gram-positive, and rod shaped. Carabao rumen fluid *Actinomyces* had strains with catalase and without catalase. Physiochemical characteristics of one of the isolates from the four feedstocks and durations are presented in Table 4 to Table 7. This group with 5 isolates from the carabao rumen hydrolysis had no indole and or urease enzymes, all strains have broad sugar acid fermentation substrates except mellizitose and sorbitol sugars. Obligate anaerobes Actinomyces were identified with *Actinomyces esraelli* and the aero tolerant isolates were identified with *A. viscosus* and *A. naeslundii* (API20A taxonomy ID system). Dehority, 1993 had no classification for the Actinomyces bacteria in the ruminant. Actinomyces in the carabao rumen were first identified in carabao rumen fluid hydrolysis. Actinomyces of the carabao rumen fluid occurred at duration of 3 days and 9 days, suggested species were among the resilient bacteria of the hydrolysis.

Genus *Streptococcus* and other Cocci

Carabao rumen fluid hydrolysis had 7 strains of bacteria from genus *Streptococcus,* a non-spore forming, Gram-positive, without catalase enzyme. Physiochemical characteristics of the isolates from hydrolysates of sugarcane, sweet sorghum, rice straw and corn stover are presented in Tables 4 to Table

7. All strains have no indole, 1 strain with urease enzyme, broad sugar acid fermentation substrates except mellizitose. Streptococcus isolates showed similarities with *Streptococcus bovis* physiological features (Dehority 1993). Some of our isolates have similarities with *S. intermedius* and *S. ureolyticus* using the (API20A ID system) taxonomy of anaerobes as standard. The presence of the *Streptococcus* strains at periods of 3days, 6days and 9days was implications of resilience at low pH hydrolysates. Weimer, 1995 indicated resilience as one of the properties of rumen bacteria, wherein resilience is the ability to recover from, and resistance to and from perturbation such as low pH. Russell and Dambrownski, 2000 had indicated that *Streptoccocus* of the rumen is one of the low pH tolerant specie. Another cocci, a *Staphylococcus saccharoyticus* was isolated also only from sweet sorghum hydrolysis. The specie was Gram positive, with catalase, non-spore forming, and cocci. It has a broad fermentation substrates but isolates had urease enzyme, indicating ability of hydrolyzing urea in the carabao rumen fluid hydrolysis. Morphology and physiological characteristic of the strain genera are presented in Table 5.

Genus Bifidobacteria

Bifidobacteria isolates of the carabao novel process had no catalase, non-spore, Gram-positive and rods shaped.Physiochemical characteristics ar presented in Table 4 to Tabble 7. Strains of the genus Bifidobacteria *have* no indole or urease, broad acid fermentation substrates. *Bifidobacteria* was resilient because of occurences at day 3 then day 9 hydrolysis. Physiochemical features of *Bifidobacteria* isolates showed similarities with *Bifidobacteria* I and *Bifidobacteria* II according to the API20A taxonomy ID system. Trovalli *et al* 1976 had isolated isolated Bifidobacterium in the rumen of calves' different ration.

Genus Lactobacillus

Lactobacillus of carabao rumen hydrolysates had no catalase, Gram-positive, non-spore forming and rod shaped. This group that was isolated only in sweet sorghum hydrolysis. Physiological characteristics showed that *Lactobacillus* have wide array of sugar acid fermentation substrate except sorbitol, had no indole but specie had urease enzyme (Table 5). Physiochemical characteristics showed the bacterium had similarities with *L. acidophilus* and *L ruminis* according to rumen bacteria classification (Dehority, (1993). *Lactobacillus* occurred at 3 days, 6 days and 9 days periods. Presence of the bacteria at all sampling period was implication of its resilient nature.

Table 4. Physiochemical and morphological characteristics of bacterial strain and their succession in the sugar cane bagasse hydrolysis using carabao rumen fluid

SUBSTRATES /BACTERIA ISOLATES	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE	CAT	SPORE	GRAM	COCI
3 days Hydrolysis																								
Streptoccocus constellatus 64360064 1) SC3-2	-	+	+	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	+
Bacteroides sp. 64360060 2) SC3-3	-	+	+	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-
Bacteroides ureolyticus 77776270 3) SC3-4	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-
Bacteroides ovatus	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-	-	-

47776270 4) SC3-6																							
Bifidobacterium 47777032 9) SC3-R1	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-
Bifidobacterium sp. 47756232 10) SC3-R3	-	-	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	-	-	+	-	-	+	-
6 days Hydrolysis																								
Clostridium clostridioforme 463772223 5) SC6-2	-	-	+	-	+	+	+	+	-	+	+	+	+	+	+	-	+	-	-	+	-	+	+	-
Clostridium sp. 46366023 6) SC6-3	-	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	+	-
Clostridium bifermentans 57757633 11) SC6-R1	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	-
Clostridium clostridioforme 47356073 12)SC6-R3	-	-	+	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	+	+	+	+	+	-
Bacteroides sp. 57757630 13) SC6-R6	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	-
9 days Hydrolysis																								
Clostridium sp. 46376203 7) SC9-1	-	-	+	-	+	+	+	+	-	+	+	+	-	+	+	-	+	-	-	-	-	+	+	-
Streptococcus sp. 46376246 8) SC9-3	-	-	+	-	+	+	+	+	-	+	+	+	-	+	+	-	+	-	-	-	+	-	+	+
Bifidobacterium 47756202 14) SC9-R1	-	-	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	-	-	-	-	-	+	-
Clostridium 47757633 15) SC9-R3	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	-

Identification Key IND : Indole(1) ,URE(2)- urease; GLU(4)- glucose; MAN(1)-mannitol; LAC(2)-lactose; SAC(4)-saccharose; MAL(1)-maltose; SAL(2)-salicin; XYL(4)-xylose; ARA(1)-arabinose; GEL(2)-gelatin; ESC(4)-esculin; GLY(1)-glycerol;CEL(2)-cellobiose; MNE(4)-mannose;MLZ(1)-melizitose;RAF(2)-raffinose;SOR(4)-sorbitol;Rham(1) TRE(2)-trehalose ; Cat(4)) Spore(1),Gram(2) ,Cocci(4) Negative reaction(-),Positive reaction(+)

Table 5. Physiochemical and morphological characteristics of the isolates of bacterial strain and succession in the sweet sorghum hydrolysis using carabao rumen fluid

SUBSTRATES /BACTERIA ISOLATES	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE	CAT	SPORE	GRAM	COCI
3 Days Hydrolysis																								
Bacteroides ovatus 57776030 No. 16 (SS3-2)	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-
Lactobacillus acidophilus 46366032 No. 17 (SS3-4)	-	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	+	-	+	-
Bacteroides ovatus 57776070 No. 18 (SS3- 6)	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	-	-	-
Bacteroides sp. 57776030 No.19 (SS3-8)	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	-	-	-
Actinomycetes sp. 47757232 No. 24 (SS3-R1)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	+	-	-	+	-
Actinomycetes sp. 47757232 No. 25 (SS3-R2)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	+	-	-	+	-
Clostridium sp. 67757073 No. 26 (SS3- R3	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-
6 Days Hydrolysis																								
Lactobacillus sp. 46366022 No.20(SS6-1)	-	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	-	+	-
Clostridium clostridioforme 46767223 No.21 (SS6-2)	-	-	+	-	+	+	+	+	+	-	+	+	-	+	+	-	+	-	-	+	-	+	+	-
Staphyloccocus saccharolyticus 67756676 No.27(SS6-R1)	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	-	+	+
C. clostridioforme 47757073 No.28(SS6-R4)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-
9 Days Hydrolysis																								
Streptococcus sp. 47777066	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	+	+

No. 22(SS9-1)																							
Lactobacillus sp 66776332 No.23(SS9-2)	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	-	+	-
Clostridium sp. 47757273 No.29 (SS9-R1)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	+	+	+	+	-
Clostridium sp. 47757073 No. 30(SS9-R2)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-

Identification Key IND : Indole(1) ,URE(2)- urease; GLU(4)- glucose; MAN(1)-mannitol; LAC(2)-lactose; SAC(4)-saccharose; MAL(1)-maltose; SAL(2)-salicin; XYL(4)-xylose; ARA(1)-arabinose; GEL(2)-gelatin; ESC(4)-esculin; GLY(1)-glycerol;CEL(2)-cellobiose;MNE(4)-mannose;MLZ(1)-melizitose;RAF(2)-raffinose;SOR(4)-sorbitol;Rham(1) TRE(2)-trehalose ; Cat(4)) Spore(1),Gram(2) ,Cocci(4) Negative reaction(-),Positive reaction(+)

Table 6. Physiochemical and morphological characteristics of bacterial strain and succession in rice straw using carabao rumen fluid hydrolysis

SUBSTRATES CODE / BACTERIA ISOLATES	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE	CAT	SPORE	GRAM	COCCI
3 Days Hydrolysis																								
Bacteroides butyricum 47776070 Cell No. 31/ (RS3-2)	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	-	-	-
Bacteriodes sp. 57776330 Cell No. 32 (RS3-4)	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	_	-	-	-
C. clostridioforme 47757233 Cell No. 41/(RS3-R2)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	+	-	+	+	-
Actinomyces 47777272 Cell No.42/RS3-R3	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	-
6 Days Hydrolysis																								
Clostridium beijerinki 47777233 Cell No. 33(RS6 – 1)	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	+	-
C. clostridioforme 57777233 Cell No. 34 (RS6 – 0)	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	+	-
Clostridium sordelli 77777373 Cell No 35 (RS6 – 3)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
C. clostridioforme	-	-	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	-	+	+	-	+	+	-

47376233 Cell No. 36 /(RS6 – 4)																							
Streptococcus sp. 47757276 Cell No.37(RS6 – 5)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	+	+	-	+	+
C. beinjerinki 47757271 Cell No.38(RS6 – 6)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	+	+	+	-	-
Actinomyces 47756072/cellNo.43/RS6R1	-	-	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	+	+	+	-	+	-
Clostridium sp. 47757673 Cell No.44/RS6R2	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	-
9Days Duration																								
Clostridium sp. 44336063 Cell No. 39(RS9-1)	-	-	+	-	-	+	+	+	-	+	+	-	-	+	+	-	-	-	-	+	+	+	+	-
Clostridium sp. 47376013 Cell No. 40 (RS9-2)	-	-	+	+	+	+	+	+	-	+	+	+	-	+	+	-	-	-	-	+	-	+	-	-
Clostriidium sp. 47757233 Cell No.45/RS9R3	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	+	-	+	+	-
Clostridium sp 47757673CellNo.46 /RS9R2	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	-

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Table 7. Physiochemical and morphological characteristics of bacterial strains and succession in the corn stover hydrolysis using carabao rumen fluid.

SUBSTRATES/ BACTERIA ISOLATES	*IND*	*URE*	*GLU*	*MAN*	*LAC*	*SAC*	*MAL*	*SAL*	*XYL*	*ARA*	*GEL*	*ESC*	*GLY*	*CEL*	*MNE*	*MLZ*	*RAF*	*SOR*	*RHA*	*TRE*	*CAT*	*SPOR*	*GRAM*	*COCCI*
3Days Hydrolysis																								
Clostridium clostridioforme 47757033 No.47(CS3R1)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	-
Bifidobacterium sp. 47757032 No. 48 (CS3R2)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	-	+	-
6 days Hydrolysis																								

Actinomycetes sp. 47776032 No.49 (CS6R1)	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	+	-
Streptococcus sp. 67776636 No. 50 (CS6R3)	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	-	+	+
9 days Hydrolysis																								
Clostridium sp. 47776631 No. 51 (CS9R1)	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	-	-
Streptococcus sp. 47757636 No. 52 (CS9R2)	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	+	+

Identification Key IND : Indole(1) ,URE(2)- urease; GLU(4)- glucose; MAN(1)-mannitol; LAC(2)-lactose; SAC(4)-saccharose; MAL(1)-maltose; SAL(2)-salicin; XYL(4)-xylose; ARA(1)-arabinose; GEL(2)-gelatin; ESC(4)-esculin; GLY(1)-glycerol;CEL(2)-cellobiose; MNE(4)-mannose;MLZ(1)- melizitose;RAF(2)-raffinose;SOR(4)-sorbitol;Rham(1) TRE(2)-trehalose ; Cat(4) Spore(1),Gram(2) ,Cocci(4) Negative reaction(-),Positive reaction(+)

Succession Behaviour of the Bacteria in the Carabao Rumen Fluid Hydrolysis

Rumen Bacterial Community

Table 8 data showed that initial population of bacteria and succession behavior in the four feedstocks and duration of 3days, 6 days and 9 days. Due to the number of strains within the 7 genera of isolates, Actinomyces*, Bifidobacteria, Bacteroidetes, Clostridium, Lactobacillus* and *Staphylococcus,* population was counted at the level of strain of these bacteria. Dubar *et al*. 1997, reported about 0 to 5% of rumen bacteria could be isolated and cultured from hydrolysates and fermented biomass.

Seven genera of bacteria had 52 strains with different morphology and physiochemical characteristics (API20A kit, Biomereiux) were isolated. Succession evaluation showed that only sweet sorghum has the complete profile while genera of *Lactobacillus* and *Staphylococcus* were isolated only in sweet sorghum hydrolysis (Table 4). Sugar cane had 6 isolates consisted of 2 strains of *Bifidobacterium*, four strains of *Bacteroidetes*, and *Streptococcu*s and *Clostridium*. Corn stover has *Bifidobacterium, Clostridium, Actinomyces*, and *Streptococcus*. More strains of Clostridium isolated at day 6 and day 9 with specie of low pH tolerant *Streptococcus*, *Bifidobacteria* and *Lactobacillus* in low pH hydrolysis indicating bacterial specie complementation particularly for soluble growth nutrients. Rice straw has 2 *Actinomyces*, a *Bacteroidetes* and 10 strains of *Clostridium* strains during the hydrolysis. Strains of *Bacteroidetes* non-recurrences at day 6 and day 9 in rice straw, suggested the specie was unable to acquire adequate growth nutrients, inhibited enzymes for cellulolytic activity and weak resilience to low pH hydrolysates at long incubation period. Increase in population size of *Clostridia* species in rice straw maybe contributed by resilience, conducive pH for cellulolysis and activation of spores. Improved conversion of carbohydrates at day 6 was due to high cellulolytic activity of the genera and their strain that developed before the period. Population size of bacteria mainly strains within genera of *Streptococcus, Bifidobacteria* and other complementing species were high at long duration of 9 days that maybe due to resilience to low nutrients degradation at low pH. The rumen fluid hydrolysis bacterial community was altered by changes in the composition of genera over period of incubation, resulting in strains with high resistance to low pH hydrolysis. The study showed that bacterial species interaction between genera and strains during biomass degradation had both positive and negative effects on the carbohydrates conversion into solubilized sugars. The synergy of the bacterial community was implication of bacterial succession behavior in the carabao rumen fluid hydrolysis. Popat *et al* 1995 indicated individual and collective group social interactions for beneficial goods of the bacteria community called bacterial quorum sensing. In another study of bacterial behavior, Weimer (1995)

showed that rumen bacteria has two properties that makes predominant microbes in the rumen, it is the redundancies which allow them to overlap functions among bacterial species and resilience that allow them resistance to, recover from perturberance or adverse condition.

Table 8. Population size of isolated rumen bacteria in various feedstocks and duration of carabao rumen fluid hydrolysis

Feedstock Duration	Sugarcane Bagasse	Sweet sorghum	Corn Stover	Rice straw	Mean
3 days	6.0	7.0	2.0	6.0	6.0 ± 2.94
6 days	5.0	4.0	2.0	6.0	4.0 +1.71
9 days	4.0	4.0	2.0	4.0	3.0+1.0
Mean	5.0+2.6	5.0+1.7	2.0+0	5.0+1.2	

Means ±SD. Means without letter superscript are not significantly different.

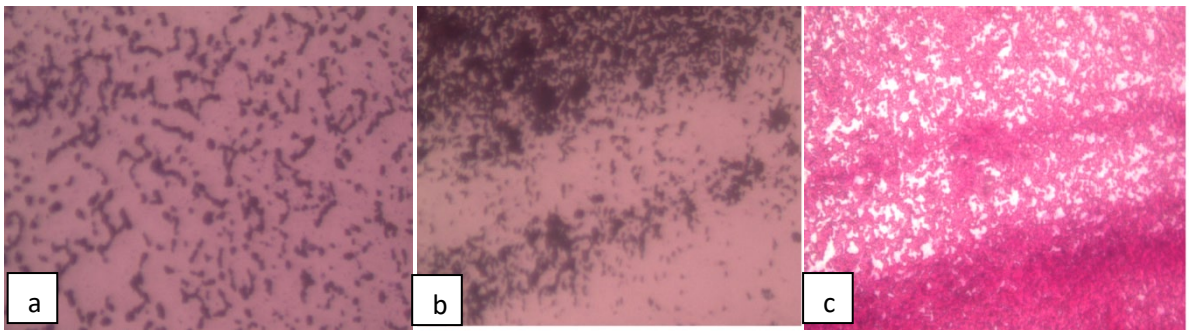

Figure 5. Morphology of carabao rumen fluid hydrolysis specie of *Lactobacillus*,) *Streptococcus* sp. and *Clostridium. Photo was* taken at low power magnification with image resolution of 680 x 340

Carabao Rumen Fluid Hydrolysis Succession Behavior of Rumen Fungi Community

Isolated fungi strains from the family *Neocallimastigacea (*Ho *et al*, 2000*)* were found in the four agriculture crop residues evaluated as alternative feedstock for cellulose ethanol production, two of species were isolated in corn stover and rice straws (Table 9 and Figure 4). Although, these species belong to the same family, the rumen fungi varied in their type's sporangium, spores that vary from large globosely to tiny spherical shaped, large hyphae to tiny massive mycelia subsurface growth, branching hyphae with cysts. Growth of the anaerobic fungi in vial of PDA had occurred only in culture medium with biomass, indicating fibrous portion of the fermenting biomass as fungi specific substrates. Zoospores and hyphae had been reported in various animals (Shridhar *et al*., 2010), Akin *etal* 1990 showed that anaerobic fungi are better than bacteria in degrading plant cell wall because the microbes can degrade phenolic contents. Fungi species biomass decomposition in co-cultures with bacteria and protozoa in vitro degradation of orchard grass showed the activity of fungus until the later stage of in vitro hydrolysis (Lee *at al*, 2000).

Table 9. Carabao rumen fluid hydrolysis isolated rumen fungi from various feedstocks.

Family of Carabao Anaerobic Rumen Fungi	Genus of Carabao Anaerobic Rumen Fungi	Sugar cane Bagasse	Sweet sorghum	Corn stover	Rice straw
Neocallimastigaceae	ID No.CRF1 Genus: *Neocalimastix sp*	+	+	+	+
Neocallimastigaceae	ID No. CRF2 Genus: *Orpinomyces sp*	+	+	+	+
Neocallimastigaceae	ID No. CRF3 Genus *Neocallimastix* sp.	-	+	+	-
Neocallimastigaceae	ID No. CRF4 Genus: *Ruminomyces sp*	+	+	+	+

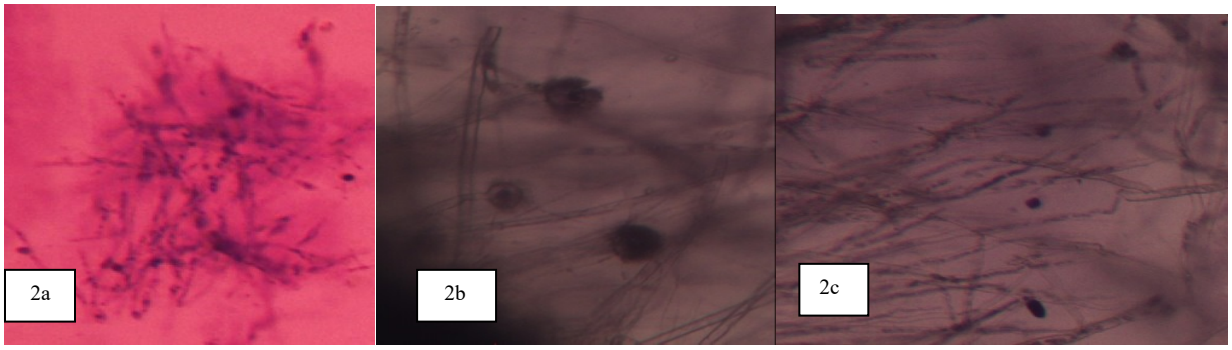

Figure 6. Subsurface growth morphology of carabao rumen fungi. Branching hyphae with constrictions,enlargement and cysts and outgrowths typical of *Ruminomyces sp*. 2b) sub-surface structure of large tubular hyphae with spherical and globose sporangium typical of *Orpinomyces sp.* and 2c) massive rhizomycelia with fusiform sporangia at interval and terminal of the mycelium typical of *Neocallimastix* sp. of carabao rumen. Photos were taken with 10X magnification compound microscope and camera with 680 x 380 resolutions.

Carabao Rumen Fluid Hydrolysis and Succession Behavior of Community of Protozoa

Carabao rumen protozoa were identified through ther morphological structures using the guide manual of Dehority, 1993. The identified species of carabao rumen fluid protozoan are presented in Table 10. Carabao rumen fluid hydrolysis of sweet sorghum had the most number of species that may be the effect of the feedstock composition and high carbohydrates conversion digestibility.

Table 10. Morphological Characteristics of Rumen Protozoa isolates in the Hydrolysis using Carabao rumen fluid

Morphology and Class of Carabao Rumen Protozoa	Sugar cane Bagasse	Sweet sorghum	Corn stover	Rice straw
With skeletal plates				
Eremoplastron	+	+	+	-
Eodiplodinium	+	+	-	-
Eodinium	++++	++	+	+
Diplodinium	-	++	++	-
Epidinium	-	+	-	-
With caudal spines				
Entodinium	+++	++++	+++	+++
Ostracodinium	+	-	+	-
Whole body ciliates				
Isotrichia	-	+	-	+
Buetchlia	-	-	+	-

Sugar cane bagasse, sweet sorghum, corn stover and rice straw biomass had *Eremoplastron, Eodiplodinium, Epidinium, Eodinium* identified in the hydrolysates because of the presence of skeletal plates while *Entodinium and Ostracondinium* hve prominent caudal spines. *Isotrichia and Buetchelia* are easily identifiable under microscope because of the ciliated body of these species.
Jabari *et al*., 2014 indicated the dominance of protozoa in terms of population and digestion ability in buffalo rumen than in cattle rumen. Dehority, (1993) mentioned about some protozoan like *Epidinium* and *Polyplastron multivesiculatum* abilities of engulfing cellulose strands. Because of solubilized cellulose engulfing behavior, protozoan in the carabao rumen fluid hydrolysis can control the population of the microbial community of bacteria and fungi that were attached to the biomass.

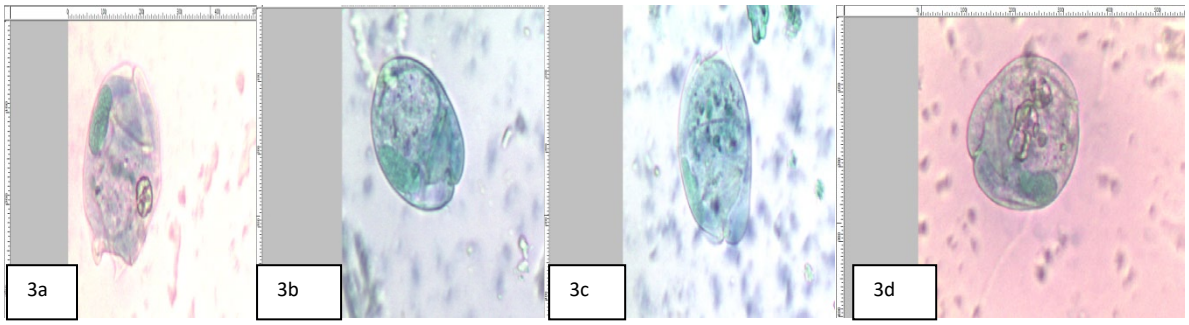

Figure 7. Morphology of, protozoa species isolated from carabao rumen fluid hydrolysis. 3a) Specie with left and right caudal spines oriented like hayline and a concretion vacuole typical of *Entodinium* sp 3b). Ovoidal shape with thick macronucleus and micronucleus with anterior thick folds and posterior right posterior lobe bigger than right typical of *Eodinium*, 3c) rectangular shape with caudal spines and thick rod-shaped macronucleus of the genus *Entodinium*, 3d) thick macronucleus and many concretion vacuoles of the genus *Diplodinium*. Protozoa isolates body sizes ranged from 20*mm* to 40*mm.* Photos were taken using a compound microscope at 10X magnification and camera with 680 x 380 resolution.

Conclusion

Bagasses of sweet sorghum and sugarcane, rice straw and corn stovers are agriculture crop residues, renewable, inexpensive, not competitive with human food and abundant supply could lower the cost of producing cellulose ethanol. Composition analysis showed a high percentage of soluble extractives in sweet sorghum hay and sugarcane bagasse compared with corn stover and rice straws. Analysis of holocellulose content, that represented both cellulose and hemicellulose showed high contents in all agriculture crop residues. Carbohydrates conversion efficiency was improved in feedstock with moderate amount of soluble extractives. Carbohydrates conversion efficiency was improved by duration, but not beyond 6 days. Low pH hydrolysis can alter the composition of rumen bacteria and protozoa to resilient species. Rumen fungi composition is unaltered due to specific location in fibrous mat of the crop residues. Culture methods of isolation, identification and characterization using API20A can identify the dominant cellulolytic rumen bacteria in the novel carabao, suggested usage in selecting species with high cellulolytic activity in low pH hydrolysis. The study showed that microbial community in the novel carabao rumen fluid hydrolysis has synergy among bacteria, fungi and protozoa that their diversity and succession behavior contributes to the efficiency of carbohydrates conversion of the novel carabao. Diversity in the microbial composition of the novel carabao can be harness in the selection of rumen species that can convert fibrous biomass of agriculture into soluble sugars with higher efficiency hydrolysis.

Acknowledgements

Due acknowledgement to the Philippine Council for Industry, Energy and Emerging Technology (PCIEERD-DOST), the Department of Agriculture - Philippine Carabao Center at Central Luzon State University (PCC at CLSU) and Absolut Distillers Inc. (ADI) for fund support in this study

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