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Cryopreservation of *Bubalus bubalis* L. Rumen Bacteria: Effect on Viability and Efficiency of Conversion of Crop Residues into Soluble Sugars for Biofuel Production

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The study had determined the effect of glycerol, 100%, 80% and 60% as cryoprotectant on bacterial cell viability and efficiency of conversion of carbohydrates in bagasses of sweet sorghum and sugarcane, and rice straw hydrolysis using rumen bacteria after cryopreservation at low temperature (-20^{0}C) and ultra low temperature (-156^{0}C) vapor phase of liquid nitrogen for 54 days. Pellet of fresh culture of rumen bacteria isolate was mixed with glycerol at 1:1 ratio of culture and glycerol solution. Cryovials were kept in cryoboxes for low temperature storage while vials in cryocanes inside canisters were kept at vapor phase of liquid nitrogen tank. New Bauer Hemacytometer was used to count viable cells before and after cyrovial was thawed at 37^{0}C for 5 minutes. For the hydrolysis, recovered viable cells were sub-cultivated in seed culture liquid medium. Carbohydrates in biomass and hydrolysates were analyzed using 3,5 Dinitrosalycilic acid assay for reducing sugars. Results showed that level of glycerol had insignificant effect on the viability at prolonged storage in the vapor phase of liquid nitrogen. Rumen bacteria *Clostridium* RS91 preserved in three glycerol solutions had averaged viability 17.28% after 54 days in the vapor phase of liquid Nitrogen while corresponding conversion of carbohydrates into sugars efficiency at 36 and 48 hours hydrolysis was 57% and 53.4%, respectively. The performance of the LN2 cryopreserved carabao rumen bacteria was significantly effected by duration of hydrolysis ($p>0.05$). Three seed culture inoculants with species combinations of *Bacteroides, Clostridium, Lactobacillus,* and *Streptococcus* from low temperature cryopreservation had carbohydrates conversion efficiency that was insignificantly effected by composition of culture(MCI) while 64.84%, 38.27% and 30.52% efficiency of carbohydrates conversion of the mixture for bagasses of sweet sorghum, sugarcane, and rice straw showed that feedstock had significantly affected performances of the bacterial seed culture($p>0.05$). Periodic testing revealed ethanol was produced by *Saccharomyces cerevisiea* fermented carbohydrates of bagasses of sweet sorghum, sugarcane and rice straws hydrolyzed by frozen and thawed carabao rumen bacteria. To our knowledge, this is the first report on cryopreservation of Philippine carabao rumen bacteria and was vital in the elucidation of the nature of rumen bacteria as microbial inoculant in hydrolysis pretreatment of alternative feedstock intended for biofuel production.

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Introduction

The great impact of globally experienced fuel crisis in the Philippines and the Biofuel Act of 2006 mandates certainly had put agriculture in the sector of biofuel particularly in the generation of bioethanol from not only from sugar crops but crop residues and lignocellulosic as altenative feedstock. Agriculture crop residues in the industrialized countries were evaluated for their alcohol potentials using chemical pretreatments (Badder, 2002; Chen *et al*., 2007) prior to enzymatic hydrolysis to further solubilized celllobioses to hexoses and pentoses fermentable into alcohol by common yeast. Pretreatment shares the biggest capital investments while low output of product ethanol and problems associated with disposal of corrosive effluents into the environment were factors that motivated further research on pretreatments of lignocelluloses for bioethanol. The hydrolysis of lignocelluloses using carabao rumen fluid, that contain cellulolytic and hemicellulolytic microbes was a paradigm of the current pretreatments intended for bioethanol production (Annex A). Specifically, the rumen fluid hydrolysis had enhanced alcohol potential of rice straws(Abenes and Florendo, 2008; Marilao *et al*., 2008) sweet sorghum bagasse(Caluya *et. al*., 2008 and Damian *et.al, 2013*) and corn stover(Concepcion *et al.*, 2010) and switchgrass(Florendo *et al.*, 2017) using hydrolysis that combined physical reduction and direct enzyme production as alternative to physical and chemical reduction and pure enzyme hydrolysis prior to ethanol fermentation by common yeast. Pretreatment hydrolysis direct cellulolytic and hemicellulolytic enzyme production make use of carabao rumen bacteria(Florendo *et al.*, 2012), that included culturable species of carabao rumen *Actinomyces, Bacteroides, Bifidobacterium, Clostridium, Staphylococcus, Streptococcus* and *Lactobacillus* The identified culturable rumen bacteria showed diversity in terms of morphology and physiological characteristics using the API20A system, had displayed different growth patterns and variable responses to acidic hydrolysis conditions. Cyclicity in growth patterns in rumen protozoa in *in vitro* culture according to Dehority (2008)was traceable to cellulolytic property and predation of bacteria. Rumen protozoa that populated hydrolysis of sweet sorghum bagasse showed species and ciliates *Entodinium* (Miranda *et al.*, 2016) decreased with increasing duration of pretreatment to 15 days. Philippine carabao rumen fungi (Peralta *et al*, 2013,) such as *Orpinomyces, Ruminomyces* and *Neocallimastix* from family *Neocallimastigacedae* had indicated tolerance to acidic pH and long duration of sweet sorghum pretreatment using carabao rumen fluid hydrolysis. Khejornsart

and Wanapat, (2010) had indicated rumen fungi positive interaction with methanogens in the rumen of cattle fed urea-limed treated rice straw and concentrates while the interaction among groups of bacteria, protozoa and fungi *in vitro* were reported (Lee *et al.*, 2000). Information however, was limited to fully explain the contributions of different species, particularly rumen bacteria that showed greater genus types population in the pretreatment hydrolysis compared to fungi and protozoa. Terry *et al.*, (1969) had indicated species of bacteria with different responses to their environment like pH and temperature. Elucidation of bacteria species nature in the absence of interaction with rumen fungi and ciliates is necessary to understand how bacteria influence carbohydrate fermentation *in vitro* and the potential of manipulation rumen composition including use of preserved rumen microbes as alternative to fresh rumen fluid in the scaling up of the process hydrolysis as pretreatment of lignocellulosic feedstock intended for biofuel production. Yamasoto *et al*. (1973; 1978) had indicated survival of bacteria after cryopreservation in moderately temperature and effect of low temperature (-53^0C) cryopreservation on survival after 92 months. Liquid nitrogen preservation was used in the study of rumen protozoa(Nsabimana *et al.*, (2003), the autotrophic bioleaching bacteria (Wu *et al*. 2003) and genetically improved fermentation microbes (Sharp,1984). Thus, cryopreservation of rumen bacteria from carabao rumen fluid hydrolysis can be adapted to further elucidate their nature as specie with cellulolytic activity and function as microbial inoculants for pretreatment intended for biofuel production.

According to Hubalek (2003), several cryoprotectant additives(CPAs) were used in the frozen storage of microorganisms. Sakurada *et al.* (1995) indicated ethylene glycol and rumen fluid in the cryopreservation of anaerobic rumen fungi. In bacteria, Dimethylsulfoxide (DMSO) and glycerol(1,2,3 propanediol) were the most utilized cryoprotectants. Glycerol was used in the long term cryopreservation of *E. coli*, that 42% glycerol was contributory to long term survival at -20^0C. A higher concentration of 2 to 55% and median of 10% was widely used in the cryopreservation of different microbes. Using a simplified cryopreservation, this study aims to evaluate the nature of carabao rumen bacteria as inoculant in the pretreatment hydrolysis after cryopreservations at low temperature and ultra low temperature with the ultimate goal of improving the performance of rumen bacteria species on carbohydrates fermentation using the hydrolysis pretreatment and subsequent effect of fermentation products such as solubilized carbohydrates for bioethanol production,volatile fatty acids and methane gas production.

The general objective of the study was to elucidate the nature of the rumen bacteria after cryopreservation in freezer temperature (-20^0C) and vapor

phase of liquid nitrogen (-156^{0}C). Specifically, the study aimed on the effect of different level of glycerol as cryoprotectant on the performance of rumen bacteria cryopreserved in liquid nitrogen (-156^{0}C) and low temperature (-20^{0}C) freezer in terms of terms of bacterial cell count, viability and efficiency of carbohydrates conversion into solubilized sugars using different feedstocks and determined the effect of sweet sorghum, sugarcane bagasse and rice straw sugars hydrolyzed by bacteria as inoculant on yeast fermentation efficiency and ethanol yield by common yeast.

Materials and methods

Cultivation of rumen bacteria for preservation

Swab of TGMA culture of isolated rumen bacteria Clostridium RS91 from rice straw hydrolysis using carabao rumen fluid was inoculated in 500 ml of Thioglycollate Broth and incubated at 37^{0}C for 24 hours. After 24 hr, the fresh culture was centrifuged at 12000 rpm for 5 min, discarded the supernatant and washed the cell pellets twice with 10 ml of 1% TGBroth. Similar procedure was done in the sub-cultivation of bacteria *Actinomyces, Bacteroides, Bifidobacterium, Clostridium, Lactobacillus, Streptococcus and Staphyococcus* species. The bacterial cell pellet suspension of 175 ml Thioglycollate Broth was divided into three equal parts for cryopreservation of the rumen bacteria using the three levels of glycerol.

Method of preservation in glycerol

Three solutions of glycerol (100%, 80% and 60%) were steam sterilized at 121^{0}C for 15 minutes, cooled and stored at 4^{0}C. Glycerol solution of 50 ml was mixed with 50 ml pellet of RS91 bacterium suspension using 1:1 mixing ratio. After mixing, 2 ml of mixture was aspirated into cryovial, that was labelled with 4 numbers coded genus of bacteria isolate (#1-52), feedstock origin (#1-4), batch number (#1-2) and replication(1-10). Each treatment total of 25 replicate cryovials were stored in cryobox (9x9) prior to storage at low temperature freezer (-20^{0}C). All isolated rumen bacteria and identified using the API20A system were preserved in 4 levels of glycerol solutions and stored at -20^{0}C for long term preservation.

Evaluation of cell count and viability.

Evaluations of cell count and viability were conducted in triplicate cryovials at d0, d1, d2, d3 and d6 of storage in freezer and 45 and 54 days

preservation in liquid nitrogen. Sample vials were thawed in sterilized water maintained at 37^0C, prepared into a 10^{-1} serial solution for direct cell count using a New Bauer hemacytometer. The suspension was stained with eosin yellow at the mixing ratio of one ml of the mixture of 10^{-1} dilution diluted with eight ml of sterilized water and 1 ml of eosin yellow stain solution, inverted test tube for 10 times to ensure thorough mixing of bacterium with staining solution. The hemacytometer with the special slide cover slip was inoculated with 10 ul of the mixture and allowed 5 minutes to stabilized at the microscope stage. After duration, viable cells were counted in 5 squares within the 5 x 5 grid of the hemacytometer. Cell counting was facilitated using a built in camera in a compound microscope with program software connected to laptop. The microscope was pre-set at low power objective and camera resolution adjusted to 630 x 380. Counting was done in the two chambers while the average cell count within chamber was multiplied by 25 in order to come up with the cell count per square. Cell count per ml was calculated using the dilution factor and multiplied by factor 10,000 in order to come out with bacterial cell count per ml. Data on cell count per ml was used in the computation of initial cell count and viability prior to hydrolysis.

Cryopreservation at low temperature

Cryovials of bacteria from *Actinomyces, Bacteroides, Bifidobacterium, Clostridium, Lactobacillus, Streptococcus and Staphylococcus* were maintained at freezer for 54 days. After duration, bacteria from species *Bacteroides, Clostridium, Lactobacillus, Streptococcus* were sub-cultivated for evaluation of their nature in a hydrolysis using sweet sorghum bagasse, sugarcane bagasse and rice straw.

Cryopreservation in liquid nitrogen

Eighteen cryo vials *Clostridium* RS91 from each treatment glycerol(100%, 80% and 60%) were transferred in cryocanes, each cane containing 6 cryovials. Initial cell count was determined prior to cryocanes storage in liquid nitrogen (LN2). Prior to storage, the cryocanes with vials of rumen bacteria were immersed at the vapor phase of the LN2 tank for 5 minutes. This procedure of pre-freezing stage aimed to check damaged cryovials. Cryovials without leak were stored at the vapor phase of the liquid nitrogen (LN2) tank for 54 days. Sub-sampling was done at 45 days on storage. The cryopreservation tank was refilled with liquid nitrogen until the bottom of cryocanes. Liquid nitrogen was obtained at the PCC liquid nitrogen storage unit

during the cryopreservation study. The cryopreservation of rumen bacteria in liquid nitrogen was based on the procedure of Wu *et al.*, (2008) for bioleaching bacteria species.

Thawing procedure

Three cryovials from each treatment were withdrawn from the LN2 tank, plunged the vials into a waterbath with 37^0C sterilized water for 5 minutes. After thawing, the content of vial was transferred into 12 ml test tube for serial dilution (1:100). The thawing procedure was also followed in the evaluation of frozen bacteria from low temperature storage. The frozen and thawed suspension was used for viable cell count of thawed samples.

Preparation of seed culture medium

A seed culture medium of 100 ml was prepared by adding four ml of salt solution 1 and 2 and added 0.5 g glucose. The mineral salt solution 1 was prepared from KH_2PO_4 (0.6%), $(NH4)_2SO_4$ (0.6%), NaCl (1.2%), $MgSO_4.7H_2O$ (0.245%) and $CaCl.2H_2O$ (0.159%) and mixed to one liter of sterilized distilled water. Mineral solution 2 was prepared from K_2HPO_4 (0.6%) diluted to 1 liter. After mixing the 2 mineral salt solution, added resazurin (0.1%) at the rate of 0.5 ml per liter seed culture solution. The seed culture solution with resazurin was boiled for 5 minutes, cooled and added 50 ml of Na_2CO_3 (8%) and 0.025g of Cysteine HCl. Solution was bubbled with CO_2 gas until the yellow color had changed to light pink. After the reduction process was completed, solution pH was adjusted to 7 using 4N NaOH solution. The seed culture solution was sterilized at 121^0C for 15 min, cooled to 45^0C before Metronidazole and Ketoced was added at the rate of 100*ug* per ml of solution. This seed culture liquid media was used in the sub-cultivation of bacteria prior to periodic testing.

Monoculture of Rumen Bacteria

For the evaluation of bacteria after cryopreservation in liquid nitrogen, three vials from the three treatments at 45 and 54 days cryopreservation in LN2 vapor phase were sampled for viability and hydrolysis after sub-cultivation. Each cryovial was thawed for the preparation of solution with 10^{-1} serial dilution. Sub-sample of 5 ml of the serial dilution was aseptically inoculated in 100 ml of Thioglycollate Broth, then incubated at 37^0C for 24 hours. Once optical density at A_{600} reached 0.8, the fresh culture was centrifuged at 12000 rpm for 5 minutes. The pelleted bacterial cells was suspended in 75 ml of the

mineral enhancement buffered solution. This cell suspension was used as inoculant in the sweet sorghum, sugarcane hydrolysis.

Mix culture of rumen bacteria

Five species of rumen bacteria were prepared from the isolates cryopreserved at low temperature (-20^{0}C) for 54 days. Cryovials of the rumen bacteria isolates were withdrawn from the freezer, thawed for 5 min at 37^{0}C sterilized water and then formulated into seed cultures as component of a microbial inoculant (MCI). For microbial inoculant MC1, the bacterial culture was composed of 2 species of *Bacteroides*(B16 and B18), a *Streptococcus*(S12) sp. and four *Lactobacillus* species(*L17, L20, L23 and L23b*),all bacteria were isolates from sweet sorghum hydrolysis. For microbial inoculant group MC2, the bacterial culture was composed of 6 unique species; 2 *Bacteroides* species (*B3 and B4*), 2 *Clostridial* species (*C5 and C7*) and 2 *Streptococcus* species(*S1 and S8*) isolates from sugar cane hydrolysis. For microbial inoculant group MC3, bacterial culture MC3 was composed of 5 unique species; two from *Bacteroides* species (*B32 and B33*), two from *Clostridial* species (*C35 and C40*) and a *Streptococcus* specie (*S37*) isolates from rice straw hydrolysis. For seed cultures using mixture of frozen thawed rumen bacterium from low temperature, vial of the bacterium was sub-cultured in 100 ml TGBroth for 24 hours at 37^{0}C. The culture was centrifuged at low speed for 5 min, discarded supernatant and the cell pellet was re-suspended in ten ml of TGBroth culture medium. The fresh suspension was inoculated in 90 ml culture medium and incubated for 24 hours at 37^{0}C. The growth performance of each seed culture was optimized in terms of cell to culture medium ratio(not indicated).

Three feedstocks sweet sorghum bagasse, sugar cane bagasse and rice straw were utilized in the acclimatization of mix bacteria as seed culture. The feedstocks were steam sterilized at 121^{0}C for 30 min, shredded in food blender and steeped in boiling water for one hour. After boiling, the liquid was drained while the residue was used for the hydrolysis. Using a 359 ml capacity sterilized bottle, added 5g of the steeped biomass, 100ul/ml each of Ketoced and Metronidazole, aseptically inoculated with each of fresh bacteria seed culture (MC) using 1:10 ratio of inoculant and volume. Using mineral buffer solution the volume was corrected to 100 ml. The bottles were sealed airtight and incubated at 37^{0}C for 36 hours. After incubation,10 ml hydrolysates was aspirated for pH determination and reducing sugar analysis. The acclimatized culture of bacteria of MC1, MC2 and MC3 was used in the periodic testing of bacteria hydrolysis using feedstocks sweet sorghum, sugar cane bagasse and rice straw.

Periodic Testing of Hydrolysis Using Frozen Rumen Bacteria

Ten ml of seed culture of rumen bacteria RS91 was aseptically inoculated into 100 ml hydrolysis culture medium using a 1:10 ratio of inoculum and fermentation volume. The vial contain 5g cooked and steeped sugarcane bagasse added 100 ul/ ml of Flagyl and 100 ul/ ml Ketoced and one ml of phenol red stain solution and then corrected volume to 100 ml using sterilized mineral buffer solution. Fermentation bottles were sealed tight with rubber cap and with aluminum crimped, incubated at 37^0C for 36 hr and 48 hours. The performance of RS91 bacterium in term of carbohydrate conversions efficiency in the sugar cane bagasse hydrolysis was evaluated at 36 hr and 48 hrs of the incubation period. The performance of the bacterium in hydrolysis was re - evaluated after 54 days of LN2 storage and sweet sorghum bagasse.

For the periodic testing of hydrolysis using mixture of frozen rumen bacteria, a liter capacity bottle was loaded with 15g steeped feedstock, added with seed culture bacteria (MC1, MC2 and MC3) using 1:10 ratio of the inoculant and effective volume of 500 ml. Antibiotics Metronidazole and Ketoced were added at 100*ug* per ml and corrected volume to 500 ml with sterilized water. Hydrolysis solution was bubbled with CO2 gas and the pH was adjusted to 7 using urea solution (15% w/v). Using a low speed shaker, all fermentation bottles were agitated twice a day for 20 minutes until 48 hours. After duration, sub- sample of 10 ml hydrolysate was collected for optical density, pH and analysis of reducing sugar (Miller,1959).

For the sugar to ethanol fermentation, solubilized sugars from the sweet sorghum, sugarcane biomass and rice straw were pasteurized for 30 minutes. After cooling, sample sugars were inoculated with *Saccharomyces cerevisiea* at the rate of 5g DCM /L sugar hydrolysates. Duration of yeast fermentation was 7 days. Reducing sugar was analyzed from initial and final stage of yeast fermentation.

Statistical Analysis

Data from the experiments were analyzed following analysis of variance in factorial experiment in Complete Randomized Design using statistical software (Sirichai, Thailand). Means comparison was done using DMRT at 5% level of significance.

Results

Effect of glycerol on frozen and thawed rumen bacterial cell viability

Rumen bacterium cell count across storage period showed the effect of level of glycerol as cryoprotectant(Figure 1). Initial bacterial cell count (d0) ranged from 2.8 to 3.3x10^{9}/ ml had declined to 2.3x10^{9} using Treatment 1, 2.1x10^{9} using T2 and 1.9x10^{9}/ ml using 60% glycerol as cryoprotectan for carabao rumen bacteria.

After 45 days, bacterial RS91 had averaged cell count of 0.6x10$^{9/}$/ ml in T1, cell count of 0.7x10^{9} cell/ml in T2 and 0.5x10^{9} cell /ml in using 60% glycerol. At prolonged duration of 54 days, data had revealed that bacterium cell count had dramatically decreased, with insignificant difference among treatments.

Mean viability of RS91 rumen bacterium at d1 of preservation in glycerol was 81.22%, 77.03% at day 2 (d2) and 68.73% at day 6 of storage revealed insignificant effect of glycerol on cell viability at -20^{0}C freezer ($P<0.05$). Bacterium viability after storage at vapor phase of LN2 showed RS91 with viability of 26.82% at 45d had decreased to 15.99% at 54d of storage. Prolonged duration had reduced the viability of bacterium using the cryopreservation in vapor phase of liquid nitrogen (Figure 1).

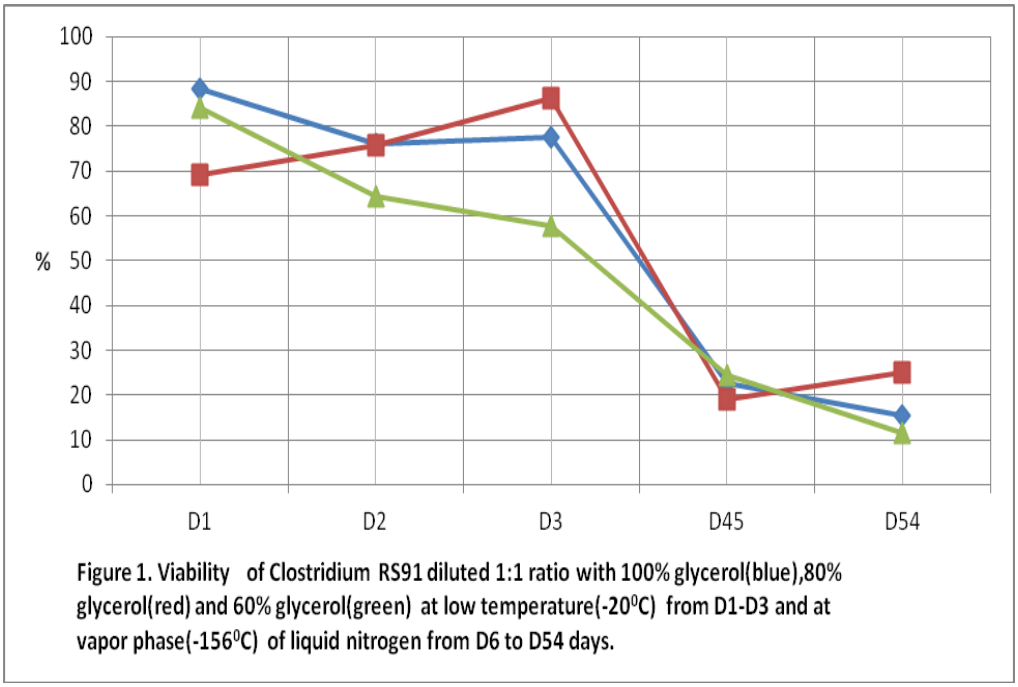

Figure 1. Viability of Clostridium RS91 diluted 1:1 ratio with 100% glycerol(blue),80% glycerol(red) and 60% glycerol(green) at low temperature(-20^{0}C) from D1-D3 and at vapor phase(-156^{0}C) of liquid nitrogen from D6 to D54 days.

The decreasing pattern of viability at different cryopreservation temperature indicated that carabao rumen bacterium Clostridium survival was affected by duration of cryopreservation. Insignificant effect of level of glycerol at prolonged durations of 54 days showed that neither higher level of glycerol at 100% to lowest 60% glycerol and diluted 1:1 ratio with cell pellets had not completely protected cells from osmotic shock. According to Ray *et al.*, (1975), glycerol mix with water results in a disrupted hydrogen bonded water network hindering the formation of extended water network that form into ice crystals. As such the network of glycerol water is providing cryoprotection in either at intracellular and extracellular part of the bacteria cell. Glycerol water mixture decreases the freezing point of water and biological fluid by coligative action, that lessens the concentration of salts in solution which in turn inhibited cellular osmotic shock. Further, Ray (1978) had indicated that cell membrane liposaccharides was protected by glycerol against freezing injury. While liposaccharide is characteristic of gram negative bacteria, the gram positive rumen bacteria specie may be affected by osmotic shock due to cryopreservation and cryoprotectant used. Compared to the concentrations of 2% to 55% for routine cryopreservation of bacteria (Postgate and Hunter, 1961; Ray *et al.*1975; Hubalek, 2003), the level of glycerol 100%, 80% and 60% had preserved carabao rumen bacteria in vapor phase of liquid nitrogen for 54 days. As result of the evaluation of glycerol as cryoprotectant for rumen bacteria, the use of 60% glycerol was recommended for viability and lower input. And since the objective was to use cryopreservation as venue for upscaling the use of rumen microbes as inoculants in the hydrolysis of alternative feedstock, cryopreservation should aim for higher viability and long term storage in low temperature or ultra low temperature. Cryopreservation of the rumen bacteria after our first report had considered Hubalek (2003) suggested control of cryopreservation of microorganism.

Effect of glycerol on carbohydrates conversion efficiency of frozen thawed rumen bacteria.

Table 1 showed that carbohydrates conversion efficiency of the frozen and thawed rumen *Clostridium* RS91 preserved in 100% glycerol was 48.88%, 53.60% efficiency in bacterium preserved at 80% glycerol and 68.49% for 60% glycerol at hydrolysis duration of 36 hours, while higher efficiencies were observed in 60% glycerol followed by 100% than at 80% glycerol in the prolong hydrolysis of 48 hours.

Table 1. Carbohydrates conversion efficiency in sugarcane hydrolysis using frozen and thawed Clostridium RS91

Level of Glycerol as	**Duration of Hydrolysis**	
Cryoprotectant	36 hr	48 hr
100%	48.88(2.54)	51.06(3.19)
80%	53.60(1.36)	48.23(4.68)
60%	68.49(6.88)	60.89(3.52)
Average	57.0(9.62) *a*	53.4(6.62) *b*

Means within row with different letters indicate significance at 5% DMRT. Enclosed value was standard deviation of mean

Statistically, efficiency of the carabao rumen Clostridium RS91 in the conversion of sugarcane bagasse into solubilized sugars was insignificantly different between level of glycerol used ($p<0.05$).

Evaluation of the duration effect showed that Clostridium RS91 efficiency of conversion of carbohydrates into solubilized sugars had 57% and 53.4% for durations of 36 hr and 48 hours respectively. Statistically, the efficiency was significantly effected by duration of hydrolysis ($p>0.05$).

Table 2 showed the performance of mixture of bacteria previously preserved at low temperature in the hydrolysis of different feedstock. The highest efficiency of 64.84% in the conversion of carbohydrates into soluble sugar was obtained in the hydrolysis of sweet sorghum bagasse, 38.27% with sugarcane bagasse and 30.52% using rice straw hydrolysis. Statistically, feedstock had significantly effected carbohydrates conversion efficiency by the mixture ($p>0.05$).

Table 2. Carbohydrates conversion efficiency of different feedstock Hydrolysis using mixture culture of frozen thawed rumen bacteria

Feedstock/ Bacteria Culture	Efficiency %
Sweet sorghum	66.80(25.44) *a*
Sugar cane bagasse	38.22(20.21) *b*
Rice straw	30.52(6.36) *b*
Bacteria culture MC1	51.31(26.50) a
Bacteria culture MC2	50.35(28.40) a
Bacteria culture MC3	39.34(20.50) a

Mean within column with different letter indicate significance by DMRT
Enclosed data in parenthesis was standard deviation of the mean

Difference in the efficiency of the bacteria mixture as inoculant was attributed to the composition of feedstock hydrolyzed. This nature of the bacteria in the hydrolysis of different feedstock was coherent with the results of Florendo *et al.*, (2012) for feedstocks using carabao rumen fluid hydrolysis and Sindhu *et.al.* (2016), that indicates that feedstock composition was a factor important in the carbohydrates conversion efficiency of white rot fungi as biological pretreatment. Data on hydrolysis efficiency due to the bacterial seed culture shows decreased efficiency in the order of MC1>MC2>MC3. Statistically, conversion of carbohydrates efficiency due to the bacterial cultures was insignificant ($p<0.05$). Insignificant difference in the carbohydrates conversion efficiency was not due to bacterial composition but may be indirectly due to the effect of interaction among bacteria with different nature within the group.

Findings on the performances of frozen thawed rumen bacteria as mixture inoculants of the hydrolysis using sweet sorghum, sugarcane bagasse and rice straw as alternative feedstock intended for soluble sugar for bioethanol production were in agreement with Coen and Dehority (1970) that indicated performance of bacterial mixture utilization of carbohydrates was dependent on composition of carbohydrates either from intact forages or isolated hemicelluloses. Prior to those results, Dehority and Scott (1967) indicated that the extent of cellulose degradation *in vitro* by pure culture of rumen bacteria was attributed by composition of the biomass. Accordingly, the results implied the importance of carbohydrate fermentation ability of rumen bacteria as degraders, utilizers or both in the formulation of microbial inoculant for a specific feedstock. Our study showed that carbohydrates conversion efficiency by carabao rumen bacteria was due to individual nature of the bacterial species and interactions within a mixture. The nature of species included difference in morphology, fermentation substrates (Dehority, 1993) while low pH resistance, interactions among bacteria that may be complementation and possible antagonisms for substrate acquisition.

Ethanol and efficiency of yeast fermentation of sugars

Table 3 showed yeast efficiency in fermenting sugars from rice straw was higher than efficiency in fermented sugars from bagasses of sugar cane and sweet sorghum. Statistically, efficiency of the yeast was insignificantly effected by hydrolyzed sugars from different feedstock ($p<0.05$).

Table 3. Efficiency of and theoretical yield of ethanol from lignocelluloses

Yeast Performance	Sweet sorghum	Sugarcane bagasse	Rice straw
Efficiency,%	47.02(0.92)	47.79(0.28)	47.49(0.14)
Ethanol Yield,g/L	0.55(0.004)	0.57(0.059)	0.67(0.033)

Mean with different letter indicate significance by 5% DMRT. Enclosed in parenthesis is standard deviation of the mean

Difference in ethanol yield was not due to the feedstock fermentable sugars alone but may be due to acidic fermentation end products of hydrolysates. According to Florendo *et al*. (2017), hydrolysates from switchgrass contain reducing sugars, acetate, butyrates and isobutyrates.The hydrolysis of switchgrass using cattle rumen microbes also produced methane gas with the passing of time. In sweet sorghum bagasse hydrolysis by carabao rumen microbes, hydrolysates sugars contain lactic acids and acetic acids (Geminiano *et al.* (2016).

Our findings showed that theorethically, ethanol was obtained from carbohydrates hydrolyzed by rumen bacteria after cryopreservation. Previous studies on alcohol content of hydrolyzed carbohydrates using rumen fluid hydrolysis and fermented by *Saccharomyces cerevisiea* and distillate analyzed by gas chromatography and mass spectrometry (GC/MS assay) showed that rice straw (Baltazar *et.al.* 2008) and corn stover (Concepcion *et.al.* 2010) distillates contain variety of alcohol. These characteristics of distillate from rumen fluid hydrolyzed carbohydrates implied that different alcohols can be derived from the lignocelluloses of alternative feedstock.

Based on our research findings, viability and consideration for low input would be our criteria in choosing 60% glycerol as cyoprotectant for future cryopreservation of carabao rumen bacteria. The nature of rumen bacteria as efficient cellulolytic microbes in the pretreatment was unaffected by cryopreservations at low temperature or vapour phase of nitrogen. Performance of the frozen and thawed bacteria was influenced by duration of hydrolysis and the composition of feedstock, which confirmed previous study on efficiency of the hydrolysis using carabao rumen fluid microbes composed of bacteria, protozoa and fungi (Florendo *et al.*, 2012). Bacterial mixture (MCI) as inoculants showed variability in efficiency in converting carbohydrates into soluble sugars. Although results did not result to significant effect, interactions of species within mixture of rumen bacteria (MCI) could explain variations. This included but not limited to difference in morphology and physiochemical

characteristics of individual species, difference in fermentation substrates, and low pH sensitivity of frozen and thawed bacterial specie.

Frozen and thawed rumen bacteria with average viability of 17.29% after 54 days of storage in the vapor phase of liquid nitrogen had produced active population by sub-cultivation in seed cultures liquid medium. To our knowledge, this is the first report on cryopreservation of carabao rumen bacteria at -20^{0}C freezer and vapor phase of liquid nitrogen (-156^{0}C) and to optimize rumen bacteria uses as microbial inoculant in the pretreatment intended for biofuel production, the recommendation is to introduce improvements in factors associated with viability for long term cryopreservation of rumen bacteria.

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