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Cattle Rumen Microorganisms Hydrolysis for Switchgrass Saccharification, Volatile Fatty Acids and Methane Production

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Absrtact This study had determined the effect of duration on processes of saccharification, volatile fatty acids and methane gas in switchgrass hydrolysis using cattle rumen microorganisms. Inputs to fermentation were 2g milled switchgrass, 10ml strained Holstein cattle rumen fluid and 88ml distilled water with headspace using a 150 ml capacity serum bottle. Hydrolysis pH was neutralized with alkaline solution; sealed fermentation bottles with butyl cap and aluminium crimp and incubated at 37^{o}C with periodic shaking of 75 revolutions per minute. Fermentation was stopped at duration 6, 14, 38, 44, 68 and 92 hours. Contents of saccharides in hydrolysates were evaluated using High pressure liquid chromatography and Dinitrosalycilic acid assay while volatile fatty acids and methane gas produced at different durations were estimated by Gas chromatography. Results showed duration's significant effect on saccharification hydrolyzed carbohydrates, highest rate of 3.97 %/hr at 6 hr and highest carbohydrates conversion efficiency of 71.2% at 92 hr($p>0.05$). Acetate, iso-butyrate and butyrate were gradually produced at different periods while volatile fatty acids total content of 14.35mM obtained at 92 hr implied duration's significant impact on volatile fatty acid production in switchgrass using cattle rumen microbes($p>0.05$). Methane gas showed significant and highest yield of 16.38mM/ ml at 44 hr and lowest yield at 92 hr duration($p>0.05$). In conclusion, switchgrass hydrolysis using cattle rumen microorganisms' fermentation processes produce hydrolyzed carbohydrates, acetate, isobutyrate and butyrate, total volatile fatty acids and methane gas. Duration of the pretreatment of switchgrass with rumen microbes is a vital factor that affects the processes of saccharification, volatile fatty acids and methane production. Information generated in the study are essential in the development of rumen microorganisms as pretreatment of switchgrass as animal feeds, biomass for bioethanol, volatile fatty acids and methane gas production.

Keywords: livestock, fermentation, grass, secondary feedstock, biofuel, feeds

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Introduction

Switchgrass (*Panicum virgatum L*), a wild species of temperate grass on North Carolina has 3 types of germplasms. Result of the varietal study showed variety ST6 - 3F with high digestibility index and dry matter yield that makes it suitable as cattle feed while varieties of ST6 - 3E and ST6-I high content of dry matter were potential biofuel feedstocks (Burns *et al,* 2008a;2008b; Akuzawa *et al.,* 2001; Bals *et al.,* 2010). Yang *et al*., in 2009 reported that switchgrass varieties had dry matter with almost 50% carbohydrates in the form of glucan, xylan and arabinan, soluble extractives that represented the waxes, fats, resins, gums, starches, essential oils ranged from 4.87% to 6.40% of dry matter and ash content that ranged from 2.98% to 3.88%. Other components that could be organic compounds such as uronic acids, acetyl groups and other minerals represented 22.10% to 25.48% of the dry matter of switchgrass. Lignin content of the three varieties ranged from 17.74% to 19.23% and its acid soluble lignin portion ranged from 3.30% to 4.04% on dry matter basis. The high content of lignin in switchgrass led to the presumption that switchgrass needs pretreatment in order to enhance degradation. Since the grass survives in non-arable lands, feedstock requires less production inputs and with the available germplasms, switchgrass has high potential as alternative feedstock in the production of cellulosic ethanol. Further, cellulosic ethanol as gasoline blends was reported with low carbon emissions than other resources like corn grain ethanol and gasoline (CERES, 2008).

Because switchgrass is lignocellulosic, it requires pretreatment like chemical, physical or combinations of chemical and physical processes. Several pretreatments have been examined previously for their effect (Bals *et al*., 2010; Jin *et al*., 2010; Garlock *et al.*, 2012; Serate *et al*., 2015) on chemical composition, enzymatic hydrolysis and ethanol production. These pretreatment works to increase surface area of carbohydrates in the lignocelluloses prior to enzymatic hydrolysis usin cellulolytic enzymes, that breakdown β1-4 linkages between and within strands of celluloses and hemicelluloses, cleaving the carbohydrates from the lignocellulose complex into oligosaccharides slurry(Zhao *et al.,* 2012). This process of saccharification using pretreatments and cellulases was normally done in submerged fermentation to facilitate dissociation of hydrolyzed carbohydrates from the unhydrolyzed materials. Among the pretreatments that were used in the bioethanol plants were Iogen protocol that pretreated wheat straws with acids and hydrolyzed by cellulases. Other protocols of bioethanol production from crop residues including grasses reviewed by Mosier *et al.,* (2005) showed that the criteria for process upscaling were mostly dependent on optimum pretreatments, enzyme hydrolysis and

yeast fermentation conditions(Chen *et al.,* 2007b). Among the process stages, pretreatment was the most expensive at cost of 30cents per gallon of ethanol from cellulosic biomass. Hence, one of the goals of new development in pretreatment was to reduce cost of production from improvement in efficiency and output.

The development of rumen as alternative pretreatment of biomass started with physical treatment like chopping and shredding to increase surface areas for rumen microbes attachment and use of directly produced microbial hydrolytic enzymes synthesized from sugars of cellulose and hemicelluloses of the lignocelluloses complex. By means of shear and tear during fermentation, agitation process stimulate the rumen microbes association and interactions and further degrade carbohydrates in breaking the β(1-4) linkages, resulting energy from glucans enhance microbial growth and colonization in undegraded carbohydrate substrates. The process of saccharification in the rumen hydrolysis is characterized by microbial particle reduction and enzyme solubilization by groups of rumen bacteria, fungi and protozoa (Jalaludin *et al*., 1992; Orpin (1983) and Stumm and Stewart, 1986; Hidayat *et al*., 1993). Compared to chemical and enzymatic pretreatment of lignocelluloses, the rumen microbes hydrolyze cellulose with combined physical, chemical and enzymes activities of rumen microbes. As the current rumen hydrolysis process efficiency is being improved, the biodegradable property of fermentation by-products and spent biomass potential as animal feeds and soil conditioner are additional advantages to its hydrolyzed sugars for cellulosic ethanol production(Florendo, 2008, personal communication).

There are commercial biogas processing plants with anaerobic fermentation and digestion processes that used large population of bacteria, protozoa and fungi species. These microbes were cultured for desirable product methane with another end product volatile fatty acids, that served as indicators of process inefficiency (Ahring, 2000; Akuzawa *et al.,* 2001; Shi *et al.,* 2009) and management of the anaerobic species in processing plant. Previous studies on rumen hydrolysis had concentrated on alcohol potential of lignocellulosic biomass rice straw, corn stovers, sweet sorghum and grasses. Rumen microbes produce volatile fatty acids and fermentation gasses in the rumen and in in vitro processes using rumen fluid. The absence of data on volatile fatty acids, gas production with the current saccharification process using different feedstocks make the hydrolysis pretreatment comparable to a black box. In-dept study of the different fermentation process could unlease the potential of the hydrolysis into an industrial process of pretreatment. Understanding the rumen fluid hydrolysis and the physical and chemical factors affecting carbohydrates

degradation and related fermentation activities by its resident microbes in hydrolysis are deemed empirical.

Objectives: This study had determined the effect of hydrolysis of switchgrass on the processes like saccharification, volatile fatty acids and methane production. Specifically, this study had determined the effect of duration 6, 14, 38, 44, 68 and 92 hours on the processes of hydrolysis using cattle rumen microorganisms and switchgrass as alternative feedstock.

Materials and methods

The hydrolysis was anaerobic process of pretreatment of switchgrass prior to yeast fermentation. Freshly collected rumen fluid from rumen cannulated Holstein cattle was used as source of microbial inoculants. The thermos bottle with cattle rumen fluid was immediately brought to the laboratory, filtered and strained with cheese cloth and incubated at $37^{o}C$ prior to inoculation.

Each of the 18 vials with 150 ml capacity were loaded with 2g milled switchgrass, 88 ml of deionized water and pre-warmed 10 ml of fresh strained cattle rumen fluid. The pH of the mixture was adjusted to pH 6.9 using a solution of 2 N Sodium Hydroxide. Fermentation bottles were sealed tight with butyl rubber cap and aluminum crimp, returned in water bath at $37^{0}C$ and agitated at 75 rpm. Treatment duration namely; 6, 14, 38, 44, 68 and 92 hours were replicated in triplicate bottles. After duration, sample bottles were stored at $4^{0}C$. Laboratory analysis of methane gas and volatile fatty acids were done at Department of Animal Science and Biotechnology. Sugar composition of the hydrolysate was analyzed at the Bioprocessing Laboratory of the Department of Biological and Agricultural Engineering of North Carolina State University at Raliegh.

Analysis of methane and volatile fatty acids

Aluminum cap of the fermentation bottle was opened to expose the rubber butyl cap. A 10 µL gas-tight syringe (Hamilton Co., Reno, NV) was probed into the rubber butyl cap of fermentation bottle up to the middle of the headspace. After aspirated, 10 ul gas was injected in the sample port of Gas chromatography unit (Model CP 3800; Varian, Walnut Creek, CA). It was equipped with a NUKOL Fused Silica Capillary Column (30m x 0.25m x 2.5 m film thickness) and a flame ionization detector (FID) and operated at oven temperature of $270^{0}C$ with detector temperature at $300^{0}C$. The flow rate of

Helium was set at 26ml/min and 80 psi while Hydrogen was provided at a flow rate of 30ml/min and 40 psi and air was set at 300 ml/min at 60 psi.

After methane gas was evaluated from the fermentation bottle, 2 mL hydrolysate was centrifuged at 15,000 rpm for 15 min at 4^0C refrigerated centrifuge. One ml supernatant was added to 200 µl of a meta-phosphoric internal standard mix (MIS). MIS was prepared by dissolving 25g of meta-phosphoric acid in 50 ml of deionized water and adding 0.218 ml of 2-ethylbutyric acid, the internal standard, and bringing to 100 mL using deionized water. A volatile fatty acids standard was prepared by mixing 1 ml of VFA standard that included acetate, propionate and butyrate with 200 ul of MIS. A standard curve was prepared using the VFA standards and analyzed on GC to quantitate unknown samples. Volatile fatty acids are reported in millimolar (mM) concentrations.

Analysis of Hydrolyzed Sugars

Hydrolyzed sugars were determined by 3, 5 Dinitrosalycilic acid (DNS) reducing sugar assay. DNS reagent was prepared by dissolving 10g of 3,5 Dinitrosalicylic acid in 400 ml of deionized water. Heat the solution at 50^0C and added 20.75 ml of 19.3N (50%w/w) Sodium hydroxide and 300g of Sodium Potassium Tartrate Tetrahydrate (Rochelle salt). Mix the solution thoroughly and diluted to 1 liter with deionized water. The DNS assay was stored in dark amber bottle and stored at room temperature. Citrate Buffer solution was prepared by dissolving 210 g of Sodium citrate monohydrates in 750 ml of deionized water. Deionized water was used to dilute the buffer solution to one liter. The solution pH was adjusted to 4.8 with acetic acid solution. A 0.5M solution of citrate buffer was prepared by mixing 1:19 ratio of deionized water with the citrate buffer stock solution.

A glucose standard was prepared by dissolving o.4g glucose(Sigma) in 100 ml of distilled water A glucose standard curve was developed with corresponding absorbance values of the glucose concentrations of 0, 0.2, 0.4, 0.8, 1.2 and 1.6 g glucose/L. For the sugar analysis, 15 ml of the rumen fluid hydrolysate was centrifuged at 4000 rpm for 10 minutes at 4^0C. A 0.5 ml sample was prepared by diluting 0.01 ml supernatant with 0.49 ml of deionized water in a 15 ml test tube. Prior to assay, glucose standards and 0.5 ml sample were vortexed and added reagents in the following order; 1 ml of sodium acetate buffer and 3 ml of DNS reagent. Sample tubes were boiled in a beaker of water for 5 min, cooled at 4^0C to intensify color. Absorbance reading at 540 wavelengths was done using a UV-vis spectrophotometer (Shimadzu). The amount of reducing sugars in sample was calculated using the equation;

$$\text{Reducing sugar} = \frac{\text{conc.of sugar(g/L) x Volume x Dilution x 100}}{\text{Sample x \% TS}}$$

Where: concentration of sugar(g/L) was obtained from the result of glucose standard equation Y = 0.9525(x) and x represented absorbance of sample for analysis

Volume of hydrolysis = 100 ml

Dilution Factor = Sample for analysis/ aliquot of hydrolysate (0.5/0.01)

The analysis of saccharides, 2 ml hydrolysate was centrifuged at 4000 rpm for 10 min at 4ºC using refrigerated centrifuge. One ml of supernate was filtered with a 0.25 μm Millipore® syringe filter into a HPLC vial. A sugar calibration standard with a concentration of 4 g/L was prepared by dissolving 1g cellobiose in HPLC ethanol water. The ethanol HPLC water was prepared by mixing 20.28 ml of Ethanol (200 proof, specific density of 0.789 g/mL) in one liter of HPLC grade water. HPLC water ethanol mixture with concentration of 16g ethanol/L was used in the preparation of standards with 0, 1, 2, 2.5 and 4g/L prior to the analysis. All sample vials and standards were kept at 4^0C until analysis. The HPLC analysis for sugar monomers procedure of Sluiter *et al.* (2006) was followed using a High pressure liquid chromatograpy ((HPLC). The HPLC was equipped with a refractive index detector (Shimadzu RID-10A) and a Biorad Aminex HPX-87H column maintained at 65°C with a corresponding guard column. The mobile phase used was 5 mM H_2SO_4 pumped at a flow rate of 0.6 ml per minute. Cellobiose in the hydrolysate was calculated Using the equation;

$$\text{Saccharides} = \frac{\text{conc of saccharides(g/L) x volume x DF}}{\text{Sample wt x Total Solids(\%)}} \times 100$$

Where: concentration of saccharides (g/L) that was obtained from HPLC results of standards analysis, Volume of the hydrolysis and DF means dilution used in the assay.

Data analysis and Statistics

Data were analyzed using ANOVA in Complete Randomized Design (CRD) using SAS statistical software version 9 while means comparison at 5% level of significance was conducted using Duncan Multiple Range Test for

Carbohydrates while LSD was used for the mean comparison of methane and acetate, butyrate, Iso-butyrate and total volatile fatty acids.

Results and Discussion

Saccharification

Switchgrass hydrolysis with cattle rumen fluid microorganisms had saccharifications at different duration. Lowest amount of hydrolyzed carbohydrates was calculated at 6 hours duration of hydrolysis while duration of 92 hr produced the highest sugars (Table 1). Statistically, duration of switchgrass hydrolysis using cattle rumen microorganisms had significantly effected hydrolysis saccharification. Carbohydrates hydrolyzed by rumen microbes had significant diference at 6 hr, 14 hr, 38 hr and 92 hours duration of the pretreatment.

Table1. Hydrolyzed carbohydrates and conversion rate in switchgrass hydrolysis using cattle rumen fluid microorganisms

Duration of Hydrolysis	**Hydrolyzed carbohydrates g/L**	**Conversion Rate Hr $^{-1}$**
6	2.92 *d*	3.97 *a*
14	4.81 *c*	2.98 *a*
38	5.63 *bc*	1.31 *b*
44	6.93 *ab*	1.39 *b*
68	6.71 *ab*	0.91 *b*
92	8.10 *a*	0.77 *b*

Difference in letter (a-c) means significant at 5% DMRT.

The process carbohydrates conversion into soluble sugars in terms of rumen microorganisms' rate of hydrolysis had decreased with time. Hydrolysis efficiency rate/hr showed its highest conversion rate of carbohydrate conversion with 3.97% at the 6 hr duration of hydrolysis of switchgrass. The lowest rate of conversion of carbohydrates into soluble sugars being 0.77% was obtained at 92hr duration of hydrolysis. Statistical analysis showed that duration had significantly effected rate of carbohydrate conversion by rumen microorganisms in the process hydrolysis of switchgrass ($P<0.05$). Durations of 6 to 14 hours had insignificant difference and higher than conversion rates obtained at 38 hr duration. Hydrolysis with 38 hr, 44hr, 68 hr and 92 hrs had insignificant difference in rate of conversion of carbohydrates into soluble sugars. Data showed that rate of carbohydrates conversion into soluble sugars in the switchgrass hydrolysis decreased with prolonged duration of hdrolysis

using the rumen microbes as inoculant. Difference maybe associated with fiber composition and digestibility index of switchgrass varieties (Burns *et al.* 2008a; 2008b, and Chen *et al.*, 2007a).

Switchgrass carbohydrates conversion into soluble sugar efficiency shown in Figure 1 revealed efficiency that increased with duration of the hydrolysis. At duration of 6 hours, process efficiency was 23.9%, gradually had significantly increased with 92 hours efficiency of 71.2%. Data revealed degradations cellulose and hemicelluloses from switchgrass by rumen microbes had optimum efficiency at the longest duration of hydrolysis. The significant increase in switchgras hydrolysis process efficiency using the rumen microorganisms was in agreement with Grant and Mertens, (1992) research that rumen fermentation of lignocelluloses requires long duration exposure to the resident microbes for attachment, colonization and microbial activity.

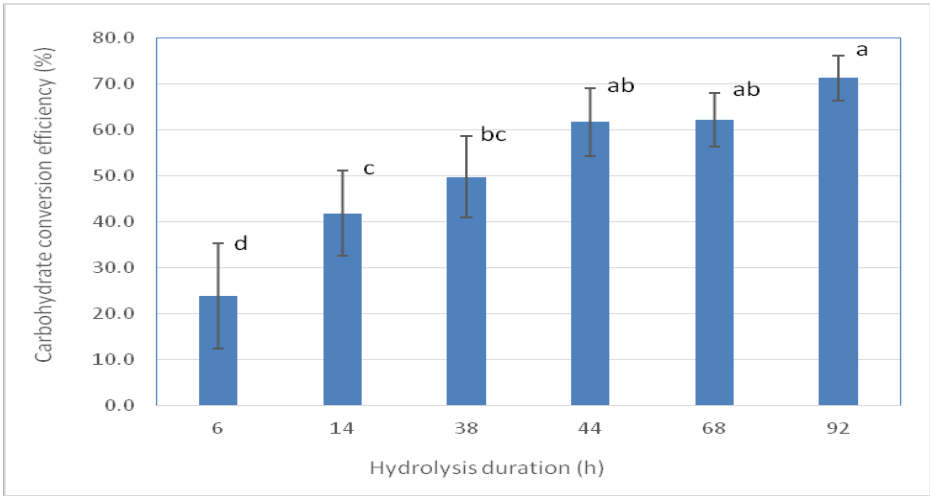

Figure 1. Carbohydrate conversion into soluble sugars efficiency by cattle rumen fluid microorganisms in switchgrass hydrolysis

Volatile fatty acids production

Table 2 showed that hydrolysis product of acetate had increased with duration of hydrolysis. Acetate was the fermentation product of hexoses derived from celluloses of switchgrass was lowest at 6 hr and and the highest acetate was obtained at 92 hrs. Duration of hydrolysis had significantly effected acetate concentrations ($p > 0.05$)

Table 2. Volatile fatty acids (mM/ml) and methane (mM/ml) in switchgrass hydrolysis using cattle rumen fluid microorganisms

Duration of Hydrolysis	Acetate	Iso-butyrate	Butyrate
6	6.42 cd	0.19 a	0.31 b
14	6.37 d	0.19 a	0.00 b
38	7.42 b	0.20 a	0.11 b
44	7.14 b	0.13 a	0.56 b
68	7.02 bcd	0.19 a	3.57 a
92	9.20 a	0.11 a	4.76 a

Difference in letter (a-c) means significant at 5% DMRT.

Data showed that acetate had significant difference between durations 6, 38, and 92 hour duration of switchgrass process hydrolysis using cattle rumen microbes. Data revealed a gradual and slow production of acetate from hydrolyzed carbohydrates over time. Iso-butyrate concentrations were low and the production results into insignificant amounts. Butyrate concentration had small and slow build up in the hydrolysis. The high amount of butyrate at 68 hours to 92 hours in the switchgrass pretreatment implied that the presence of rumen microbial interconversion metabolisms, wherein acetate can be converted to butyrates (Van Soest, 1983). Result of the process showed that acetate and butyrates accumulated at longer duration of 68 to 92 hours the hydrolysis (Figure 3).

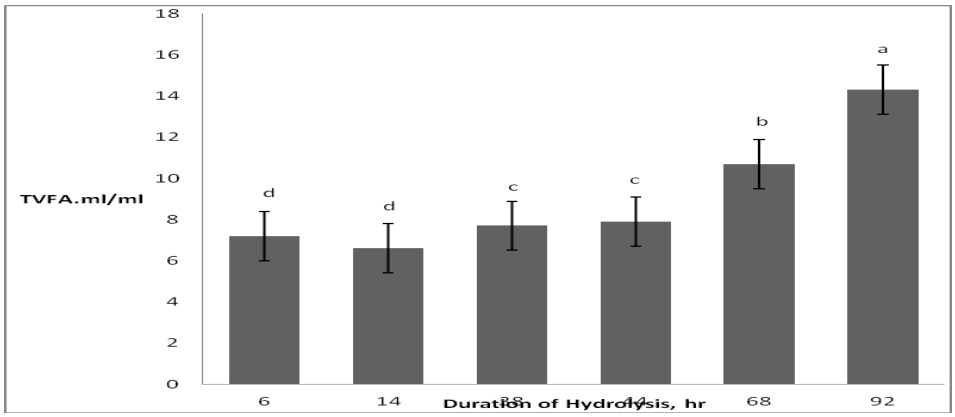

Figure 2. Total volatile fatty acid at different duration of switchgrass hydrolysis using cattle rumen microorganisms

Methane Gas Production

Figure 3 showed the methane production with durations of switchgrass hydrolysis using the cattle rumen microorganisms. Methane gas had increased to 16.38 mM per ml at 38hr and had stabilized until 68 hr duration of the switchgrass hydrolysis using rumen microbes as methane producers. However, methane gas had reduced concentrations of 13.44 mM at 92 hr duration of switchgrass hydrolysis. Statistically, duration had significantly effected methane production in the switchgrass hydrolysis using rumen microbes (P>0.05). Volume of methane gas was comparable between 38 hr to 68 hr while lowest at 92 hours durations of the hydrolysis.

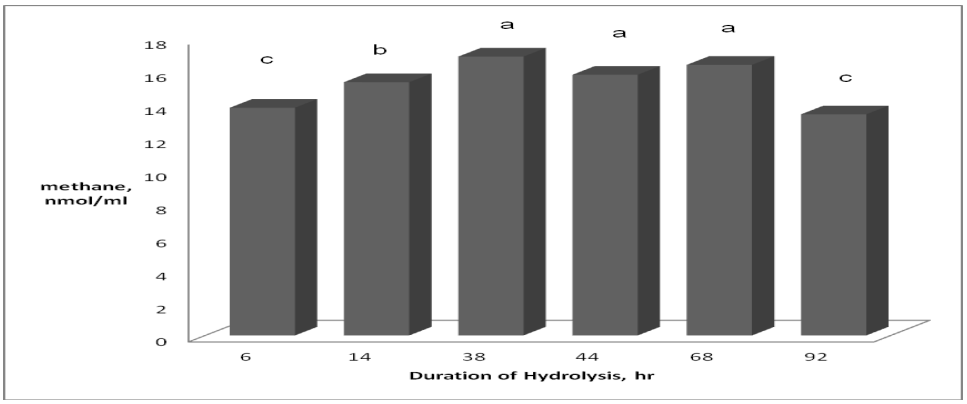

Figure 3. Methane production at different durations of switchgrass hydrolysis using cattle rumen microorganisms

Conclusion

Switchgrass as alternative feedstock for bioethanol production was pretreated in hydrolysis using cattle rumen microorganisms showed that duration of pretreatment had significantly influenced saccharification. Saccharified celluloce and hemicelluloses increased with duration of the process. Data on the significant production of hydrolyzed carbohydrates and the decreasing rate of carbohydrates conversion implied the changes in microbial composition and functional metabolisms. High amount of acetate, and butyrates over iso-butyrate at 92 hours implied that prolong duration was essential for total volatile faty acids production from the secondary fermentation process of switchgrass.

Findings on the methane gas production between 6 hr and 92 hours showed that the precursor substrates carbon dioxide and hydrogen from saccharification had decreased in switchgrass hydrolysis. The result gave the

implication that process of saccahrification over time could reduce the methane production in switchgrass hydrolysis. The study showed antagonism between methane and volatile fatty acids production at the later period, indicating hydrolysis changes in microbial rumen control of the different processes in the pretreatment of switchgrass. Fermentation by pool of anaerobic microbes in acidic anaerobic digestion or biogas production indicated the negative effect of volatile fatty acids on methane production (Williams, 2000; Wilkie, 2008). Likewise, researchers had used volatile fatty acids as by-products of biogas processing plant as parameter of inefficiency (Ahring, 2008; Akuzawa *et al*, 2001; Lana *et al.*, 1998; Menke and Steingass, 1988; Goux *et al*., 2015; Vanwonterghen *et al*. 2015). In the present study, switchgrass hydrolysis using cattle rumen microorganisms' saccharification and methane production was controlled by volatile fatty acids effect. According to rumen microorganisms' researchers, responses of rumen vary in acidic environment (Russell, 1991; Franke Whittle *et al.,* 2014).

Kirchgessner *et al.,* 1994 indicated nutrient composition of fermenting biomass as significant impact on methane gas production. In the present result of study, methane production was directly dependent switchgrass cellulose and hemicelluloses degradation for carbon dioxide (Van Soest, 1983) and the presence of interspecies hydrogen transfer as essential substrates requirements for methane and energy ATP for growth of species (Lee *et al*., 2000; Hom *et al*., 2003).

Conclusively, switchgrass hydrolysis using cattle rumen microorganisms as alternative feedstock and pretreatment process has different products from different microbial processes. Saccharification and duration effect indicated that short duration hydrolysis is important for optimum saccharification without loss of sugars to volatile fatty acids, methane production is favorable at long duration and is vital for processes intended for saccharification. Time requirement is vital in order to mitigate rumen microorganisms' control of fermentation of lignocelluloses in switchgrass.

The findings of this study are important in the development of rumen microorganisms as biological pretreatment process for switchgrass as animal feeds, biomass for bioehanol production, volatile fatty acid and methane production.

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