In vitro gas production of fermented cocoa pod (*Theobroma cacao*) added with cellulolytic inoculum from cattle rumen fluid

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ABSTRACT: This research was conducted to investigate the effect of level of cellulolytic inoculum from cattle rumen fluid in cocoa pod (*Theobroma cacao*) fermentation on *in vitro* gas production. The first stage of this experiment was getting cellulolytic microbes from rumen fluid, followed by producing microbial biomass as inoculum. In the second stage, cocoa pod as much as 300 g were fermented by addition of cellulolytic inoculum. The level of inoculum were 0%, 5% and 10% (dry matter basis). Each treatment was consisted of three replicates. Fermentation were done anaerobically, at room temperature for 21 days. The variables being measured were pH value, glucose and lactic acid content, chemical composition including dry matter (DM), organic matter (OM), crude fiber (CF), crude protein (CP), ether extract (EE), and nitrogen free extract (NFE) being produced, as well as *in vitro* gas production. The data obtained were analyzed using variance analysis of one way design. The differences between mean values were analyzed using *Duncan's new multiple range test* (DMRT). The result showed that addition of 10% cellulolytic inoculum didn't affect glucose and lactic acid content, but pH value of fermented cocoa pod with cellulolytic inoculum addition was lower than control. In general, there was no effect of cellulolytic inoculum addition on chemical composition and *in vitro* gas production were observed, except for CF and NFE. The addition of cellulolytic inoculum 5% and 10% decreased CF content by 3.91% and 5.74%, and increased NFE content by 5.24% and 10.52%, respectively compared to control. It can be concluded that cellulolytic inoculum improves chemical quality of cocoa pod by decreasing CF and increasing NFE, however it doesn't affect to *in vitro* gas production.

Key words: cocoa pod, cellulolytic inoculum, *in vitro* gas production

INTRODUCTION

Cocoa pods, a by-product of cocoa production, are utilized as a feed resource for ruminants in regions where fresh pasture is limited and seasonal well as removing cocoa-pods from the field eliminating a potential reservoir for the cocoa-pod borer (an insect pest which reduces subsequent cocoa yield and quality). Utilization of cocoa pods in the animal diet needs to be considered due to its nutritive quality i.e. high crude fiber content. It was reported that cocoa pods containts 240 to 360 g CF/kg DM (Wong *et al*., 1987 cit. Quigley *et al*, 2009) CP content of 75 g/kg DM and a NDF content of 590 g/kg DM (Quigley *et al*, 2009). Because harvesting cocoa pods is seasonal and its water and fiber containing are high, it could be ensiled to provide a continuous supply of feed for ruminants since ensiling preserves nutritive components by decreasing the pH through homofermentation of the major water soluble carbohydrates (WSC) to lactate. Cocoa pods fermentation is determined by its chemical composition and ensiling additives such as bacterial inoculum can accelerate fermentation. Mechanisms of applying inoculum to silage differ (Sun *et al*, 2009). Microbial inoculum applied to fourages at ensiling promotes homofermentation of major WSC to lactate, thereby causing a rapid pH decline (Zahiroddini *et al*., 2004). The low pH conserves WSC and decreases proteolysis and deamination by inhibiting fermentation (Muck and Pitt, 1993 *cit.* Sun *et al*, 2009). Treatment of silage with fibrolytic enzymes has been proposed as a means of directly enhancing fiber degradation to increase availability of WSC as a substrate for LAB (Weinberg *et al*., 1995).

Although the positive effects of inoculum on the preservation and nutrititive value of silage such as higher lactate:acetate ratios, lower ammonia N, decreased DM losses (Henderson, 1993), increased digestibility, improved aerobic stability and enhanced growth performance (McAllister *et al*., 1995)

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have been reported. However, utilization of cellulolytic inoculum from cattle rumen fluid in cocoa pod fermentation has been reported far less frequently than other inoculum. However, the potential value of inoculum for waste product of agriculture silage has not been extensively explored, likely due to the fact that waste product of agriculture silages tend to have naturally high CF concentrations. The present experiment was undertaken to examine the effect of inoculum from cattle rumen fluid on the cocoa pods fermentation by measuring characteristics of the fermentation and *in vitro* gas production.

MATERIALS AND METHODS

Microoganism

The microorganism used in this study, cellulolytic microbes was obtained from rumen fluid of fistulated Ongole Crossbred cattle. The rumen fluid was kept in waterbath at 39°C. Anaerob condition was maintained by flowing CO_2 into the container. A quantity of rumen fluid samples was taken out and subjected into cellulase assay (*carboxymethyl cellulase*).

Culture Conditions

Following heat sterilization (121°C for 30 min), the enrichment medium was inoculated with 10% of rumen fluid. The composition of medium were 0.2 g $(NH_4)_2SO_4$; 0.1 g $MgSO_4.7H_2O$; 0.2 g K_2HPO_4; 0.4 g $CaCO_3$; 2 ml NaCl 1 %; 1 g Sistein HCl; 2 drops of resazurin solution (0.1%); 120 ml dH_2O and 80 ml of rumen fluid. Cellulose as much as 2 g were added into medium solution as substrate for cellulolytic microbes (Omelianski, 1902 *cit.* Skinner, 1971). Microbes were grown at 39°C, pH 7 for 7 days under liquid culture condition. The primary enzymes in this product was cellulase.

The inoculum grown in the enrichment media was taken out, and it was added into the glass fermenter filled with growing medium as much as 10% from the total medium. The medium consisted of 0,2 g $(NH_4)_2SO_4$; 0,02 g $MgSO_4.7H_2O$; 0,4 mg NaCl; 1,8339 g, $K_2HPO_4.3H_2O$; 0,2 g *yeast extract*; 1 g cystein HCl; 2 drop of resazurin solution (0.1%) as an indicator, 80 ml steril rumen fluid and 120 ml dH_2O, and 4 g cellulose (Omelianski (1902) *cit.* by Skinner (1971). The fermenters was kept at 39°C for 7 days on anaerobic condition**.** The inoculum was then inoculated for fermentation of cocoa pod, and a part of them was subjected for cellulase assay.

Fermentation of Cocoa Pod

After growing for 7 h, the inoculum was mixed to 300 g of cocoa pod, and incubated aerobically at room temperature for 21 days. The inoculum was added to achieve final concentrations of 0, 5 or 10% based on DM of cocoa pod. At the end of the incubation period, pH, sugar and lactic acid content were determined. Then, sample was collected, dried at 55°C for 72 h, ground through a 1-mm screen Wiley mill and analyzed for chemical composition as well as for *in vitro* gas production.

Enzyme Assay

The cellulase activity was determined according to Halliwel *et al.* (1985) using 1% carboxymethylcellulose (CMC) as a substrate. In the first step, the inoculum was centrifuged at 10,000 g for 15 min to separate microbes from crude enzyme. The cell-free supernatant was analyzed for cellullase activity. One milliliter of resulting supernatant (crude enzyme) was incubated with 1 ml of 1% CMC in 0.1 M sodium acetate buffer (pH 5.5) at 38°C for 45 min. The reducing sugar thus released was estimated by ferricyanide reaction. The optical density was calibrated with glucose solutions of known concentration. Appropriate blanks were used. The activity was expressed in units (U), defined as the amount of enzyme required to produce 1 μmol glucose per min. The protein concentration was measured by the method of Lowry (Plummer, 1978). Bovine serum albumin was used as a standard.

Measurement of Fermentation Parameters

pH of Fermentation. pH of fermented cocoa pod was immediately recorded using a pH meter.

Sugar Content. The samples (fermented cocoa pod) as much as 1 g were added with dH_2O, filtered through filter paper to produce 10 ml crude supernatant, and then homogenized with centrifuge at 3,000 g for 15 min. Supernatant produced was added with dH_2O up to 10 ml and assayed for reducing sugar, which was analyzed by the Nelson-Somogyi method (Plummer, 1978).

Lactic Acid Content. Two g of fermented cocoa pod were deproteinized using TCA 10%, filtered through filter paper to produce 10 ml crude supernatant, and then homogenized with centrifuge at 3,000 g for 15 min. The resultimg supernatant was added with TCA 10% up to 10 ml and assayed for lactic acid content according to Baker and Summerson method (Hawk *et al*, 1976).

***Chemical* Composition**. The samples were analyzed for chemical composition including dry matter (DM), organic matter (OM), crude fiber (CF), crude protein (CP), ether extract (EE), and nitrogen free extract (NFE) according to AOAC procedure (2005). These analysis were carried out for original and fermented sample of cocoa pod to determine the effect of fermentation on chemical composition and *in vitro* gas production.

***In vitro* Gas Production**. Short-term *in vitro* incubations were carried out with rumen fluid from fistulated Ongole Crossbred. The rumen fluid was withdrawn before the morning feeding and was squeezed through four layers of surgical gauze into an Erlenmeyer flask under continuous flushing with CO_2, and efforts were made to maintain the temperature at 38 to 39°C. The fluid was then mixed with a bicarbonic buffer for *in vitro* gas production pH 6.9 in a ratio of 1:2 (v/v) as described by Menke and Steingass (1988). The substrate (fermented cocoa pod) was milled to pass through 1mm sieve and 300 mg was weighed in 100-ml glass syringes. After mixing, 30 ml of diluted rumen fluid was transferred to glass syringe containing 300 mg of each substrate. Each glass syringe was sealed with syringe cap and was incubated in a water bath at 39°C for 72 h. The total of gas production and degraded fractions were calculated using *Neway Excel* according to Chen (1994).

Experimental Design and Statistical Analysis

Treatments were arranged in a one way design, with the main factors being levels of cellulolytic inoculum (containing 0, 5 or 10% DM basis). Fermentation experiments were separately conducted for each treatment with three replicates. Cocoa pod was utilized as substrate for solid state fermentation.

The data in the main study were analyzed as a one way arrangement. The differences of mean value were analyzed by Duncan's new multiple range test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Chemical Composition of Cocoa Pod Fermentation

The addition of cellulolytic inoculum to cocoa pods fermentation decreased CF ($P<0.01$) and increased NFE ($P<0.05$), but it didn't affect DM, OM, CP and EE, as presented in Table 1. The CF content with addition of 5-15% inoculum decreased 3.91%, and 5.74% compared with control (without inoculum). It decreased due to cellulolytic activity of inoculum during fermentation. Under liquid culture conditions, the cellulolytic inoculum produced cellulase activity 0.25 U/mg in growth medium and 1.09 U/mg in enrichment medium. Fiber was preferentially degraded during fermentation. The NFE content increased 4.98% and 9.52% compared to control (without inoculum).

Cellwall degrading enzymes, such as cellulases and hemicellulases, applied to herbage before ensiling decreased the cell wall content of ensiled crops (Selmer-Olsen *et al*., 1993). Lignin was undegradable component of plant cell wall by cellulase. Nadeau *et al* (2000) repoted that cellulase treatment decreased NDF concentration by 18% in orchardgrass and by 15% in alfalfa, with no additional effects by inoculant or formic acid, compared with the controls. Averaged across plant species, cellulase degraded 25% of the cellulose and 13% of the hemicellulose. Enzymatic hydrolysis

of cellulose was 65% greater in orchardgrass than in alfalfa silage (31 vs 19% hydrolysis). Less cell-wall degradation in alfalfa is likely related to the greater lignin concentration and lower initial NDF concentration in alfalfa than in orchardgrass. Moreover, there are many cross-links between lignin polymers and polysaccharides in the plant cell wall via phenolic acids, predominantly ferulic and p-coumaric acids, which provide cell wall integrity and resist enzymatic attack by microorganisms (Trzciñska *et al*., 2005).

Table 1. Effects of addition of cellulolytic inoculum from cattle rumen fluid on chemical composition (% DM basis) of cocoa pod fermentation

Level of cellulolytic inoculum, %	Chemical composition					
	DM[ns]	OM[ns]	CP[ns]	EE[ns]	CF	NFE
0	15.80	92.42	8.65	6.03	33.99[a]	43.71[p]
5	15.68	92.65	7.96	6.11	32.66[b]	46.00[pq]
10	14.73	94.08	7.68	6.01	32.04[c]	48.31[q]

[abc] Means in the same column within CF with different superscripts differ very (P<0.01).
[pq] Means in the same column within NFE with different superscripts differ (P<0.05).
[ns] not significant

Nutrient Loss During Ensiling Fermentation

Losses of CP and CF during ensiling ranged from 5.54 to 7.47% DM basis and 21.97 to 27.74 % DM basis of initial content, while lossesd of NFE ranged from 17.51 to 27.74% DM basis, and increased when level of cellulolytic inoculum increased. This effect was dose dependant, with the higher level of cellulolytic inoculum, the higher losser of CP, CF and NFE. Less cell-wall degradation in cocoa pods is likely related to the greater lignin concentration.

Table 2. Nutrient loss (% DM basis) during ensiling fermentation of cocoa pods

Level of cellulolytic inoculum (%)	CP	EE[ns]	CF	NFE
0	5.54[a]	3.78	21.97[a]	17.51[p]
5	6.04[a]	3.60	22.61[a]	14.99[q]
10	7.47[b]	4.48	27.74[b]	27.74[p]

[ab] Means in the same column within CP or CF losses with different superscripts (P<0.01).
[pq] Means in the same column within NFE losses with different superscripts (P<0.05).
[ns] not significant

Sun *et al* (2009) found there were nutrient loss during ensiling fermentation including CP, aNDF, WSC and DM of whole maize stover. When the loss of each component was expressed as a proportion to its initial content prior to ensiling, WSC loss was 489 g/kg, while DM, aNDF, and CP losses were 107, 84 and 98 g/kg respectively. The losses of components of fermented cocoa pods were lower than those fermented maize stover as reported by Sun *et al* (2009).

pH, Glucose and Lactic Acid Content

Fermented cocoa pods had low pH (i.e., 5.28–5.66), good texture, odour and colour. The pH was decreased when the fermentation was added with cellulolytic inoculum 5 and 10%, as presented in Table 3. Cocoa pods, however, is not as easily ensiled as grass because of its greater buffering capacity, as indicated in this study by higher CP, and, consequently, higher pH. Addition of cellulase to cocoa pods fermentation was necessary to stimulate lactic acid production and to decrease pH. Eventhough in this study, addition of cellulolytic inoculum to cocoa pods fermentation didn't affect glucose and lactic acid contents, but it could decreased pH of fermentation. It was reported by Nadeau *et al* (2000) silage fermentation depleted reducing sugars in control alfalfa silage and left only trace amounts in control orchardgrass silage, because alfalfa had greater lignin concentration and lower initial NDF concentration than in orchardgrass. Enzymatic hydrolysis of NDF to soluble sugars supplied as much sugar as was fermented during ensiling of orchardgrass.

In order to further understand the influences of each component to silage quality, correlations between pH in silage and losses CP, CF and NFE among the treatments. Results showed a negative correlation between pH and CP, CF and NFE loss. Thus it was CF content in the plant material, and its loss during fermentation, which drove acid production and decreased pH of fermented cocoa pods.

Cellulolytic inoculum produce cellulase, that can hidrolyzed cellulose to oligomer and become its monomers (Nelson and Cox, 2000), WSC is generally fermented to lactic acid and other products (Merry *et al*, 2006). Microbial inoculum applied to fourages at ensiling promotes homofermentation of major WSC to lactate, thereby causing a rapid pH decline (Zahiroddini *et al*., 2004).

Table 3. Effects of cellulolytic inoculum from cattle rumen fluid on pH, glucose and lactic acid content of fermented cocoa pods

	Level of cellulolytic inoculum (%)		
Parameters	0	5	10
Glucose (mg/ml)[ns]	0.04	0.02	0.02
Lactic acid (%) [ns]	0.96	0.97	0.99
pH	5.66[p]	5.42[q]	5.28[q]

[pq] Means in the same row within pH with different superscripts differ significantly ($P<0.05$).
[ns] not significant

Colombatto *et al*. (2003) found that additions of enzymes reduced pH, NDF and ADF contents of maize silage. Including cell wall degrading enzymes in silage additives has been used to increase WSC available to LAB (Weinberg et al., 1995), presumably due to degradation of NDF. Sun *et al* (2009) reported that addition of cellulase reduced losses of a NDF and CP, while increasing loss of WSC, thereby indicating that cellulase accelerated WSC fermentation. It may be that addition of cellulase increased substrate for LAB from NDF degradation, and that propagation of LAB could be promoted in the early stage of ensiling, which resulted in a rapid increase in lactic acid, and a drop of pH, so inhibiting activities of the non-lactic acid bacteria and plant enzymes for proteolysis. The time required to reach an optimum pH could be shortened, leading to less degradation of fiber and protein in ensiled forage.

Effects on In Vitro Gas Production

Table 2 shows that fermentation using cellulolytic inoculum up to 10% has not affected the *in vitro* gas production of cocoa pods. Even though there were declining of its CF content, but it could not increased the *in vitro* gas production.

Table 4. Effects of cellulolytic inoculum from cattle rumen fluid on *in vitro* gas production of fermented cocoa pods

	Level of cellulolytic inoculum (%)		
Parameters	0	5	10
Cumulative gas production (ml/300 mg DM)[ns]	20.765	17.526	19.619
Fraction a (ml/300 mg DM) [ns]	0.403	-0.362	-0.700
Fraction b (ml/300 mg DM) [ns]	24.161	20.558	23.788
Fraction c (ml/h) [ns]	0.027	0.029	0.028

[ns] not significant

Cumulative gas production of fermented cocoa pods was lower than fermented king grass using 0, 2, 4, 6,or 8% chitinolytic inoculum as reported by Widyastuti (2006), those were 59.80, 53.60, 55.90, 56.50, and 51.90 ml/ 300 mg DM, whereas the CF content of fermented king grass was 29.89, 29.38, 30.72, 28.20, and 28.94 % DM basis. The low gas production of fermented cocoa pods was affected by its high CF content.

Based on these in vitro experiments, it does not seem that cellulolytic inoculum plays an important role in improving forage fiber degradation by cellulolytic ruminal bacteria.

CONCLUSIONS

Addition of cellulolytic inoculum from cattle rumen fluid improved cocoa pods quality by accelerating degradation of CF components, reduced pH, and promoted loss of nutrients during ensiling, apparently in a dose-dependent manner.

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