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Full Length Article

Digestibility and Protein Content Improvement of Corncob Silage Using Chicken Feather Partially Digested by *Bacillus subtilis* G8

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Abstract

Chicken feather digestions by *Bacillus subtilis* G8 culture and crude keratinase were comparatively investigated. Digestion by crude keratinase released the soluble protein corresponding with the enzyme quantities used. The maximum soluble protein released by crude keratinase were 27 and 23% by weight from milled chicken feather (MCF) and un-milled chicken feather (UCF), while bacterial digestion released 51 and 43% from MCF and UCF, respectively. Partially digestion by bacterial culture was selected as the appropriate process based on total crude protein remaining, practicability and economic reasons. Using bacterial digested chicken feather (BDCF) as a protein source for corncob silage (CS) fermentation by mixing 5, 10 and 15% BDCF generated the BDCF mixed silages as CS-BDCF-5%, CS-BDCF-10% and CS-BDCF-15%, respectively. Proximate analysis revealed the proportionally increase of crude protein with the BDCF added. Crude protein of 33.46% was found in CS-BDCF-15%, while those of CS-BDCF-5% and CS-BDCF-10% were 14.12% and 25.02%, respectively. Gas production values from *in vitro* digestibility test of all BDCF mixed silages were not different significantly at 24 h, while *in sacco* digestibility of CS-BDCF-15% showed the highest value of dry matter degradation. This research confirms the advantage of BDCF and strongly supports potentiality to utilize CS-BDCF for ruminant feeding. © 2015 Friends Science Publishers

Keywords: *Bacillus subtilis*; Keratinase; Chicken feather protein; Corncob silage

Introduction

Maize (*Zea mays*) is an economic plant in tropical area particularly Thailand and approximately 4,000-4,900 MT was produced annually. The plant residual particularly corn stover and corncob is commonly generated as the main by-products. In many tropical countries, fresh corn stover/stalk is either utilized as an alternative roughage or raw material in silage production for ruminant feeding especially in dry season, while a huge of stiff and high fiber by-product as corncob is abundantly remained. Production and management of corn stover silage was well established (Elferink *et al*., 2000; Mohd-Setapar *et al*., 2012), however, rare of silage fermentation using corncob as the main fiber component was reported. Corncob was pretreated by fungal culture for improving of protein content (Olagunju *et al*., 2013). Silage prepared by using corncob as the main fiber source with supplementation of cassava ship and sugar cane molasses was preliminary investigated; however, the corncob silage obtained has limitation for use regarding very low protein content was detected (Cheepudom, 2010). Poultry feather is the most abundant keratinous material in nature accumulated as a byproduct residual from the poultry processing industry and contains protein in high quantity up to 90% (Karthikeyan *et al*., 2007; Gupta *et al*., 2012; Saravanan and Dhurai, 2012). According to the high content of protein, poultry keratinous waste has been successfully utilized as protein source for feed ingredient. Traditional methods for feather meal production are occupied physical and chemical treatment processes such as steam, pressure, strong alkali or acid. Those required significant energy and resulted in destruction of some essential amino acids (Wang and Parsons, 1997). However, biotechnological processing of feathers for feather meal production is preferred as it is non-polluting processes and preserves the essential amino acids as methionine, lysine and histidine (Riffel *et al*., 2003; Gupta *et al*., 2012). To solve the problem of low protein content of corncob silage mentioned previously, utilizing chicken feather as a cheap protein source in corncob silage production is reasonably expected. *Bacillus subtilis* G8 is a bacterium isolated from soil sample and capable of keratinase producing. Crude keratinase from this bacterium is able to digest pig bristle and release the soluble protein when incubated at 37°C. Chicken feather was also previously revealed to be an efficient substrate for

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keratinase production by *B. subtilis* G8 (Cheepudom, 2010).

This report describes the comparison of chicken feather digestion by *B. subtilis* G8 culture and its crude keratinase. The results from utilizing of bacterial digested chicken feather as the additional protein source in corncob silage fermentation and the digestibility of newly formulated corncob silages are also explained.

Materials and Methods

Microorganism and Seed Preparation

B. subtilis G8 was maintained on nutrient agar (NA) at 4°C. Seed culture was prepared by transferring one single colony into nutrient broth (NB) and incubated at 37°C with 180 rpm aeration for 6–8 h. The bacterial culture obtained was used as seed inoculum.

Preparation of Crude Keratinase

Mineral medium containing gram per liter of 1.5 K_2HPO_4, 0.05 $MgSO_4.7H_2O$, 0.025 $CaCl_2$, 0.015 $FeSO_4.7H_2O$ and 0.005 $ZnSO_4.7H_2O$ (pH 7.5) was used as the enzyme production medium. Seed culture of *B. subtilis* G8 (10 mL) was transferred into sterile 1 liter mineral medium containing 10 g chicken feather and incubated at 37°C with 180 rpm rotary shaking for 4 days. The culture medium was taken and determined for keratinase activity. Enzyme solution (100 µL) was mixed with 100 µL of a solution of 3.2 mg/mL azokeratin (Sigma-Aldrich, USA) in 50 mM sodium phosphate buffer, pH 7.5. The reaction mixture was incubated at 37°C for 45 min and terminated by adding 500 µL of 0.1 M trichloroacetic acid (TCA). Then, the mixture was centrifuged at 10,000×g for 15 min and a clear supernatant was measured for the absorbance at 450 nm. The assay was conducted in triplicate. One unit of enzyme was defined as the amount of enzyme that resulted in an increase of the absorbance at 450 nm of 0.1 per min from the assayed condition (Wang *et al*., 2008).

Comparison of Chicken Feather Digestion by *B. subtilis* G8 Culture and Its Keratinase

Digestion of chicken feathers by *B. subtilis* G8 culture was performed in 250 mL Erlenmeyer flask, each flask containing 1 g of substrates (milled chicken feather, MCF and un-milled chicken feather, UCF). Then, mineral medium was added and sterilized at 121°C for 20 min. Bacterial inoculum of 0.1, 0.5, 1.0 and 2% (v/v) was separately inoculated into each flask and incubated at 37°C with 180 rpm rotary shaken for 7 days. Samples were collected with 24 h interval and centrifuged at 10,000×g, 4°C for 15 min, the supernatants were then measured for soluble protein by Lowry method (Stoscheck, 1990). For enzymatic digestion, 1 g each of MCF and UCF was separately mixed with 25 mL of 50 mM phosphate buffer pH 7.5 in 250 Erlenmeyer flasks and then sterilized at 121°C for 20 min. Aseptic karatinase prepared by filtering crude enzyme through 0.2 µm millipore filter in aseptic condition was added and the final volume was adjusted to 50 mL by sterile 50 mM phosphate buffer pH 7.5, then incubated at 37°C for 7 days. Samples were collected at 24 h interval and centrifuged at 10,000×g, 4°C for 15 min. The clear supernatants were measured for soluble protein. Total soluble protein released from all treatments was presented after subtraction by the soluble protein value at 0 day incubation. Total crude protein quantities of MCF and UCF prepared by both digestion methods, *B. subtilis* G8 culture and its keratinase, were measured by Kjeldahl method (Persson *et al*., 2008).

Applying the Bacterial Digested Chicken Feather (BDCF) in Corncob Silage

BDCF was used to formulate corncob silage as a supplementary protein source. The formula for corncob silage fermentation was consisted of by weight 81.25% corncob, 8.25% molasses, 10% cassava chips, 0.5% urea, water was added to get 45.28% moisture (Cheepudom, 2010). BDCF mixed corncob silage was prepared by replacing corncob with various amounts of 5, 10 and 15% (w/w) BDCF. The mixture was incubated at room temperature (25–35°C) for 5 days (Cheepudom, 2010). The nutritional quality of corncob silages was then measured by proximate analysis to determine moisture, protein, fiber, fat, nitrogen free extract and ash contents (AOAC, 1984). Corncob silages fermented with 5, 10 and 15% (w/w) BDCF were assigned as CS-BDCF-5%, CS-BDCF-10% and CS-BDCF-15%, respectively, while CS was corncob silage without BDCF addition.

Digestibility Test

An *in vitro* digestibility test was performed by gas production technique to investigate the digestibility of CS-BDCF-15% compared to CS and corncob silage fermented with 15% undigested chicken feather (CS-UDCF-15%). All samples were dried at 60°C for 48 h and ground into the powder form. Rumen fluid mixture was freshly prepared as described by Menke and Steingass (1988), mixing 400 mL rumen fluid, 400 mL distilled water, 200 mL ammonium carbonate buffer pH 8.0, 200 mL macromineral, 1 mL 0.1% (w/v) resazurine, 0.1 mL micromineral and 40 mL reduction solution. For the *in vitro* digestibility test, 230 mg of silage powder was mixed with 30 mL of the rumen fluid mixture in a glass syringe and then incubated in rotating water bath at 39°C. The gas production was recorded at 0, 2, 4, 8, 12, 24, 48, 72 and 96 h. The gas production values were used to calculate for organic matter digestibility (OMD), metabolizable energy (ME) and net energy of lactation (NE_L) as described previously by Menke and Steingass

(1988).

The *in sacco* digestibility test using nylon bag technique was also performed on rumen fistulated dairy cow (Holstein Friesian) to investigate the digestibility test of CS, CS-BDCF-15% and CS-UDCF-15%. Initially, nylon bags were dried at 60°C for 24 h and the bags weight were recorded (W1). Three grams of milled samples were put into nylon bag and then weight were recorded (W2). The incubation was carried out in the rumen for 2, 4, 8, 12, 24, 48, 72 and 96 h, using the bags incubated in water bath at 39°C for 30 min as the control (0 h). At the end of incubation periods, all bags were collected and washed in washing machine for 15 min and dried at 60°C for 48 h. Then, the remaining weight (W3) was recorded and the percentage of dry matter and crude protein disappearance was calculated as described by Ørskov and McDonald (1979).

Results

Chicken Feather Digestion by Culture of *B. subtilis* G8 and Its Keratinase

The maximum soluble protein of milled chicken feather digestions by *B. subtilis* G8 culture was 5.05 mg/mL at 2 days incubation, while the maximum soluble protein of un-milled chicken feather of 4.31 mg/mL was found at 3 days incubation (Fig. 1). Those soluble protein quantities were calculated to be 51 and 43% by weight of milled and un-milled chicken feather, respectively (Fig. 3). Chicken feather digestion by various quantities of aseptic crude keratinase 100, 250, 500 and 1000 units, the maximum of soluble protein was found from milled chicken feather at 2.73 mg/mL while those from the lower enzyme loaded were lower corresponding with the enzyme quantity used (Fig. 2A). Similar result was found with un-milled chicken feather, the maximum of soluble protein was 2.27 mg/mL when incubated with 1,000 units crude karatinase (Fig. 2B). The maximum quantities of soluble protein were found to be only 27% and 23% total weight of MCF and UCF, respectively (Fig. 3A). Comparing the quantity of the soluble protein per total weight of chicken feather, the released soluble protein by crude keratinase was clearly lower than that released by *B. subtilis* G8 culture, however those were markedly stable after reaching the maximum levels.

Applying BDCF in Corncob Silage as the Supplementary Protein

The results of proximate analysis of corncob silage fermented with various amounts of digested chicken feather are presented in Table 1. The result clearly proved that increase of total crude protein (CP) in silage was observed when the BDCF was mixed. The observed CP in corncob silage mixed with various amounts of BDCF (5, 10 and 15%) were 14.12, 25.02 and 33.46%, respectively, which is significantly different in each treatment at $P<0.05$.

Table 1: Proximate analysis of corncob silage (CS) and corncob silage fermented with 5, 10 and 15% bacterial digested chicken feather (CS-BDCF) on dry matter basic.

Characters	CS	CS-BDCF-5%	CS-BDCF-10%	CS-BDCF-15%
Moisture (%)	54.99^{a} ±0.76	54.79^{a}±0.34	52.07^{b}±0.77	50.65^{c}±0.67
Crude protein	5.03^{d}±0.22	16.12^{c}±1.55	27.02^{b}±1.00	33.46^{a}±0.31
Ether extract	0.30^{b}±0.04	0.64^{a}±0.07	0.62^{a}±0.05	0.60^{a}±0.080
Crude fiber	30.50^{a}±0.60	17.08^{b}±1.53	13.25^{c}±0.89	10.90^{d}±0.56
Ash	3.77^{b}±0.06	5.19^{a}±0.65	5.18^{a}±0.60	4.06^{b}±0.32
Nitrogen free extract	5.41	6.18	1.86	0.33

$^{a, b, c, d}$ means significantly different within rows ($P< 0.05$)

Table 2: The values of *in vitro* gas production, organic matter digestibility (OMD), metabolizable energy (ME), net energy of lactation (NE_L) of CS-BDCF-15% mixed silage compared to CS and CS-UDCF-15%

Characters	GP (mg/200 mg DM, 24 h)	OMD	ME	NE_L
CS	38.05^{a}±7.62	53.30^{b}±6.44	7.68^{a}±1.03	3.32^{a}±0.73
CS-BDCF-15%	34.48^{a}±4.81	67.16^{a}±4.06	8.80^{a}±0.65	4.04^{a}±0.46
CS-UCCF-15%	32.31^{a}±3.79	65.54^{a}±3.20	8.54^{a}±0.51	3.85^{a}±0.36

$^{a, b}$ means significantly different within column ($P< 0.05$)

In vitro and *in sacco* Digestibility Test

Gas production of CS, CS-BDCF-15% and CS-UDCF-15% were not significantly different ($P<0.05$) at 24 h. However, the higher gas production was clearly observed in CS after 24 h compared to CS-BDCF-15% and CS-UDCF-15%, especially at 72-96 h incubation (Fig. 4). The values of organic matter digestibility (OMD) of CS-BDCF-15% and CS-UDCF-15% were significant higher than that of CS, while metabolizable energy (ME) and net energy of lactation (NE_L) were not different significantly in all silages (Table 2). In contrast to the results from in gas production test, the digestibility value in rumen by *in sacco* of CS-BDCF-15% was the highest and significantly different ($P<0.05$) from those of CS-UDCF-15% and CS, especially at 72 h incubation (Fig. 5).

Discussion

Based on the fact of the most abundant keratinous poultry feather accumulated as a byproduct residual from the poultry processing industries incorporated with low protein content of corncob silage, this research aimed to find the appropriate way to utilize chicken feather as protein source for corncob silage fermentation. However, natural feather needs pretreatment before use (Wang and Parsons, 1997; Tiwary and Gupta, 2012) and our research aimed to investigate the biological digestion either by bacterial cell or its keranolytic enzyme. The result revealed that chicken feather digestion by *B. subtilis* culture released the soluble

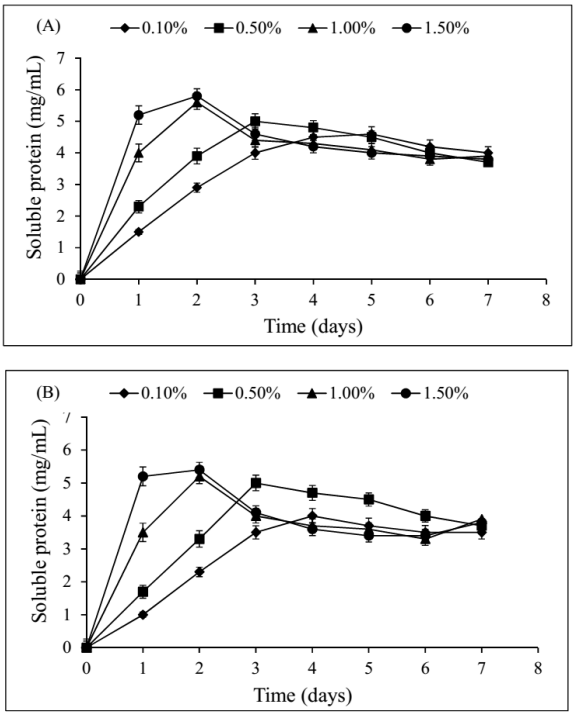

Fig. 1: Soluble protein released from digestion of milled chicken feather (A) and un-milled chicken feather (B) by *Bacillus subtilis* G8 culture using 0.1, 0.5, 1.0 and 1.5% (v/v) inoculi

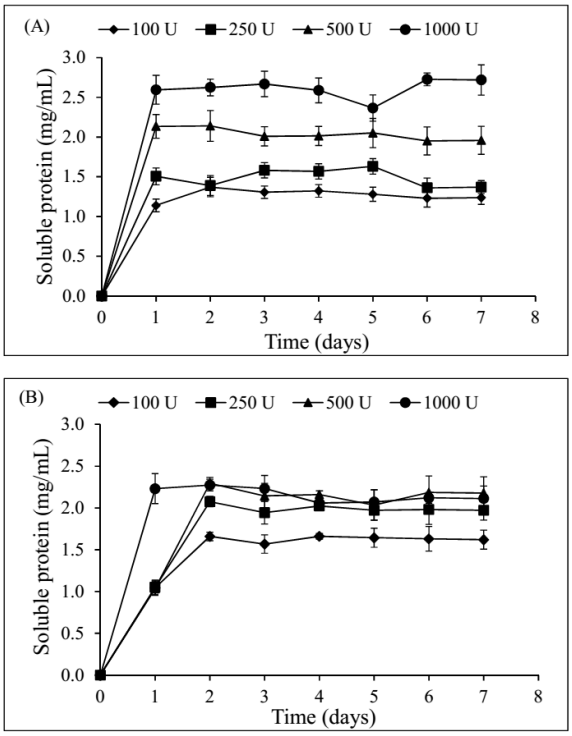

Fig. 2: Soluble protein released from digestion of milled chicken feather (A) and un-milled chicken feather (B) by various amounts of crude keratinolytic enzyme (100, 250, 500, 1000 U)

protein from both MCF and UCF increasingly to the maximum corresponding with inoculum size and the fastest soluble protein release was found with 1.5% (v/v) inoculum. However, the maximum soluble protein was almost the same level as that of 1.0% (v/v) inoculum at 2 days of incubation. Furthermore, similar to all treatments, the slowly decrease was observed after 2 or 3 days incubation. This might be due to the utilization of soluble protein by *B. subtilis* G8 for cell growth and metabolism as suggested by Zerdani *et al*. (2004). Additionally, a slight decrease of soluble protein due to the utilization of bacteria for growth during chicken feather digestion by *Bacillus* sp. FK46 was also observed (Suntornsuk and Suntornsuk, 2003). In comparison to the enzymatic digestion by crude keratinase, the rate of soluble protein release was corresponding to the enzyme quantities. MCF substrate markedly gave the higher soluble protein compared to those of UMF. This can be explained by the general principle of solid substrate and enzyme interaction, smaller particle size results in the larger surface area and commonly provides the higher reaction rate as found in enzymatic hydrolysis of lignocellulosic substrate in corn bran (Agger and Meyer, 2012). The decrease of soluble protein release was not observed in all treatments of enzymatic digestion which were performed in aseptic condition. This also supports the loss of soluble protein due to the utilization by microbial cell during the digestion by *B. subtilis* G8 as mentioned previously. However, even though the variation of either inoculum size or keratinolytic enzyme quantities showed the difference in soluble protein release, the total crude protein quantities analyzed by Kjeldahl method of both digestion processes were not significant different (Fig. 3B). Regarding the reasons based on scientific results of degrading capability, the simple preparation, remaining total protein quantity and an economic concern on the production cost for the larger scale preparation of BDCF, digestion by *B. subtilis* G8 culture was selected as the appropriate process. Additionally, the safety concern on feed contaminated harmful microbe is clarified because *B. subtilis* is worldwide accepted as the generally regarded as safe (GRAS) organism by the Food and Drug Association (Schallmey *et al*., 2004). Therefore, it is safe for feed application.

In applying of BDCF by mixing with corncob silage, the results from proximate analysis indicated the proportionally increase of crude protein content in all treatments along with the increase of BDCF added (Table 1). It is markedly observed that after subtraction by 5.03% of total crude protein found in CS with BDCF addition, the total crude protein increased approximately 2 times (1.82–1.99) of BDCF added. This reasonably explained as the BDCF was added by replacing the corncob mass which is an important source of dry matter (DM) containing ingredient.

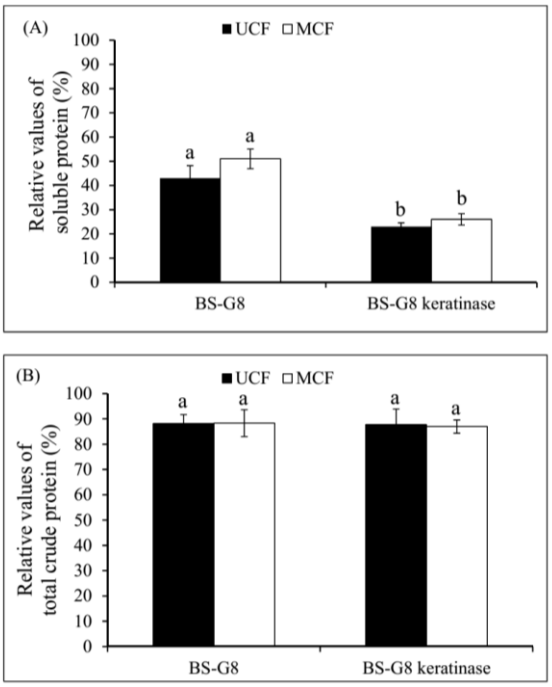

Fig. 3: Comparison of protein quantities after digestion at 37°C for 2 days by *B. subtilis* G8 culture (BS-G8) and 1,000 U crude keratinase (BS-G8 keratinase); Soluble protein released (A) and total crude protein (B)

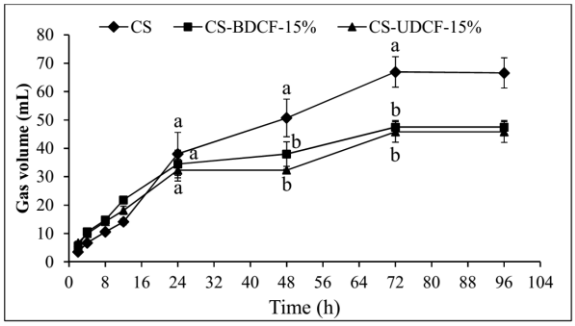

Fig. 4: *In vitro* gas production of corncob silage (CS), mixed corncob silages with 15% digested chicken feather mixed corncob silage (CS-BDCF-15%) and 15% undigested chicken feather (CS-UDCF-15%)

The appropriate utilization of corncob silage mixed with BDCF for ruminant feeding needs to be considered. Corncob silage without any addition of BDCF with 5.03% crude protein might be qualified enough for general feeding of ruminant, however, in case of daily cow feeding, either CS-BDCF-5% or CS-BDCF-10% should be more suitable as the high crude protein content is normally required. The effect on milk production depends on the sufficiency of supplementary protein (Campbell and Marshall, 1975; Perry *et al*., 2004). Usually, the protein need of a dairy cow depends on its size, milk production and step of pregnancy.

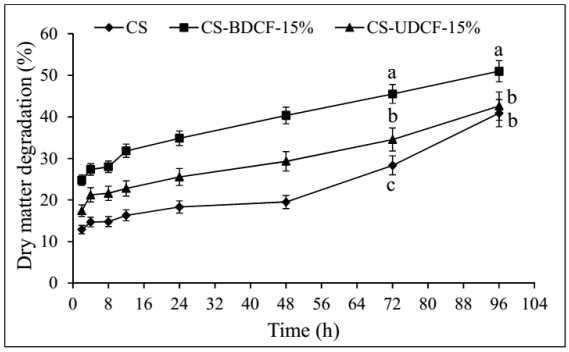

Fig. 5: Dry matter degradation efficacy of corncob silage in rumen (CS), Corncob silage fermented with 15% bacterial digested chicken feather (CS-BDCF-15%) and corncob silage fermented with 15% undigested chicken feather (CS-UDCF-15%)

Crude protein needs at different levels of milk production are 16-18% for early lactation, 14-16% for mid lactation and 12-14% for late lactation (Moran, 2005).

The difference of gas production observed after 24 h incubation between CS and CS-BDCF15% or CS-UDCF-15% (Fig. 4) might be due to the difference in carbohydrate content derived directly from corncob quantity as gas production ability was investigated and confirmed to have a positive correlation with carbohydrates content (Coelho *et al*., 1988). Besides gas production, microbial fermentation process in rumen also produces short chain volatile fatty acids (VFAs) and microbial proteins (Blümmel *et al*., 1997; Megías *et al*., 2014). In addition, the higher value obtained from *in sacco* digestibility test since 24 h until entering the 72-96 h incubation time which was recommended to be the best period for prediction of OMD by Fonseca *et al*. (1998). Regarding the faster disappearance of organic matter, the higher efficiency of feed degradation and turnover rate in ruminant rumen can be predicted. Therefore, the highest value of *in sacco* digestibility obtained from CS-BDCF-15% clearly confirms the distinguished positive effect of chicken feather digestion by *B. subtilis* G8 culture compared to CS and CS-UDCF-15%.

According to the high protein content up to 33.46% and the highest digestibility observed by *in sacco* digestibility test of the CS-BDCF-15%, more interesting challenge is expected to the utilizing of CS-BDCF-15% for replacing the use of expensive and costly commercial feed concentrate which normally contains crude protein more than 20%. This idea may become the helpful factor for cost reduction particularly for the commercial ruminant farming.

Conclusion

In comparison to chicken feather digestion by *B. subtilis* G8 keratinolytic enzyme, partial digestion by bacterial culture was simpler, lower production cost and no significant

difference in total crude protein content. Therefore, partial digestion of chicken feather with *B. subtilis* G8 was more appropriate than the use of *B. subtilis* G8 crude keratinase. Utilizing of 15% bacterial digested chicken feather as a protein source in corncob silage fermentation could increase crude protein comparable to the protein level in the commercial concentrates. Therefore, the chicken feather digested by *B. subtilis* G8 has a high potential to be utilized as an additional protein source in corncob silage for ruminant feeding.

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