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Research Article
Cellulase Production by *Fusarium graminearum* and its Application in Ruminant's Diets Degradation

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Abstract

Background and Objective: Cellulase as a fibrolytic enzyme is a highly effective tool for agricultural waste treatments. Production of cellulase enzyme on medium of agricultural wastes by *Fusarium graminearum* to be used in ruminant feeding was the main objective of this study. **Materials and Methods:** Impact of initial pH of growth medium, different nitrogen sources and variety of agriculture by products as a carbon sources on cellulase production have been studied. Electron microscope was used for investigate the impact of the resultant cellulase on corn stover degradation, while batch culture technique was used for investigate impact of different levels of the produced and commercial cellulases on total mixed ration digestibility by rumen microorganisms (*in vitro*). **Results:** Cellulase maximum production by *F. graminearum* was obtained at 20% corn stover, initial pH of growth medium 5.0 and peptone as a nitrogen source. All addition levels of the produced cellulase increased dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose and hemicellulose degradability of the treated diets, but the maximum produced cellulase efficiency% for dry matter degradability was obtained at 1200 IU kg^{-1} DM reached 23.19% over the control. **Conclusion:** Utilization of the produced cellulase in enrichment of the feeding value of the agricultural by-products may help in overcome of the feed gap with good impact on environment and public health.

Key words: Feed resources, agricultural by-products, fungal cellulase production, *in vitro* batch culture, ruminal fermentation, agricultural wastes

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The continuous elevation of prices of feed ingredient and the wide gap between animal's requirements and available feeds forced the nutritionists for searching for alternative feed resources[1]. The Egyptian agricultural sector is trying to cover the shortage in feedstuffs by importation which representing a burden on the Egyptian economy[2]. However, the agricultural by-products can play an important role for minimizing this feed gap[3]. The annual production of agricultural by-products estimated to be around 30 million t of dry material/year[4]. These abundant wastes are mostly left in the road sides or burnt in the fields which lead to environmental pollution and health hazards[5]. The main problem facing in the utilization of agricultural by-products as feed resources is their high content of crude fiber which limits their nutritive value[6]. Thus, to improve the nutritive value of these residues, it is important to breakdown the lignocellulosic bonds in it[7]. Cellulase as a fibrinolytic enzyme plays a vital role in saccharification of agricultural by-products to glucose, cellobiose or cellooligosaccharides[8]. Also, microbial cellulase represents a valuable tool for improving ruminant's ability for fibrous feed digestion[9,10]. Many of *in vitro* studies showed that supplemented ruminal bacterial cultures with cellulase led to improve ruminal fermentation through increase diet's dry matter (DM), organic matter (OM), neutral detergent fiber (NDF) and acid detergent fiber (ADF) digestibility, alter ruminal pH with increase microbial protein synthesis and volatile fatty acid production[11-13].

Recent advances in biotechnology have allowed for large production of microbial active cellulases, but its production cost is still high[14]. Production of extracellular cellulase from anaerobic bacteria (*Acetivibrio cellulolyticus*, *Ruminococcus albus* and *Fibrobacter succinogenes*) and aerobic fungi (*Fusarium*, *Aspergillus*, *Trichoderma* and *Penicillium*) have been reported by Murad and Azzaz[7]. Bacterial cellulases are constitutively produced, whereas fungal cellulase is produced only in the presence of cellulose. Therefore, agro-industrial residues may play an important role in commercialization of new sources of fungal cellulases. Inclusion of agricultural by-products as carbon sources in fungal growth medium may reduces the cost of cellulose production[7,15].

This study was conducted for production of cellulase by *Fusarium graminearum* under the optimum fermentation conditions and investigated the effect of the resultant cellulase on ruminant's diet's degradation (*in vitro*). This study would advance a new knowledge through, optimization of cellulase production using unfamiliar fungal strain and unfamiliar agricultural residues as a carbon sources in the growth medium with new techniques act for decreasing the cost of production by 50%.

MATERIALS AND METHODS

This study was conducted in the Laboratories of Dairy Department-National Research Centre, Egypt. *Fusarium graminearum* was obtained from laboratory of dairy microbiology and the agricultural by-products were obtained from suburbans of Giza province. This study has been extended for 4 months from 10 January, 2018 till 10 April, 2018.

Sample collection: For cellulase production, around 100 samples were taken. For the *in vitro* study around 250 samples were taken. *Fusarium graminearum* was obtained from laboratory of dairy microbiology, the agricultural by-products (substrate) were obtained from suburbans of Giza province, scanning electron micrographs were taken to observe the effect of the produced cellulase on corn stover fibers and the rumen contents were collected from the rumen of slaughtered rams , then moved directly to the laboratory in separate warmed oxygen-free plastic jars to observe the effect of the produced cellulase on ruminant's diets degradability.

Media and inoculum preparation: Spores of *Fusarium graminearum* was transferred from surface of the actively growing slants of potato dextrose agar medium to 250 mL conical flasks each containing 50 mL of malt medium (malt extract (30 g L^{-1}), yeast extract (5 g L^{-1})). After incubation on a rotary shaker (120 rpm) at 29±1°C for 48 h, the grown culture has been employed as inocula (5% v/v) for 1000 mL conical flasks each containing 100 mL of cellulose powder medium (CPM) which composed of (g L^{-1}) NaCl; 6.0, $(NH_4)_2So_4$; 1.0, K_2HPO_4; 1.0, $MgSO_4$. $7H_2O$; 0.05, $CaCl_2$; 0.1, Yeast extract; 0.5, Peptone; 0.5, Glucose; 4.0, Cellulose powder; 2.0 and adjusted to pH 6.0 as reported by Khattab *et al.*[16].

Cellulase production culture conditions: Effect of initial pH of growth medium in a range between 3 and 8, nitrogen sources included NH_4CL, $(NH_4)_2SO_4$, urea, yeast and peptone at level of 0.33 g N L^{-1} and different agriculture by products (corn stover, bean straw, pea pods peel, palm fronds, wheat straw and rice straw) as a carbon sources at level of 10% on cellulase production have been studied.

Cellulase assay: One cellulase unit was defined as the amount of enzyme that liberates reducing sugar at the rate of 1 μmole min^{-1} under the conditions of the assay. The carboxymethyl-cellulase activity (CMC) for resultant enzyme was determined according to Mandels *et al.*[17]. The reducing sugar liberated was determined by modified dinitrosalicylic acid method (DNS) of Miller[18].

Scanning electron micrographs for corn stover fibers: Corn stover fibers were dried and then treated with the produced cellulase (50 U cellulase/1 g DM/100 mL buffer acetate at pH 6.5) for 24 h at 40°C and 110 rpm in a rotary shaker. The enzymaticly untreated corn stover (control) was kept in flask at the same condition. Finally, scanning electron micrographs of both treated and untreated corn stover fibers were taken to observe the effect of enzyme treatment.

***In vitro* study:** *In vitro* dry matter, neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose and hemicellulose degradability were determined for the experimental diet. A 400 mg sample of the control diet powder was weighed into 120 mL serum bottles. The control diet was consisted of 50% concentrate feed mixture (CFM), 25% Berseem hay and 25% corn stover. The bottles (3 replicates) were separately supplemented with rumen liquor, buffer solution and Pan-Zyme and the produced cellulase solutions at different levels (0, 600, 1200, 1800 and 2400 IU kg^{-1} DM of the diet). Rumen contents were collected from the rumen of slaughtered rams fed berseem hay ration, then moved directly to the laboratory in separate warmed oxygen-free plastic jars. Rumen liquor contents were strained through 4 layers of cheese-cloth and the obtained liquor was mixed with the buffer solution at 39°C under continuous flushing[19] of CO_2. The bottles were sealed and maintained at 39°C in a shaking water bath (20 oscillations min^{-1}) for 24 h. After 24 h of incubation the pH value, total gas production (TGP) volume, NH3 and total volatile fatty acids (TVFA) concentrations were determined according to the method of Azzaz *et al.*[20].

Statistical analysis: Data obtained from this study were statistically analyzed by IBM SPSS Statistics for Windows[21] using the following general model procedure:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where, Y_{ij} is the parameter under analysis ij, μ is the overall mean, T_i is the effect due to treatment on the parameter under analysis, e_{ij} is the experimental error for ij on the observation, the Duncan's multiple range tests was used to test the significance among means using probability level less than 0.05 ($p<0.05$) for significance expression[22].

RESULTS

Effect of different agricultural by products (carbon sources) on cellulase production: As shown in Fig. 1, corn stover as cellulolytic substrate gave the highest cellulase activity with *Fusarium graminearum* (1.01 μmole mL^{-1} min^{-1}), while rice straw gave the lowest cellulases production (0.31 μmole mL^{-1} min^{-1}). Therefore, corn stover selected for conducting further studies on cellulase production by *Fusarium graminearum*.

Effect of carbon source concentration on cellulase production: Data illustrated in Fig. 2 Showed effect of different concentration of corn stover powder ranged from 2.5-20% (w/v) on cellulase production by *Fusarium graminearum*. Maximum cellulase activity reached

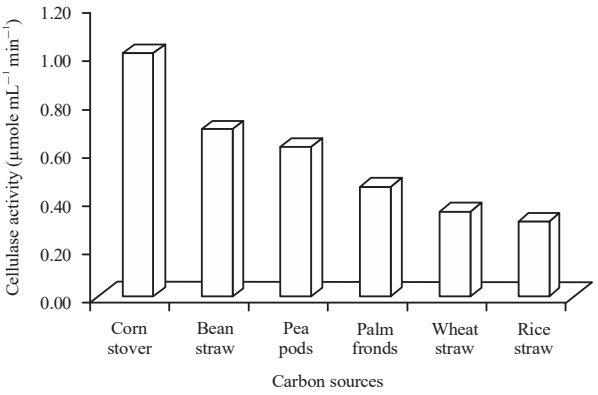

Fig. 1: Effect of different carbon sources on cellulase production

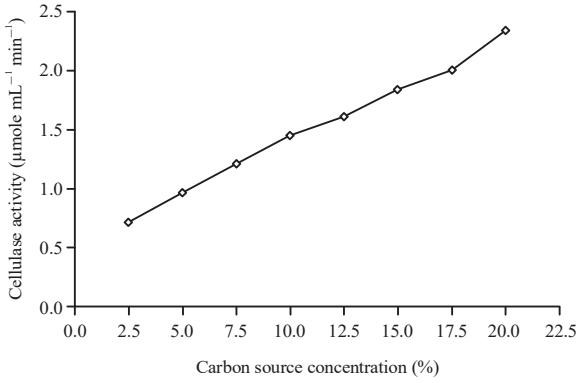

Fig. 2: Effect of carbon source concentration (20 w/v) on cellulase production

(2.34 μmole mL^{-1} min^{-1}) was obtained at 20% (w/v) corn stover concentration, while the minimum activity (0.71 μmole mL^{-1} min^{-1}) was obtained at 2.5% of corn stover concentration of modified cellulose powder medium (CPM). Based on these data, corn stover concentration at 20 (w/v) was chosen for further studies on the modified CPM.

Effect of initial pH of growth medium on cellulase production: The cellulase production by *Fusarium graminearum* in varying initial pH of the growth medium showed that pH 5.0 was the optimum (Fig. 3). Moreover, when pH level increased, the enzyme production was decreased. Accordingly, the initial pH for the modified CP medium was adjusted to pH 5.0 in the subsequent experiments.

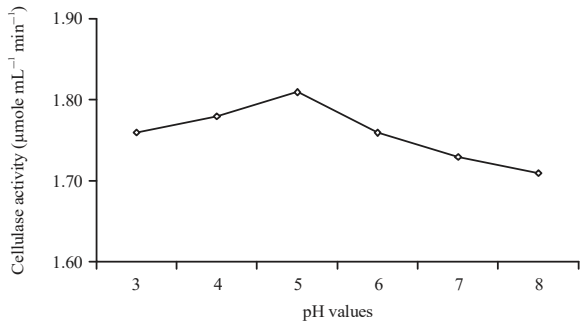

Fig. 3: Effect of initial pH of growth medium on cellulase production

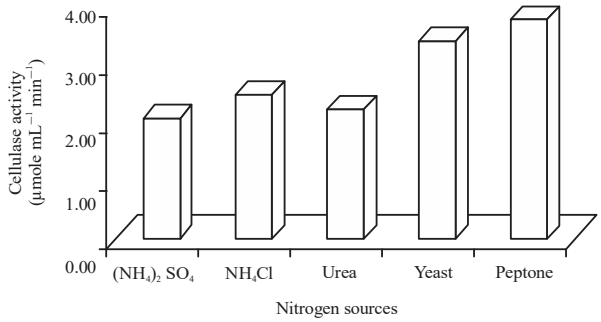

Fig. 4: Effect of nitrogen sources on cellulase production

Effect of nitrogen sources on cellulase production: Data of Fig. 4 showed that among 5 nitrogen sources tested for screening their effect on cellulase activity, peptone was found to be the best nitrogen source producing the highest level of cellulase activity reached 3.79 (μmole mL^{-1} min^{-1}) by *Fusarium graminearum*. These data indicating that the source of nitrogen should to be organic for better cellulase production.

Scanning electron micrographs for corn stover: Electron micrographs showed that the fibers of corn stover were separated after cellulase treatment as a result of cellulose hydrolysis (Fig. 5a and b).

***In vitro* rumen fermentation:** The obtained results showed that all levels of the produced cellulase and Pan-Zyme increased DM, NDF, ADF, cellulose and hemicellulose degradability of the treated diets compared with the control one, which gave the lowest values of diet degradability parameters (Table 1). The maximum produced cellulase efficiency percentage for dry matter degradability was obtained at 1200 IU kg^{-1} DM reached 23.19% over the control:

$$\underset{(\%)}{\text{Enzyme efficiency}} = \text{DM}(\%)\,\frac{\text{Treated diet-Control diet}}{\text{Control diet}}\times 100$$

Also, the Pan-Zyme takes the same trend and its efficiency percentage for dry matter degradability reached 25.15% over the control. There are no marked changes in the ruminal parameters pH and total gas production (TGP) due to treatment with the cellulases (Table 2). While marked changes have been found in total volatile fatty acids (VFA) and NH_3

Table 1: Cellulases effects on degradability parameters of experimental diets

		Diet degradability parameters (%)					
Treatments	Enzyme level (IU kg^{-1})	DM	NDF	ADF	Cellulose	Hemicellulose	Enzyme efficiency
Control	0	52.01^{c}	33.05^{c}	30.11^{e}	32.92^{c}	32.34^{e}	0.00
Produced cellulase	600	60.10^{b}	38.85^{b}	35.14^{d}	38.58^{b}	45.91^{c}	15.56
	1200	64.07^{a}	46.43^{a}	43.24^{b}	40.85^{b}	57.85^{a}	23.19
	1800	63.18^{a}	38.88^{b}	39.88^{c}	39.33^{b}	48.81^{b}	21.48
	2400	62.24^{a}	39.43^{a}	33.74^{d}	36.96^{b}	41.86^{d}	19.67
Pan-Zyme	600	61.51^{ab}	42.08^{a}	42.73^{b}	39.68^{b}	48.01^{b}	18.27
	1200	65.09^{a}	45.58^{a}	50.80^{a}	48.04^{a}	50.28^{b}	25.15
	1800	63.68^{a}	41.81^{a}	39.93^{c}	41.58^{b}	47.53^{c}	22.45
	2400	63.23^{a}	37.40^{b}	38.40^{c}	35.05^{b}	46.48^{c}	21.58
SE		0.93	1.18	1.42	1.10	1.57	0.00

DM: Dry matter, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, SE: Standard error, $^{a-e}$Significantly different ($p<0.05$)

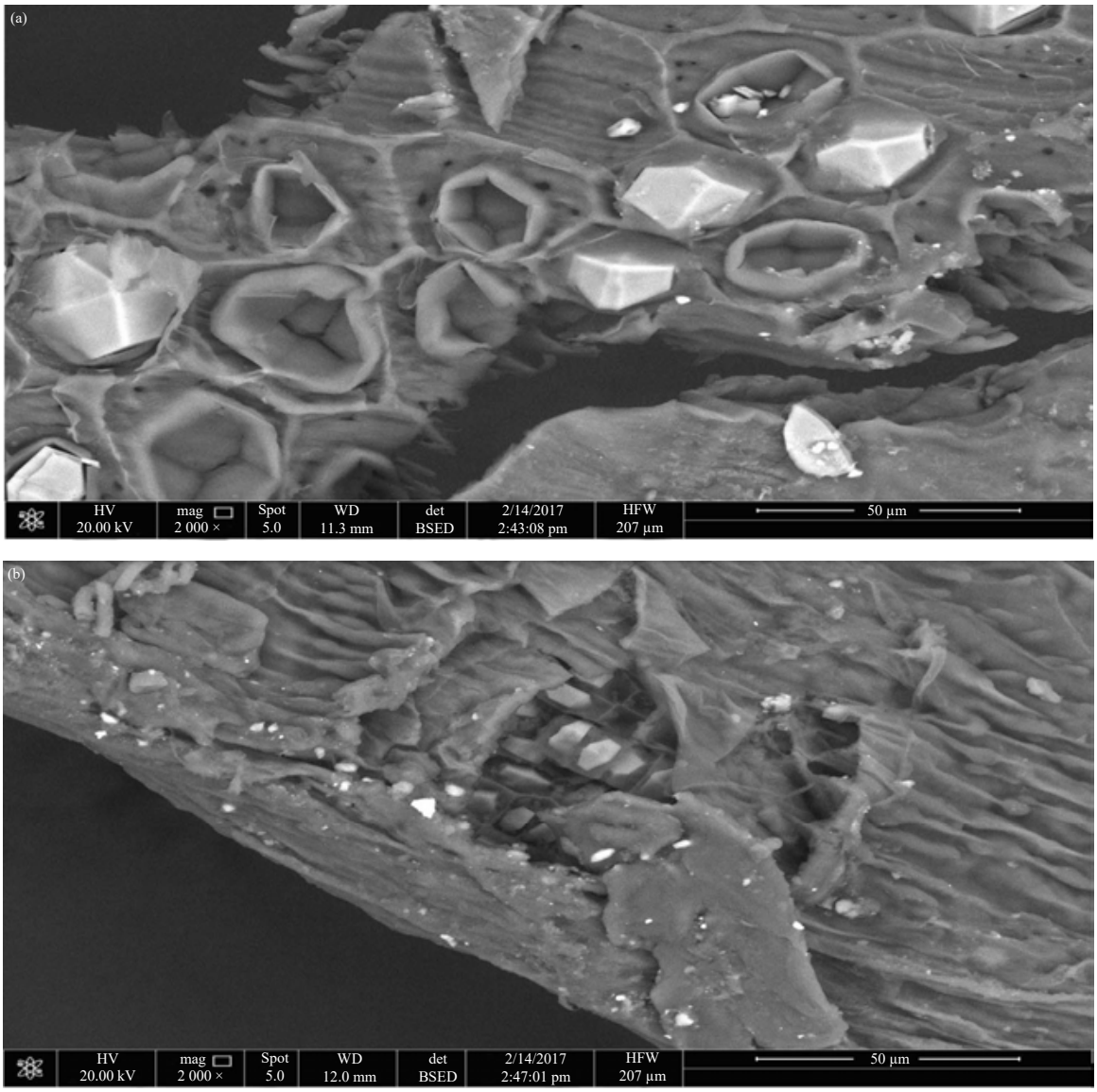

Fig. 5(a-b): Electron micrographs, (a) Before and (b) After treatment of corn stover fibers with the produced cellulase

Table 2: Cellulases effects on ruminal parameters (*in vitro*)

	Ruminal parameters				
Treatments	Enzyme level (U kg^{-1})	TGP (mL)	pH	NH_3 (µmol L^{-1})	TVFA (mEq dL^{-1})
Control	0	159.50	6.70	33.84^{c}	6.15^{b}
Produced cellulase	600	159.50	6.66	37.03^{a}	6.25^{b}
	1200	161.00	6.78	37.51^{a}	6.80^{a}
	1800	163.50	6.72	33.61^{c}	6.65^{a}
	2400	160.50	6.81	33.08^{c}	6.60^{a}
Pan-Zyme	600	156.00	6.65	35.60^{b}	6.25^{b}
	1200	164.50	6.72	37.44^{a}	6.75^{a}
	1800	157.00	6.79	36.18^{ab}	6.45^{b}
	2400	152.00	6.82	36.13^{ab}	6.45^{b}
SE		1.15	0.02	0.47	0.06

TGP: Total gas production, TVFA: Total volatile fatty acids, SE: Standard error, $^{a\text{-}c}$significantly different ($p<0.05$)

concentrations due to cellulases supplementation (Table 2). The maximum increase in TVFA and NH3 concentrations has been noticed after diet treatment with the cellulases at 1200 IU kg^{-1} DM.

DISCUSSION

Production of cellulase by *Fusarium graminearum* requires optimal condition for their growth. The superiority of corn stover over the other agricultural wastes for cellulase production may be due to that corn stover contain growth factors (ex, minerals) or may be it act as a carbon and nitrogen source at the same time. Therefore the carbohydrate part of the lignocellulosic agricultural residues has received considerable interest in cellulase production process. Production of cellulase under the optimum fermentation conditions using the agricultural by-products as the substrate (carbon source) may give us highly effective feed additive product with low cost. The utilization of the produced cellulase in enrichment of the feeding value of the agricultural by-products may help in overcome of the feed gap with good impact on environment and public health. The carbon source of production medium is critically affecting the cellulase activity[7].

It has been reported that, the impact of the agricultural wastes as carbon sources on cellulase production is vary due to their chemical nature and its effect on the growth of the cultivated fungi[15]. Cellulase production by different fungal strains on variety of agricultural residues has been reported with different yields[8,13,15]. It is will known that, fungal cellulase synthesis and secretion under submerged fermentation is mainly depending on moisture content of growth medium[13]. In the current study, the higher concentration of corn stover not negatively affects the active water of the growth medium and gave *F. graminearum* more ability for cellulase production. It seems that higher CPM moisture content (2.5% corn stover) decreases cellulase production yield due to suppression of *F. graminearum* growth through reduction in interparticle spaces and impaired oxygen transfer[23]. These findings are agree with those obtained by Aboul-Fotouh *et al.*[13], who found that minimum cellulase production was obtained by *A. niger* at 2.5% rice straw concentration of growth medium.

Initial pH of the microbial growth medium has profound effect on cellulase production. It's well known that the optimal pH for cellulase production varies with different fungal strains. In the current study, pH 5.0 was the optimum for cellulase production by *Fusarium graminearum.* Also, Sethi and Gupta[24] reported that maximum cellulase activity by *Penicillium chrysogenum* and *Aspergillus niger* was observed in medium of pH 5.0. Similar observation was reported for cellulase production by *A. tereus* QTC 828 by Ali *et al.*[25] and *Trichoderma resei* by Doppelbauer *et al.*[26].

In current study, peptone was found to be the best nitrogen source producing the highest level of cellulase activity by *Fusarium graminearum.* Many researchers have noticed that organic nitrogen sources gave better cellulase activity than inorganic nitrogen sources[8,13,15]. In accordance, peptone was found to be the most promising and effective nitrogen source for cellulase production[27] by *Penicillium waksmanii* F10-2, *A. hortai*[28] and *A. niger*[13]. It seems that the presence of the peptone at certain level in fungal growth medium was essential for high levels of cellulase production.

The produced cellulase in the current study was able to degrade the corn stover fibers as shown in electron micrographs. In accordance, Murad and Azzaz[8] noticed the same impact of cellulase produced by *A. flavus* NRRL 5521on the rice straw fibers. The electron micrographs may give evidence for occur of fiber saccharification but the degree of saccharification should be assayed on the basis of release of reducing group.

Production of cereal crop residues, especially corn stover has increased dramatically as a result of more corn production. Microbial treatments of such residues may improve its nutritive value and gave solution for feed gap in Egypt[29,30]. In current study, diets containing 25% corn stover have been treated with the produced fungal cellulase and commercial enzyme source. In fact, the increase DM, NDF, ADF, cellulose and hemicellulose degradability is reasonable as cellulases supplementation led to more hydrolysis and separation for fibers of feed particles (see electron micrographs). Positive effects of fibrolytic enzymes supplementation on the diets degradability have been observed in many of the *in vitro* studies by Azzaz *et al.*[9], Aboul-Fotouh *et al.*[13] and Azzaz *et al.*[20]. Aboul-Fotouh *et al.*[13] suggested that cellulases supplementation might alter the carbohydrates structure of the treated diets during the interaction period, making it more amenable for rumen microorganisms. In current study, although ruminal TVFA and NH_3 concentrations have been increased after cellulases addition, the ruminal pH and TGP are not affected. This might be due to increase extent of dietary amino acids deamination by rumen microorganisms and enhancement of microbial protein synthesis which may drive the pH toward alkalinity. In the same time, increase extent of dietary carbohydrate hydrolysis after cellulases addition let for more acids production especially TVFA which opposite action of amino acids deamination process. This may give an

explanation for why the ruminal pH was not change. The current data supports the findings of Ribeiro *et al.*[31], who noticed that *in vitro* DM, NDF and ADF disappearance tended to increase for barley straw treated with different types of fibrolytic enzymes with no effect of enzyme on the ruminal pH. Also, Addition of fibrolytic enzymes to a total mixed ration (TMR) in a RUSITEC did not affect pH[32]. On the other hand, the rapid fermentation of the concentrate portion the teasted diet may lead to higher propionate production with no effect on CH_4 production. Propionate acts as alternative hydrogen sink in the rumen diverting hydrogen away from the reduction of CO_2 to CH_4, while the production of butyrate and acetate promotes methanogenesis[33]. This may give an explanation for why the ruminal TVFA production increased with no effect on TGP production in this study.

CONCLUSION

Finally, it could be concluded that production of cellulase under the optimum fermentation conditions using the agricultural by-products as the substrate (carbon source) may give us highly effective feed additive product with low cost. On the other hand, the utilization of the produced cellulase in enrichment of the feeding value of the agricultural by-products may help in overcome of the feed gap with good impact on environment and public health.

SIGNIFICANCE STATEMENT

This study discovered the potential production of cellulase by *Fusarium graminearum* under the optimum fermentation conditions using the agricultural by-products as the substrate. This may give feed factories highly effective feed additive product with low cost. The utilization of the produced cellulase in enrichment of the feeding value of the agricultural by-products can be beneficial for livestock breeders who suffering high prices of traditional feed stuff. This study will help the researchers to uncover the critical areas of using of biotechnology for enrichment of the feeding value of the agricultural residues and their impact on environment and public health.

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