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Asian Journal of Animal and Veterinary Advances

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OPEN ACCESS Asian Journal of Animal and Veterinary Advances

ISSN 1683-9919
DOI: 10.3923/ajava.2016.824.831

Research Article
Fungal Cellulase Production Optimization and its Utilization in Goat's Rations Degradation

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Abstract

Objective: Fungal cellulase production under the optimum conditions and investigates the impact of the produced cellulase on degradation of goat's rations compared with commercial cellulase source. **Materials and Methods:** *Asperigillus niger*, *Asperigillus flavus*, *Asperigillus fugimatus*, *Trichoderma viride* and *Penicillium chrysogenum* were separately grown as stand cultures in 250 mL conical flasks containing 50 mL of cellulose powder medium for screening their ability for cellulase production. In the *in vitro* trial, degradation of dry matter and organic matter were determined for goat's ration. The ration was supplemented separately with locally produced cellulase (Asperozym) and commercial cellulase source (Phytabex plus®) at 4 levels (500, 1000, 1500 and 2000 U kg^{-1} DM) compared with the control. **Results:** *Asperigillus niger* had the highest cellulase activity reached 0.44 U mL^{-1}. The maximum production of cellulase by *A. niger* was achieved at 10% rice straw, inoculum size of 4%, initial pH of growth medium 6.0 and peptone as a nitrogen sources at a concentration of 0.33 g N L^{-1}. Increasing the Asperozym and Phytabex plus® addition levels up to 1000 U kg^{-1} DM gave the maximum ($p<0.05$) values of dry matter and organic matter degradation. **Conclusion:** The locally cellulase production for feeding animals may reduce the cost of importation and encourages self-reliance.

Key words: Cellulase production, *A. niger*, rice straw, peptone, goat's rations

Received: August 02, 2016 Accepted: September 16, 2016 Published: November 15, 2016

Citation: G.E. Aboul-Fotouh, G.M. El-Garhy, H.H. Azzaz, A.M. Abd El-Mola and G.A. Mousa, 2016. Fungal cellulase production optimization and its utilization in goat's rations degradation. Asian J. Anim. Vet. Adv., 11: 824-831.

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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

A long cherished dream of ruminant nutritionists has been to manipulate the ruminal fermentation to guarantee more feed utilization efficiency. In this concern, the compact nature of plant fiber cell walls may influence the efficiency of feed utilization by ruminants[1]. As reported by Yang *et al.*[2] the digestion of fibrous substances in the rumen is slow, incomplete and may limit the animal productivity. Thus, to increase the availability of nutrients in fiber-rich feed stuff it is necessary to destroy the compact nature of the cellulytic tissues[3]. Recent advances in fermentation technology have allowed for large quantities production of enzymes (e.g., cellulase) which can be used as livestock feed supplements[4]. Cellulase is one of fibrolytic enzymes family; which acts collectivity to hydrolyze cellulose to glucose, cellobiose or cellooligosaccharides[1]. Microbial cellulase production has been largely studied during the last decade[1,4,5]. Madhavan and Mangalanayaki[6] reported that fungi are the main cellulase producing microorganism and *Aspergillus*, *Penicillium* and *Trichoderma* sp. are the higest cellulase producing fungal strains. However, the production of cellulase varies according to the type of strain, cultivation conditions (initial pH, inoculums size and incubation period) and the growth medium composition particularly carbon and nitrogen sources[5]. Low yields over prolonged fermentation are the major limiting factor for cellulases production[7]. Therefore, using high yielding strains, optimal fermentation conditions and cheap raw materials as a carbon source is the way for maximum and economical cellulase production[4]. Concerning with the utilization of cellulases in ruminants feeding, there are many *in vitro* studies have been done[8-10]. These studies showed that supplementing ruminant's rations with cellulytic enzymes can improve feed utilization through increase the rate of fiber degradation. Morgavi *et al.*[11] attributed this positive effect to increase numbers of ruminal fibrolytic microbe's colonies on the surface of feed particles as direct response to cellulases addition. In contrast, it has been reported that fibrolytic enzymes treatment did not improve the rate and amount of degradability[12]. Therefore, the main objectives of this study were to optimize fungal cellulase production and evaluate the impact of the produced cellulase (Asperozym) and the commercial cellulase (Phytabex plus®) on the *in vitro* degradation of goat's rations.

MATERIALS AND METHODS

Rice straw as substrates: The air-dried rice straw was cut into 0.5-1 cm then dried at 70°C for 24 h in air-circulation oven and ground to powder from 5-10 mm in an electric grinder then packed and stored in dry place at room temperature till use.

Fungal cultures, media and inoculum preparation: Five fungal cultures were used for screening their ability for utilizing cellulose as main carbon source for cellulase production; *Asperigillus niger*, *Asperigillus flavus*, *Asperigillus fugimatus*, *Trichoderma viride* and *Penicillium chrysogenum* were obtained from Dairy Microbiology Laboratory-National Research Center, Cairo, Egypt. These fungal cultures were cultivated and maintained on potato dextrose agar medium (PDA). Malt medium containing malt extract (30 g L^{-1}), yeast extract (5 g L^{-1}) was used for preparing the activated fungal inocula; Cellulose Powder Medium (CPM) recommended by Fadel and Foda[13] was used for growth and cellulase production. The medium has the following composition (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 medium pH was adjusted to pH 6.0. Spores of fungi were transferred from surface of the actively growing slants of (PDA) medium to 250 mL conical flasks each containing 50 mL of malt medium. After incubation on rotary shaker (120 rpm) at 29±1°C for 48 h, the grown cultures were employed as inocula for experimental 250 mL conical flasks each containing 50 mL of CPM at rate of 5% (v/v) inoculum size.

Culture conditions for cellulase production: The effect of fungal cultures on cellulase production was studied. After 3 days of incubation at 29±1°C the filtrate of each fungal culture was used for cellulase activity detrmination. Effect of substrate concentration was investigated through replacing of cellulose powder in CPM by 2.5, 5, 7.5, 10, 12.5 and 15% (w/v) of rice straw. The fermented rice straw for each flask was mixed with 25 mL of 0.02 M acetate buffer (pH 5.0) by shaking in a rotary shaker (120 rpm) for 1 h at room temperature to extract the enzyme and the extracted mixture was filtered and collected for cellulase activity assay. Effect of inoculum size ranged from 2-10% (v/v) on cellulase activity was studied. Effect of the initial pH of growth medium was studied through adjusting the initial pH values in a range between 3 and 8 using either NaOH or HCL 0.1 N. Effect of nitrogen source included the use of three inorganic salts (ammonium sulphate, ammonium chloride and sodium nitrate and three organic sources (meat extract, yeast extract and peptone) were studied. Various nitrogen sources were used separately at an equivalent concentration of 0.33 g N L^{-1} media as recommended by Murad[14] these nitrogen sources

replaced the original nitrogen present in the CPM. The level of a parameter optimized in an experiment was maintained in the subsequent studies.

Enzymes source: Phytabex plus® is a commercial cellulolytic enzyme source produced by EN BIO., TECH CO., LTD-China and purchased from the company of IBEX International LTD (United Kingdom). Each gram of it contains 500 U of cellulase and 0.75×10^{10} CFU of *Bacillus subtilis*, while Asperozym is laboratory produced cellulase from *Asperigillus niger* and each gram of it contains 240 cellulase units.

Enzymes assay: The carboxymethyl-cellulase activity (CMCase) for Asperozym and Phytabex plus® were determined according to Mandels *et al.*[15]. A 0.5 mL of 1% carboxymethyl cellulose in 0.1 M citrate buffer pH 5 was placed in a test tube and 1.0 mL of both enzymes selutions were separately added. The test tube was incubated at 40°C in a water bath with shaker for 30 min. The reaction was terminated by adding 4.0 mL of 3,5-dinitrosalicylic acid (DNS) reagent to the reaction mixture, boiled for 5 min[16]. The absorbance of the appropriately diluted reaction mixture was read at 540 nm using a spectrophotometer. One cellulase unit is defined as the amount of enzyme that liberates reducing sugar at the rate of 1 μmol mL^{-1} min^{-1} under assay condition.

***In vitro* study:** *In vitro* dry matter and organic matter degradation (IVDMD and IVOMD) for the tested goat's rations were determined. The tested ration consisted of 50% concentrates feed mixture, 25% Egyptian clover and 25% wheat straw. Samples of 1 g of tested ration powder were accurately weighed into 250 mL incubation bottles. The experimental ration was supplemented separately with locally produced cellulase (Asperozym) and commercial cellulase source (Phytabex plus®) at 4 levels (500, 1000, 1500 and 2000 U kg^{-1} DM) compared with the control using 5 bottles per each supplementation level. Both enzymes solutions, buffer solution, macro-mineral and trace mineral solution were directly added to each bottle before 24 h of starting the incubation with rumen liquor and the reduction solution as described by Fondevila and Perez-Espes[17]. According to the farm management system, rumen contents were collected from rams fed Egyptian clover hay ration before the morning feeding, then moved directly to the laboratory in separate warmed oxygen-free plastic jars. Rumen liquor contents were strained through four layers of cheese-cloth and the obtained liquor was mixed with the previously mentioned solutions at 39°C under continuous flushing with CO_2. The bottles were sealed with rubber stoppers and incubated at 39°C for 48 h.

Statistical analysis: Data obtained from this study were statistically analysed by IBM SPSS (Version 20.0.) Statistics for Windows[18] using the following general model procedure:

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

where, Y_{ij} is the parameter under analysis of the ij flasks of cellulase production trails or bottles of *in vitro* rumen liquor trails, μ is the overall mean, T_i is the effect due to treatment on the parameter under analysis and 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[19].

RESULTS AND DISCUSSION

Impact of fungal cultures on cellulase activity: The capability of the tested fungal cultures on cellulase production on CPM was shown in Fig. 1. *Aspergillus niger* gave the highest ($p<0.05$) cellulase activity, while *Asperigillus fugimatu* gave the lowest cellulase activity. The supperurity of *A. niger* for cellulase production can be attributied to its elongated hyphae which produce mechanical pressure on the cellulose structure[7]. In our opinioun, the ability of *A. niger* for addapting with the change in the culutring conditions makes it more capable of growth and cellulase production than the other fungi. The findings of the present study mathcing the most recent studies which concluded that *A. niger* the best fungal strain for cellulase production[20,21]. So, *A. niger* was chosen for further studies on the cellulase production on CPM.

Effect of substrate concentration on cellulase production: Results illustrated in Fig. 2 showed the effects of different concentration of cellulytic substrate (rice straw) ranged from 2.5-15% (w/v) on cellulase production by *A. niger*. Maximum cellulase activity ($p<0.05$) reached (0.67 U mL^{-1}) was obtained at 10% (w/v) rice straw concentration, while the minimum cellulase activity reached (0.31 U mL^{-1}) was obtained at 2.5% rice straw concentration of production medium. The moisture content of the fungal growth media is a critical factor as it influences the growth, synthesis and secretion of enzymes and different metabolites[22,23]. The obtained results are agreed with those stated by Castilho *et al.*[24] that enzymes production by *A. niger* decreased when level of moisture increased, while Madhavan and Mangalanayaki[6] found that cellulase production give its maximum value by *A. niger* when banana

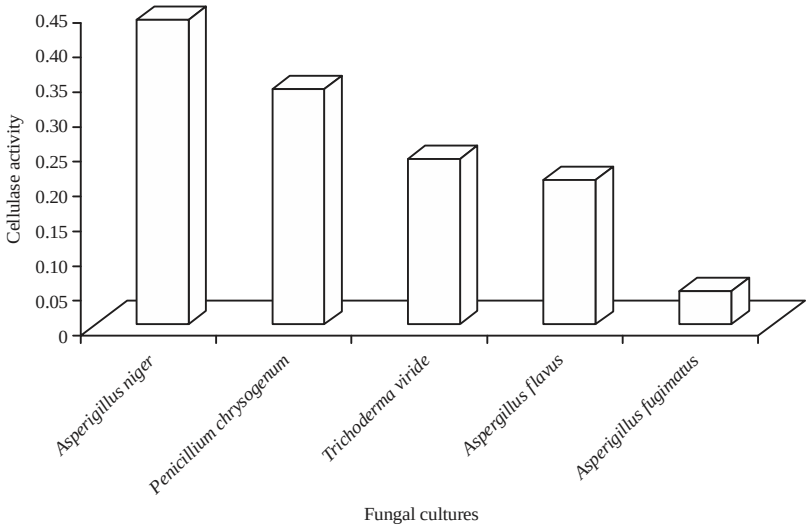

Fig. 1: Fungal cultures tested for cellulase production

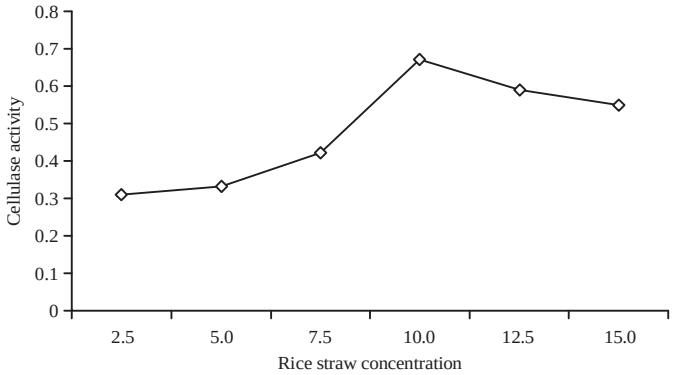

Fig. 2: Effect of rice straw concentration on cellulase production by *Asperigillus niger*

wastes were used as substrate at level of 2%. In the present study, higher moisture levels (2.5% rice straw concentration of CPM) can decrease the rate of enzyme production due to suppression of fungal growth through interparticle spaces reduction of the solid matrix which in turn negatively affects oxygen transfer[25]. In contrast, increase substrate concentration of the enzyme production media can cause decrease nutrients solubility and high water tension[26]. In addition, Acuna-Arguelles *et al.*[27] reported that, low water-availability media make fungi suffer cell membrane modifications which may affect the mechanism of nutrients transportation and subsequently affect the metabolism negatively. This may be the reason for reduction of cellulase enzyme activity at 15% of rice straw concentration of CPM. Based on the present results, rice straw concentration at 10% (w/v) was chosen for further studies on the cellulase production medium.

Effect of inoculum size: *Aspergillus niger* has exhibited different responses to variations in inoculum size from 2-10% (v/v). The results of Fig. 3 showed that the maximum cellulase productivity by *A. niger* was given at 4% inoculum size (0.48 U mL^{-1}) and further increase in the inoculum size led to decrease cellulase productivity. In this concern, Zhang *et al.*[28] stated that impact of inoculant amount on cellulase production by *Trichoderma viride* was small and 5% inoculum was the most suitable. Moreover, Alam *et al.*[29] reported that the maximum cellulase yield was obtained with 5% (v/w) of inoculum size when fermented oil palms biomass by *Trichoderma harzianum*. Nadagouda *et al.*[30] reported that lower inoculum size makes substrate utilization and cellulase formulation takes longe time. While the highest one can ensure rapid fungal biomass synthesis to certain limit, after it the enzyme production could decrease. This because of nutrients depletion due to biomass enhancement, which

would result in a decrease in metabolic activity. In this concern, Omojasola *et al*.[31] stated that inoculum sizes above 6% decreased cellulase yield when pineapple peels and pineapple pulp fermented by *A. niger* and attributed this to clumping of cells which causing reduction of sugar and oxygen uptake rate and in turn enzyme release. In the light of these results, 4% inoculum size was selected for conducting further studies on modified CPM by *A. niger.*

Effect of initial pH of growth medium: Medium initial pH has a great effect on the growth of the microorganism, permeability membrane, as well as on the biosynthesis and stability of the enzymes[32,33]. Figure 4 shows, cellulase yield by *A. niger* showed the maximum at pH 6.0 (0.45 U mL^{-1}), the further increase in pH of the growth medium causes decrease production of cellulase. This finding support the earlier data of Yadav *et al*.[34] who found that maximum cellulase production by fungi was obtained at pH 6 and the further increase in pH reduced the cellulase activity. In contrast, Mahalakshmi and Jayalakshmi[35] found marked increase in the cellulase yield by *A. niger* till pH 8, then cellulase yield gave its minimum at pH 9 in all treated substrates. Concerning with the optimum pH for cellulase production, the obtained results confirmed the previous data of Parry *et al*.[36] who found that optimal pH for CMCase from *A. niger* was 6-7, while Akiba *et al*.[37] found that pH 4.0-4.5 was optimal pH for cellulase production by *A. niger.* Based on the obtained results, the initial pH of cellulase production medium will adjusted to be pH 6 in subsequent experiments.

Effect of nitrogen source: The results of Fig. 5 showed that cellulase yield gave its maximum value (0.79 U mL^{-1}) when peptone was the source of nitrogen in *A. niger* growth medium. This data support earlier results of Acharya *et al*.[38] who found that peptone is the best nitrogen source for cellulase production by *A. niger* 1433. Furthermore, Enari and Markenan[39] found that maximum cellulase activity can be achieved by using yeast extract or peptone as organic nitrogen sources. In addition, Chuwech *et al*.[40] found that cellulase production by *Pycnoporus coccineus* gave its maximum activity when the growth medium nitrogen source was organic. Also, Abou-Taleb *et al*.[41] found that organic nitrogen sources more suitable for cellulase production by *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C23

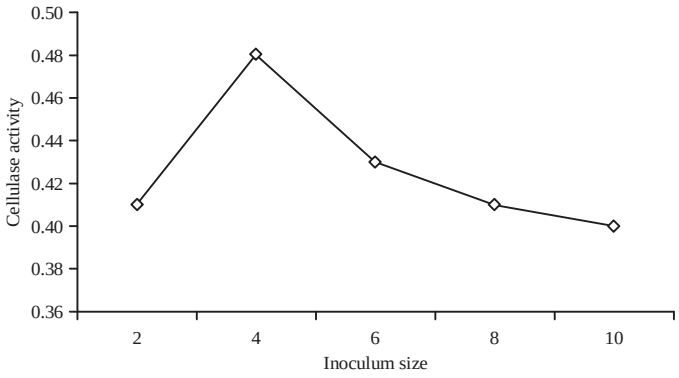

Fig. 3: Effect of inoculum size on cellulase production by *Asperigillus niger*

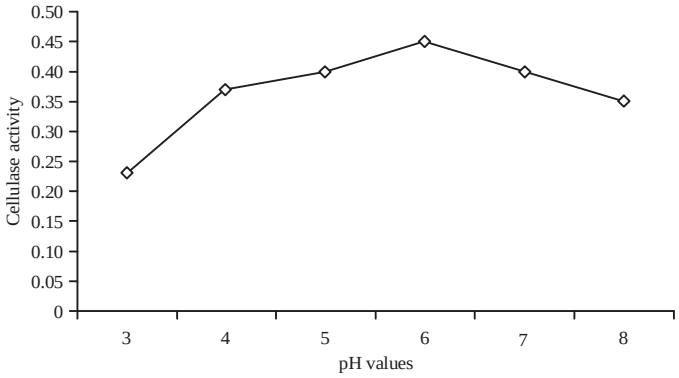

Fig. 4: Effect of initial pH of growth medium on cellulase production by *Asperigillus niger*

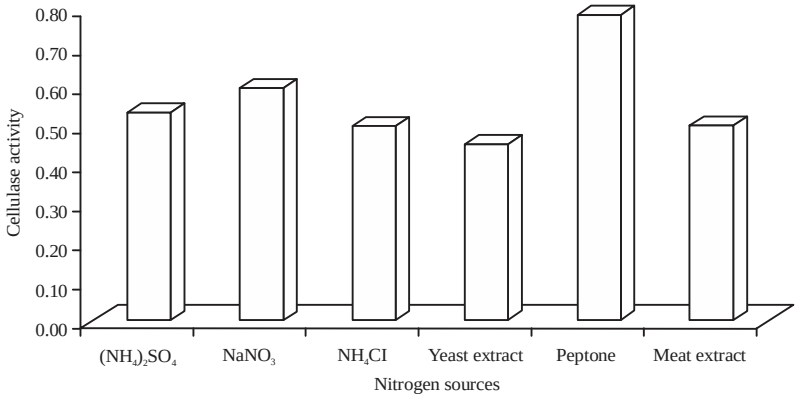

Fig. 5: Effect of nitrogen source on cellulase production by *Asperigillus niger*

Table 1: Cellulases effect on *in vitro* dry matter and organic matter degradation of tested ration

Enzymes source	Enzyme level (U kg^{-1} DM)	IVDMD (%)	Mean±SE	IVOMD (%)	Mean±SE
Control	0	34.99^{d}	1.86	40.87^{d}	1.73
Asperozym	500	40.55^{c}	1.55	47.95^{cd}	1.97
	1000	56.75^{a}	1.98	65.08^{a}	1.99
	1500	51.04^{b}	1.67	59.02^{b}	1.86
	2000	39.86^{cd}	1.51	47.03^{cd}	1.69
Phytabex plus®	500	39.77^{cd}	1.77	47.12^{cd}	1.89
	1000	57.05^{a}	1.85	65.96^{a}	1.70
	1500	50.99^{b}	1.96	58.71^{b}	1.79
	2000	40.43^{c}	1.09	48.06^{cd}	1.21

$^{a-d}$Different superscripts in the same column are significantly different at ($p<0.05$)

than inorganic sources. In contrast, Xavier and Lonsane[42] reported that inorganic nitrogen sources are the best for cellulase production by fungi. The presences of cellulase activity differences in the different studies may be due to use of different materials as substrate, different cultural practices and different microorganisms[20].

***In vitro* study:** Results of Table 1 showed that all Asperozym and Phytabex plus® supplementation levels increased ($p<0.05$) the *in vitro* DM and OM degradation for the tested ration compared to control one. Increasing the Asperozym and Phytabex plus® supplementation levels up to 1000 U kg^{-1} DM gave the highest values of *in vitro* DM and OM degradation. Further increase in both enzymes supplementation level up to 2000 U kg^{-1} DM led to decrease the DM and OM degradation of the tested ration. Many of *in vitro* studies reported the positive effects of fibrolytic enzymes supplementation on the nutrients digestibility[8,9,43]. There are many assumptions illustrate how fibrolytic enzymes improve feed utilization by rumen microorganisms including the following: (1) Enzymes-feed interaction period is necessary for any significant increases in ruminal digestion[44]. Kung *et al.*[45] reported that enzyme-feed interaction period give the chance for creating a stable enzyme-feed complex which protects the supplemented enzymes from proteolysis by the rumen microorganisms. (2) Increasing ability of the ruminal microorganisms to attachment and/or access to feed particles and subsequently accelerate the rate of digestion[46]. (3) Enhancing the hydrolytic activity of the ruminal microbes due to added enzyme activities and/or synergy with rumen microbial enzymes[11,47]. In the present study Asperozym and Phytabex plus® might have also altered the structure of the tested ration during the interaction period, making it more amenable to rumen microorganisms.

CONCLUSION

In light of the obtained data it can be concluded that using high yielding fungal strains, optimal fermentation conditions and cheap raw materials as a carbon source is the way for maximum and economical cellulase production. The laboratory produced cellulase enzyme (Asperozym) and the commercial one (Phytabex plus®) supplementation enhanced the *in vitro* rumen fermentation through increased amount of dry matter and organic matter degradation.

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