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Research Article
Optimizing Production of Tannase and *in vitro* Evaluation on Ruminal Fermentation, Degradability and Gas Production

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Abstract

Background and Objectives: Enhancing poor quality roughages by biological treatments has interested for many researches in last years. So, in the current study was carried out to production of tannase enzyme by *Aspergillus terreus* and its impact on ruminal fermentation, degradability and gas production were evaluated through *in vitro* trials. **Materials and Methods:** *Aspergillus terreus* was grown as stand cultures in 100 mL conical flasks containing tannic acid powder medium. The maximum production of tannase by *Aspergillus terreus* was achieved at inoculum ratio of 4% (v/v), 72 h of incubation period, initial pH 5.0, urea as a nitrogen sources at a concentration of 0.33 g N L^{-1} and pomegranate peel as a carbon source at a concentration of 10% (w/v). For animal feeding experiments *in vitro* dry matter, NDF, ADF, cellulose and hemicellulose disappearance and rumen fermentation were determined for the experimental diets. The concentrate:roughages ratio was 50:50 on DM basis, experimental diets plus different levels of tannase enzyme being 0, 5250, 10500, 15750, 21000 and 26250 IU kg^{-1} DM were studied. **Results:** Tannase enzyme increased DM, NDF, ADF, cellulose and hemicellulose degradability of the treated diets compared with the control diet (0.0 IU kg^{-1} DM), which gave the lowest values of diet degradability parameters. The maximum produced tannase efficiency percentage for dry matter degradability was obtained at 15750 IU kg^{-1} DM. Production of tannase enzyme by *Aspergillus terreus* strain under solid state fermentation was superior over tannase production from other fungal strains under the submerged cultures. **Conclusion:** In conclusion tannase enzyme had positive improving of feed digestion in the current *in vitro* study.

Key words: Tannase enzyme, *Aspergillus terreus*, *in vitro*, digestibility, rumen fermentations

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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

Tannase enzyme (tannin acyl hydrolase EC, 3.1.1.20) is present in animals, plants and it is mainly produced by various microorganisms like bacteria, yeast and fungi[1-3]. The enzyme is composed of two separated enzymes: an esterase that catalyzes the cleavage of ester and a depsidase that hydrolyzes the depside bonds[4]. Tannase enzyme can be catalyzed the hydrolysis of ester and peptide bonds present in complex tannins, gallotannins and gallic acid esters[5]. While, the enzyme has not affect the carbon-carbon bonds that found on condensed tannins[6-8]. Tannase completely hydrolyzes hydrolysable tannins, releasing gallic acid and glucose[9,10]. Also, Tannase enzyme hydrolyzes various substrates such as digallic acid, propyl gallate, epicatechin gallate, methyl gallate and epigallocatechin gallate-releasing gallic acid[11,12].

Tannase enzyme is used in several industrial processes such as; manufacture of instant tea, manufacture of coffee-flavored soft drinks, clarification of fruit juices and as an analytical probe for determination the structure gallic acid esters[13,14]. Tannins can also shift site of protein digestion (increase ruminal escape protein) and nitrogen excretion (from urine to feces) in ruminants, due to their ability to bind and precipitate protein[6]. Tannins also bind carbohydrates to some extent, which also have effect on fermentation and nutritional value of some diets[6,15,16]. Also, the enzyme was used as additive to reduce tannins effects in ruminant diets[15,16]. The present study was devoted for production of tannase enzyme by *Aspergillus terreus* in solid-state fermentation under the optimum condition and evaluates the impact of the resultant enzyme on rumen fermentation characteristics (*in vitro*).

MATERIALS AND METHODS

Tannase production experiment

Fungal strains: Nine Fungal strains were used for screening their abilities of utilizing tannic acid as main carbon source for production of tannase enzyme. The cultures included *Fusarium avenaceum*, *Fusarium oxysporum*, *Cephalosporium acremonium*, *Trichoderma viride*, *Asperigillus niger*, *Aspergillus fumigatus*, *Penicillium chrysogenum* and *Aspergillus terreus*. These cultures were obtained from laboratory of plant pathology of National Research Center, Cairo, Egypt and *Asperigillus flavus* NRRL 5522 (non-aflatoxin producer strain) was obtained from National Centre for Agriculture Utilization Research, Microbial Genomics and Bioprocessing Research Unit, Department of Agriculture, Peoria, Illinois, USA. The work was carried out in the dairy production laboratory at National Research Centre, Giza, Egypt, in April, 2018.

Microorganism maintenance and inoculum preparation: Fungal cultures were grown on potato dextrose agar medium (PDA). It grows rapidly at room temperature 25-37°C. The slant cultures were then used for further work or stored in refrigerator at 4°C. Malt extract medium containing malt extract (30 g L^{-1}), yeast extract (5 g L^{-1}) was used for preparing the activated fungal inocula, Tannic acid powder medium (TAPM) recommended by Murad *et al.*[17] was used for growth and tannase production.

Solid state fermentation was used for tannase enzyme production; the medium composition was as the follows: ammonium sulphate 1.7%, sodium chloride 0.1%, sodium phosphate 2.0% and pomegranate peels 10% with pH adjusted to 7.0. Spores of fungi were transferred from surface of the actively growing slants of (PDA) medium to 250 mL conical flasks which contained 50 mL of malt extract medium. After incubation on a rotary shaker (120 rpm) at 32°C for 48 h, the grown cultures were employed as inocula for experimental flasks (250 mL) contained the previous media at rate of 4% (v/v) inoculum size.

Optimization of fermentation process for tannase enzyme production: Enzyme production was carried out in 250 mL conical flasks containing 50 mL each from the previous medium. Static cultures were used for studying fungal tannase production under variable environmental condition as follows:

Effect of inoculum ratio: Inoculum ratios ranged from 1-8% (v/v) were used with the tested fungal cultures.

Effect of incubation period: Tannase assay was performed after various incubation periods i.e., 1, 2, 3, 4, 5 and 6 days (24-144 h) and the tannase activity was determined according to Sharma *et al.*[18].

Effect of initial pH: The influence of different initial pH values was studied through adjusting pH values at 3, 4, 5, 6, 7 and 8 using citric buffer.

Effect of nutrients sources

Effect of nitrogen sources: Various nitrogen sources were used separately at an equivalent concentration of 0.33 g (N L^{-1}) media as recommended by Murad[19]. The nitrogen

source included three inorganic salts (ammonium sulphate, ammonium chloride and urea) and three organic sources (meat extract, yeast extract and peptone). These sources replace the original nitrogen source in the test medium.

Effect of carbon sources: Influence of various carbon sources on tannase enzyme production were studied by testing different tannins containing waste materials including moringa cake, palm kernel, palm fronds, wheat straw, olive cake, pomegranate peels, range between 5-30% (w/v) to the fermentation media.

Assay of tannase: Tannase enzyme activity was determined by the method of Sharma *et al.*[18]. One unit of the tannase enzyme was defined as the amount of enzyme, which liberated 1 µmol of gallic acid in 1 min.

Stander curve: Stander curve was designed according to Sharma *et al.*[18]. Figure 1 represents the calibration curve for gallic acid, presenting linearity between 100 and 1000 µg L^{-1}.

In vitro study

Enzyme sources: Tannase was laboratory produced from *Aspergillus terreus*. Each kg contains 5250 international units (IU). Tannase activity for the produced enzyme was determined according to method of Sharma *et al.*[18].

Experimental diets: The experimental diets were formulated to meet the dairy animal's requirements. The concentrate to roughage ratio was 50:50 on DM basis. The concentrate feed mixture (CFM) consisted of 60% corn grains, 22.6% soybean meal, 15% wheat bran, 1% limestone, 0.4% minerals and 1% NaCl. The control diet was 50% concentrate feed mixture (CFM), 25% berseem hay and 25% date seeds powder. The control diet plus tannase enzyme at levels 5250, 10500, 15750, 21000 and 26250 IU kg^{-1} DM (T1, T2, T3, T4 and T5 respectively). The feed ingredients samples were analyzed according to the AOAC[20] methods to determine crude protein (CP), ether extract (EE) and ash contents. Organic matter (OM) and non-fiber carbohydrate (NFC) contents were calculated by difference. OM = 1000-Ash content, while NFC = 1000-(NDF+CP+EE+Ash contents). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined using the methods described by Van Soest *et al.*[21]. The chemical composition of feed ingredients and the control diet are shown in Table 1.

***In vitro* experiment:** *In vitro* dry matter, NDF, ADF, cellulose and hemicellulose disappearance were determined for the experimental diets using batch culture technique as described by Khattab *et al.*[22]. A sample of 500 mg of the control diet powder was weighed into 120 mL serum bottles. The bottles (3 replicates) were separately supplemented with rumen liquor, buffer solution and tannase enzyme at different levels 0, 5250, 10500, 15750, 21000 and 26250 IU kg^{-1} DM. 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 of CO_2. The bottles were sealed and maintained at 39°C in a shaking water bath (20 oscillations/min) for 48 h. After 48 h of incubation the pH value, total gas production volume. Substrates and substrate

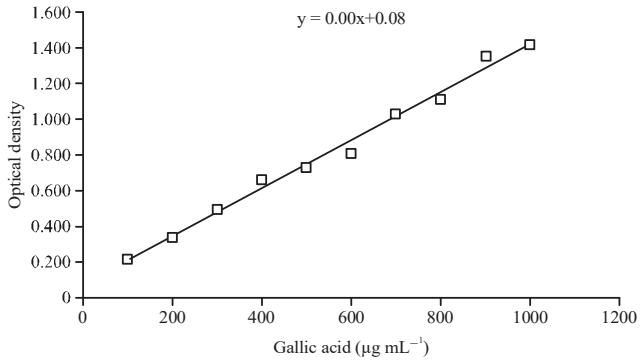

Fig. 1: Stander curve of gallic acid

Table 1: Chemical composition of feed ingredients and control diet

	DM (g kg^{-1})							
Feed ingredients	DM	OM	NDF	ADF	CP	EE	Ash	NFC
Corn grain	884.5	985.5	184.4	35.9	82.5	53.15	14.5	665.5
Soybean meal	888.8	932.7	150.6	64.6	387.6	47.80	67.3	346.7
Wheat bran	893.3	956.0	352.1	98.3	152.6	37.60	44.0	413.7
Date seeds powder	907.8	969.5	570.5	471.0	68.1	65.90	30.5	265.0
Clover hay	924.0	867.9	409.4	268.8	174.1	39.80	132.1	244.6
Control diet	889.8	944.2	343.6	210.3	140.2	50.50	55.8	409.9

DM: Dry matter, OM: Organic matter, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, CP: Crude protein, NFC: Non fiber carbohydrate, EE: Ether extract

residues after 48 h of incubation were dried at 70°C and analyzed for the amount of DM (DM digestibility) according to AOAC[20]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed by Ankom200 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY) according to Van Soest *et al.*[21].

Statistical analysis: Data were statistically analyzed using GLM procedure of SAS software (Version 9.2). Significant differences between means of treatments were carried out by the Duncan's test and the significance threshold was set at $p<0.05$.

RESULTS AND DISCUSSIONS

Effect of fungal strains on tannase enzyme production: Data presented in Fig. 2 Illustrated the difference between fungal strains employed for production of tannase enzyme. *Aspergillus terreus* exhibited the highest tannase activity than that of the other fungal strains, being 10643.54 IU mL^{-1} min^{-1}. These results are in agreement with those reported by Reddy and Reddy[23] who suggested that *Aspergillus terreus* which selected out of eight fungal isolates have the ability to grow on the presence of tannic acid as a carbon source by Submerged Fermentation.

Effect of inoculum size (%) on tannase production: Fungal culture exhibited different responses to variations in inoculum size from 1-8% (v/v). Data presented in Fig. 3 illustrated that the production of tannase enzyme by *Aspergillus terreus* was increased significantly by increasing inoculum ratio up to 4% (v/v) being 4208.20 IU mL^{-1} min^{-1}. Further increasing in inoculum ratio up to 8% led to decrease in tannase production by fungal cultures.

Effect of incubation period on tannase production: The effect of different incubation periods (24-144 h) on tannase enzyme production from *Aspergillus terreus* is shown in Fig. 4. The tannase production was gradually increased with rise of incubation period until 72 h and then decreased. Maximum tannase yield was obtained after 72 h reaching 6508.3 IU mL^{-1} min^{-1} of incubation. These results are in agreement with those reported by Reddy and Reddy[23], who reported that maximum tannase production by *Aspergillus terreus* was reached after 72 h of incubation. While, Lal *et al.*[24] found that maximum tannase production from *A. niger* was shown at 7th day. Paranthaman *et al.*[25] reported that the decreased enzyme yield on prolonged incubation could also be due to inhibition and denaturation of the enzyme. It has been reported before that tannase was produced during the primary phase of growth and thereafter the activity decreased either due to the decrease in production or due to enzyme degradation.

Effect of the initial pH values on tannase production: The medium was adjusted to different initial pH values i.e., from pH 3.0-8.0 and tannase production was studied. The highest value of tannase activity was recorded at pH 5.0 (8234.46 IU mL^{-1} min^{-1}) (Fig. 5).

These results are in line with those reported by Reddy and Reddy[23], who confirmed that the tannase activities from *Aspergillus terreus* peaking at a pH of 5.0. Also, Murad *et al.*[17] and Lal *et al.*[24] found that optimum pH for tannase enzyme production by *Aspergillus niger* was shown at 5.0.

In this study, a low level of enzyme production was noted with increasing pH values up to pH 5.0. This may be due to the increased activity of tannase enzyme at acidic pH and this activity was decreased as the pH approached the alkaline range[17,26]. Any change of pH value affects the protein

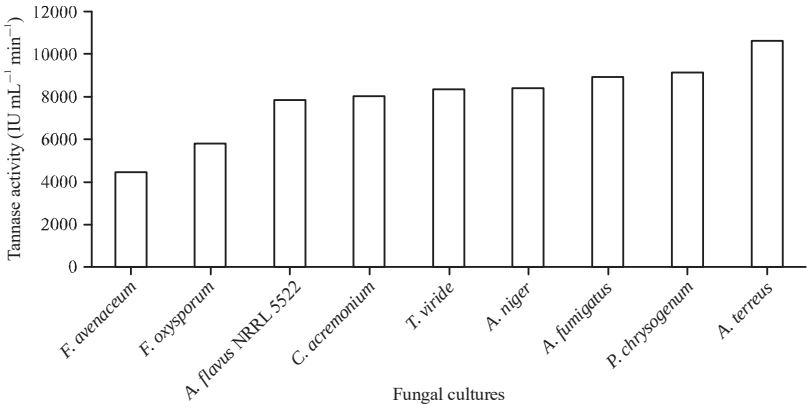

Fig. 2: Effect of fungal strain on tannase production

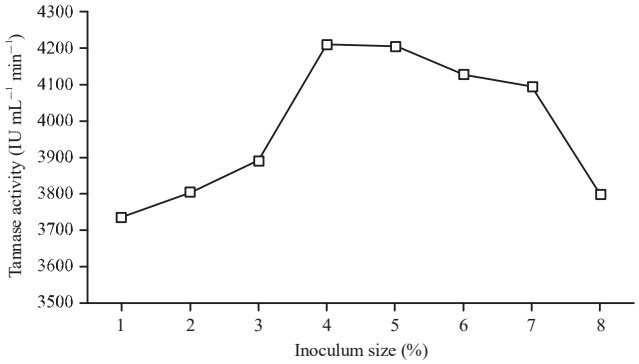

Fig. 3: Effect of inoculum size (%) on tannase production

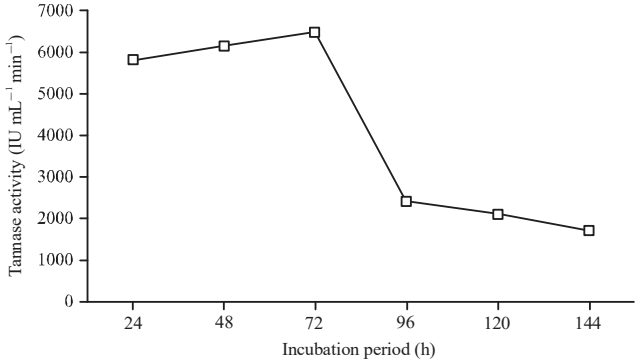

Fig. 4: Effect of incubation period on tannase production

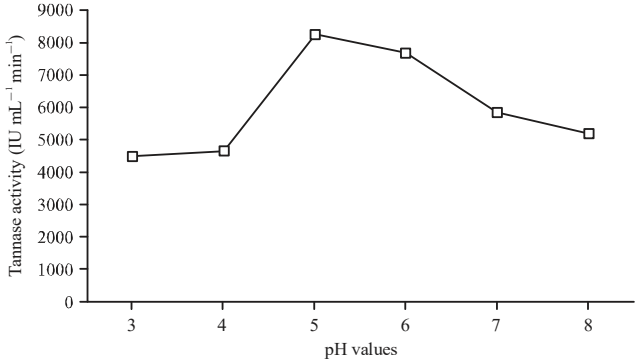

Fig. 5: Effect of the initial pH values on tannase production

structure and a decline in enzyme activation or its instability nature[27]. It could be concluded from the results that tannase from the *Aspergillus terreus* needed an acidic environment to be active. In general, fungal tannase is an acidic protein[28].

Effect of nitrogen sources on tannase production: The effect of supplementation with different nitrogen sources (meat extract, yeast extract, peptone, ammonium chloride, ammonium sulfate and urea) on the fungal growth and tannase enzyme production were studied and results are shown in Fig. 6. Maximum enzyme production was observed with urea as an inorganic nitrogen source (7772.92 IU mL^{-1} min^{-1}). while, meat extract was found to be the best organic nitrogen source producing the highest level of tannase enzyme activity by *Aspergillus terreus* being 6979.08 IU mL^{-1} min^{-1}. Similar results were reported by Kulkarni *et al.*[29], who found that the addition of beef extract yielded the highest tannase enzyme activity. But, Reddy and Reddy[23] reported that the maximum tannase enzyme production was observed with yeast extract. This result indicate that the source of nitrogen should be inorganic for obtaining better tannase enzyme activity. The nitrogen is very important limiting factor in the microorganism production of enzymes. Addition of nitrogen source to the substrate may have promoted cell growth and enzyme production[30].

Effect of different substrates on tannase production: Data in Fig. 7 Illustrated the use of available agriculture wastes as a carbon sources in the growth medium. It was known that using agriculture wastes are generally considered the best substrates for the process of enzyme production based on solid stat fermentation and reduce the costs of enzyme production[31]. Among of the available substrate materials tested Pomegranate peel gave the maximum tannase production gave 7198 IU mL^{-1} min^{-1} with using *Aspergillus terreus* flowed by olive cake with activity reached 1496 IU mL^{-1} min^{-1}, while, Moringa cake produced the lowest activity (487 IU mL^{-1} min^{-1}). These results indicated that tannase enzyme production was varied with the type of waste used as substrate[17]. This could be attributed to that solid materials have dual roles of supply nutrients to the microbial culture[23].

Effect of pomegranate peel concentration in the growth medium tannase production: The effect of moisture content on the growth and production of tannase enzyme was studied at different moisture levels (Fig. 8). The moisture level content is one of the most critical factors which affecting of tannase enzyme production[32]. In general, results showed that maximum tannase production was observed at 10% (w/v) of pomegranate peels. The activity reached 3654.36 IU mL^{-1} min^{-1}.

Nutrients digestibility: The obtained results showed that all levels of the produced tannase increased DM, NDF, ADF, cellulose and hemicellulose degradability of the treated diets compared with the control diet, which gave the lowest values

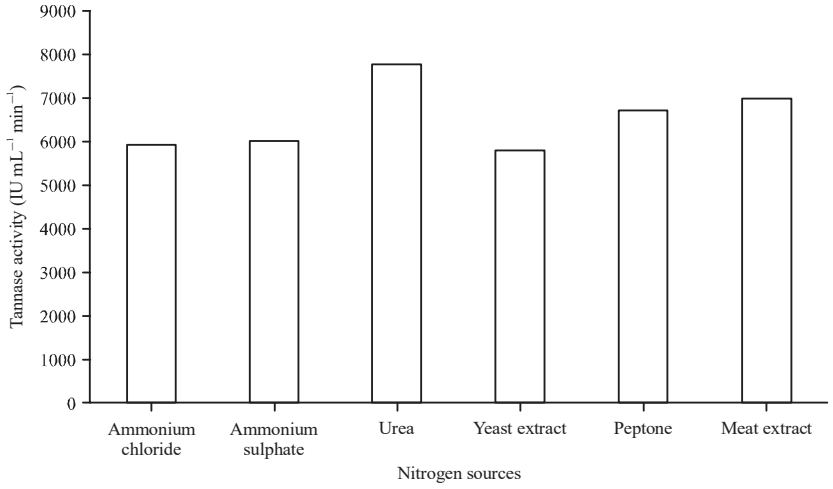

Fig. 6: Effect of nitrogen sources on tannase production

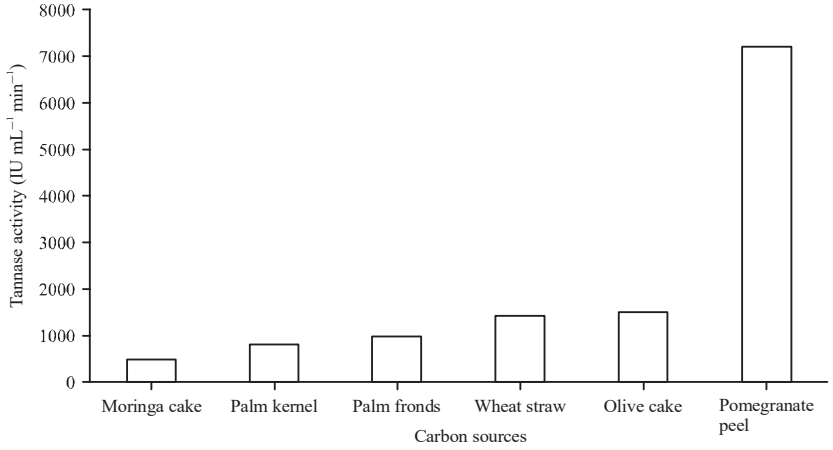

Fig. 7: Effect of different substrates on tannase production

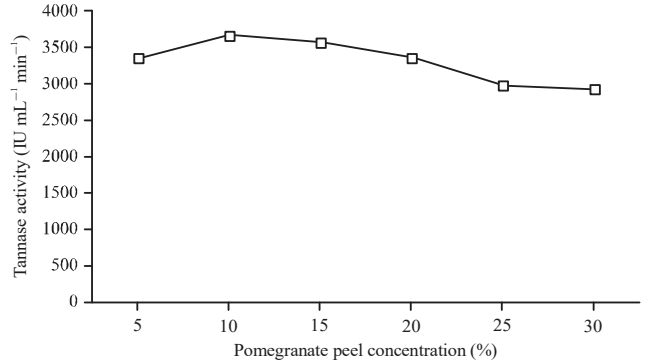

Fig. 8: Effect of pomegranate peel concentration in the growth medium on tannase production

of diet degradability parameters. The maximum produced tannase efficiency percentage for dry matter degradability can be calculated as:

$$\text{Enzyme efficiency (\%)} = \frac{\text{DMD (\%) (treated diet)-DMD (\%) (control diet)}}{\text{DMD (\%) (control diet)}} \times 100$$

The maximum produced tannase efficiency percentage for dry matter degradability was obtained at 15750 IU kg^{-1} DM (Table 2). Improvement of diet degradability parameters could be attributed to effective action of tannase enzyme (catalyzes the hydrolysis of ester and depside bonds present in the tannins) on tannins contents by reduce their toxic effects on the rumen microbes and allow for more proteins, carbohydrates and minerals liberating. Similar results were reported by Abd El Tawab *et al.*[6,15], who found that adding tannase enzyme increased dry matter, organic matter and crude fibre degradability. Also, the use of tannase as an ingredient of animal feed would improve the digestibility of the feed[28].

Table 2: Tannase effects on degradability parameters of experimental diets (*in vitro*)

Treatments	Enzyme levels (IU kg^{-1})	Diet degradability parameters (%) DM	NDF	ADF	Cellulose	Hemicellulose	Enzyme efficiency (%)
Control	0	55.67±0.83^{d}	34.20±2.36^{c}	31.11±0.61^{b}	37.64±0.94^{bc}	38.51±4.74^{a}	0.00
Produced tannase	5250	57.56±0.10^{cd}	37.10±2.57^{bc}	31.78±4.50^{b}	34.11±5.45^{c}	44.32±0.253^{a}	3.40
	10500	58.52±0.23^{cd}	41.28±2.66^{ab}	40.05±2.68^{a}	45.97±2.83^{ab}	42.98±2.745^{a}	5.12
	15750	65.23±1.37^{a}	47.61±1.54^{a}	47.40±2.84^{a}	51.78±5.10^{a}	47.92±0.759^{a}	17.17
	21000	62.77±0.23^{ab}	43.89±2.00^{ab}	44.03±1.55^{a}	51.77±1.13^{a}	43.68±2.723^{a}	12.75
	26250	60.03±1.67^{bc}	43.16±0.43^{ab}	43.10±2.09^{a}	50.28±1.38^{a}	43.37±3.218^{a}	7.83

DM: Dry Matter, MDF: Neutral detergent fiber, ADF: Acid detergent fiber

Table 3: Tannase effects on ruminal parameters (*in vitro*)

Treatments	Enzyme levels (IU g^{-1})	TGP (mL)	pH	NH_3 (μmol L^{-1})	TVFA (mEq dL^{-1})
Control	0	158.00±2.645^{a}	6.44±0.030^{a}	40.85±2.591^{a}	6.13±0.033^{a}
Produced tannase	5250	150.00±1.154^{b}	6.46±0.045^{a}	32.75±2.057^{bc}	6.10±0.435^{a}
	10500	157.00±1.527^{a}	6.41±0.011^{a}	31.87±2.361^{c}	6.43±0.796^{a}
	15750	157.33±2.333^{a}	6.45±0.006^{a}	38.98±1.183^{abc}	6.50±0.650^{a}
	21000	160.67±2.185^{a}	6.47±0.047^{a}	40.49±1.710^{ab}	6.03±0.120^{a}
	26250	160.00±0.577^{a}	6.45±0.029^{a}	37.84±3.440^{abc}	6.10±0.200^{a}

Rumen fermentations: There are no marked changes in the ruminal parameters (pH, ammonia (NH_3) concentration and the total gas production (TGP)) after treatment with the produced tannase compared with the control (Table 3). The total volatile fatty acids (TVFA) concentration show slight increase after treatment with the produced tannase at addition level of 15750 IU kg^{-1} compared with the control. This may attribute to the enhancement effect of the produced tannase on proteins and carbohydrates degradability of treated diet[6,15,33].

CONCLUSION

Summarizing the data presented in this study, it can be concluded that production of tannase enzyme by *Aspergillus terreus* strain under solid state fermentation was superior over tannase production from other fungal strains under submerged cultures. Also, the results indicated that tannase enzyme had a positive effect on feed digestion in the *in vitro* study.

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