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Fungal Pectinase Production Optimization and its Application in Buffaloe's Diets Degradation

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Abstract

Pectinase production for improving buffalo's diets digestion is the main objective of this work. Effects of fungal strains and different cultivation conditions on pectinase production have been studied. *In vitro* batch culture technique was used for investigate impact of the produced pectinase compared with commercial pectinase (SMIZYME®) on rumen fermentation parameters and diet degradation. *Penicillium chrysogenum* exhibited the highest pectinase activity at 3 days of incubation period, initial pH 4 of the growth medium, yeast extract as a sole nitrogen source and pomegranate peel as a carbon source at a concentration of 15 % (W/V). Three (g/kg) of the both enzymes supplementation significantly increased treated diet's dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF) degradability with increase total gas production (TGP) and short chain fatty acids (SCFA) concentration. The enlargement of pectinase production locally will lead to animal production improvement, encourage self-reliance and reduce the cost of enzymes importation.

Keywords: Pectinase Production; *Penicillium Chrysogenum*; Pomegranate Peel; Rumen Microorganisms and Buffaloes' Diets

Abbreviations: DM: Dry Matter; NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber; TGP: Total Gas Production; SCFA: Short Chain Fatty Acids; PDA: Potato Dextrose Agar; PPPM: Pomegranate Peel Powder Medium; CMC: Carboxymethyl-Cellulase; BPPM: Beet Pulp Powder Medium; TMR: Total Mixed Ration; VFA: Volatile Fatty Acids.

Introduction

Pectinase is a generic name group of enzymes that catalyze break down of the glycosidic bonds of the galacturonic acid long chain residues in the pectin rich plants [1]. Pectinases make up almost 25% of the global food enzyme market because of its wide range of applications in food, feed and pharmaceutical industries

[2]. It is expected that value of food enzymes market will increase up to $ 41.4 billion by 2020, with a compounded annual growth rate of 6.7% [3]. The major constraint for commercialization of new sources of enzymes is higher cost of the production [4]. It is thought that define the optimal microbial cultivation conditions for capable microbial strains and cheap raw substrate may reduce the cost of enzymes production [5]. A large number of bacteria, yeasts and many filamentous fungi are potential pectinase producers [6]. Fungi (ex: *T. viride, A. flavus, A. niger, F. oxysporum, A. terreus, and P. chrysogenum*) have attracted the most attention as enzymes producers because of its prolific yield and its long history in fermentation industries [1,5,7-9]. Submerged fermentation technique is still the most favorable system for microbial enzymes production in large-scale, despite a lot of research papers recommended solid state fermentation for microbial enzymes (ex: pectinase) production [1,7]. Utilization of agricultural residues as carbon sources in pectinase production media has yielded good enzyme activity with reduction of the production cost [6,7].

Utilization of microbial pectinases in agriculture waste treatments or as livestock feed supplements was expanded in the last two decades [6]. Many studies reported good impact of pectinase as a fibrolytic enzyme on ruminant's diets degradation *in vitro* and *in vivo* [4,10-12]. Addition of pectinase to ruminant's feeds can reduces the feed viscosity, which increases absorption of nutrients, liberates nutrients, either by hydrolysis of non-biodegradable fibers or by liberating nutrients blocked by these fibers, and reduces the amount of feces [7]. It is well known that pectin constitute the main component of cell wall of orange peel, sugar beet pulp, and pomegranate peel, that become essential source for livestock feeding in Egypt. Therefore, this study was carried out to optimize production of fungal pectinase, and evaluates impact of the produced pectinase for improving degradation of buffaloe's diets rich in pectin.

Material and Methods

Fungi and Inoculum Preparation

Fusarium avenaceum, *Asperigillus fugimatus*, *Cephalosporium acremonium*, *Trichoderma viride*, *Aspergillus niger, Fusarium oxysporum, Aspergillus terreus,* and *Penicillium chrysogenum* were obtained from Dairy Microbiology LAB - National Research Centre, Giza, Egypt. These fungi were cultivated and maintained on potato dextrose agar medium (PDA). Malt medium containing malt extract (30 g/l); yeast extract (5 g/l) was used for preparing the activated fungal inocula; beet pulp powder medium (BPPM) was used for growth and pectinase production. The medium has the following composition (g/l) 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; beet pulp powder, 10.0 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 a rotary shaker (120 rpm) at 29 $^+$ 1^{o}C for 48 h, the grown culture was employed as inocula for experimental 1000 ml conical flasks containing 100 ml (BPPM) at rate of 5 % (V/V) inoculum size.

Culture Conditions Affecting Pectinase Production

Static cultures were used for studying pectinase production by *Penicillium chrysogenum* under variable condition including substrate (pomegranate peel) concentration effect, incubation period, initial pH and nitrogen source. The general procedure included use of triplicate of 1000 ml conical flasks each containing 100 ml of pomegranate peel powder medium (PPPM).

Effect of different substrate sources includes pea pods**,** pomegranate peel, sugar beet pulp and orange peel on pectinase production by *Penicillium chrysogenum* was investigated.

Effect of substrate (pomegranate peel) concentration ranged from 2.5 % to 20% (W/V) on pectinase production by *Penicillium chrysogenum* was investigated. The fermented pomegranate peel for each flask was mixed with 100ml of 0.02M acetate buffer (pH 5.0) by shaking in a rotary shaker (120 rpm) for one hour at room temperature to extract the enzyme and the extracted mixture was filtered and collected for pectinase activity assay.

The influence of incubation period was studied through determination of pectinase activities after 24, 48, 72, 96, 120, 144 and 168h. 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 0.1N NaOH or HCl.

Effect of nitrogen source included the use of two inorganic salts (ammonium sulphate, and ammonium chloride; and three organic sources (Peptone, yeast extract and urea) were studied. Various nitrogen sources were used separately at an equivalent concentration of 0.33 g N/l media as recommended by Murad, et al. These

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

Enzymes Sources

The produced enzyme: Laboratory produced fibrolytic enzymes from *Penicillium chrysogenum.* Each gram contains 200 units of pectinase and 98.82 unit of cellulase. It worth to mention that, the pectinase units of each gram of the produced enzyme was adjusted to be equivalent to that found in the commercial enzyme product (SMIZYME®).

SMIZYME: A commercial enzymes source from AGRI-VET Company, Egypt. Each gram contains 200 unit of pectinase, 100 unit of cellulase, 2500 unit of α- amylase, 10000 unit of protease, 5000 unit of xylanase, 300 unit of mannanase, and 3000 unit of beta-glucannase.

Enzymes assay: The pectinase and carboxymethyl-cellulase activities (CMC) for resultant crude enzyme extract (the produced enzyme) and commercial enzyme source (SMIZYME®) were determined according to Buga, et al. [13] Mandels, et al. [14] respectively. One unit of pectinase activity was defined as the amount of enzyme that produced one µmole of D-galacturonic acid per minute at 40°C and pH 5.0 [15], while one cellulase unit was defined as the amount of enzyme that liberates reducing sugar at the rate of one µmol/ ml/min under assay condition [16].

***In vitro* trial of the tested rations:** Batch fermentation culture experiment was conducted according to Ismail, et al. [17] to evaluate impact of the produced and commercial fibrolytic enzymes sources addition to ruminant's diets on rumen fermentation characteristics. A total mixed ration consisted of of 50% concentrates feed mixture, 20% Egyptian clover, 20 % Sugar beet pulp and 10% dried orange pulp was used as a substrate. The concentrate feed mixture consisted of 55% corn, 20 % soybean meal, 21.5 % wheat bran, 1.5 % limestone, 0.5 % di calcium phosphate, 0.2 % yeast, 0.3 % bicarbonate, 0.5 % premix and 0.5 % NaCl. For obtaining of the rumen microorganisms (inoculum), rumen fluid was collected from rumen of slaughtered rams fed clover hay ration. The produced and the commercial fibrolytic enzymes were added to control ration at the following levels: 0, 1, 2, 3, 4 and 5g/kg on DM basis. Each treatment was tested in 3 replicates accompanied by 3 blank vessels (no substrate). The tested rations (400 mg) were added separately to the 125 ml incubation vessels. Each vessel was filled with 40 ml of mixture of 1:3 (v/v) rumen fluids: buffer solution**.** All vessels were sealed and incubated at 39˚C for 24 h. After 24 h of incubation, all vessels were filtered in fiber filter bags 25 micron porosity (ANKOM-USA). The residues in the bags were dried at 70˚C in oven for 48 h to analyse dry matter (DM), neutral detergent fiber (NDF) and acid detergent fiber (ADF) digestibility. Rumen fluid pH was measured using (pH-meter). Overall volume of the produced gases was determined using Hohenheim Syringes (100 ml) as described by Navarro-Villa, et al. [18]. Quantitative analysis of ammonia concentration was carried out by a modified Nessler's method [19]. The short chain fatty acids (SCFA) concentration was calculated according to equation of Makkar, et al. [20].

$$SCFA\ (mmol) = 0.0222\ Gas - 0.00425$$

Where, Gas: is gas production at 24 hours incubation (ml/200 mg DM).

Statistical Analysis

Statistical analyses were conducted by the general linear model procedure adapted by IBM Corp [21]. Released according to the following model:

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

Where Y_{ij} is the dependent variable, µ is the overall mean, T_i is the effect of treatment and e_{ij} is the residual error. Duncan's multiple test Duncan, et al. [22] was carried out to separate among means.

Results and Discussion

Impact of Fungal Cultures on Pectinase Production

The capability of *Fusarium avenaceum, Trichoderma viride, Asperigillus flavus NRRL 5522, Cephalosporium acremonium, Fusarium oxysporum, Aspergillus niger, Asperigillus fugimatus, Penicillium chrysogenum and Aspergillus terreus* on pectinase production on beet pulp powder medium (BPPM) was shown in Table 1. *Penicillium chrysogenum* gave significantly ($P<0.05$) the highest pectinase activity (10.87 µmole/ml/min) followed by *Aspergillus terreus* (9.37 µmole/ml/min) while the other fungal cultures gave low activity especially *Fusarium avenaceum* which gave the lowest pectinase activity (8.09 µmole/ml/min). Pectinases produce by many of microorganisms including fungi, yeasts and bacteria [23-25]. However, almost all the commercial preparations of pectinases are produced from fungal sources [26,27]. Pectinase production by filamentous fungi varies according to the type of strain, cultivation conditions and the growth medium composition [1]. On

the light of these results, *Penicillium chrysogenum* was chosen for further studies for pectinase production.

Table 1: Effect of fungal cultures on the produced pectinase activity (µmole/ml/min).

Fungal cultures	Pectinase activity (µmole/ml/min)	± SE
Trichoderma viride	8.24^{d}	0.05
Asperigillus flavus NRRL 5522	8.54^{cd}	0.08
Aspergillus niger	8.77^{c}	0.28
Fusarium oxysporum	8.86^{c}	0.13
Aspergillus terreus	9.37^{b}	0.08
Penicillium chrysogenum	10.87^{a}	0.12
Fusarium avenaceum	8.09^{d}	0.15
Cephalosporium acremonium	8.19^{d}	0.09
Asperigillus fugimatus	8.12^{d}	0.06

Average in the same column having different superscripts are differ significantly (P<0.05) for a, b, c and d.

Table 2: Effect of different carbon sources, pH value, concentration of pomegranate peel, incubation period and nitrogen sources on produced pectinase enzyme (µmole/ml/min) from *Penicillium chrysogenum*.

Different carbon sources								± SE
Pea pods		pomegranate peel		Sugar beet pulp		Orange peel		0.27
8.79^{c}		10.53^{a}		8.29^{c}		9.79^{b}		
pH value								0.36
3	4	5	6	7		8		
27.01^{b}	28.44^{a}	26.18^{c}	25.77^{c}	25.72^{c}		24.7^{d}		
Concentration of pomegranate peel (%)								1.002
2.5	5	7.5	10	12.5	15	17.5	20	
15.49^{e}	17^{e}	20.9^{d}	22.33^{d}	25.4^{c}	29.53^{a}	27.57^{ab}	26.36^{bc}	
incubation period (day)								0.442
1	2	3	4	5	6	7		
26.6^{d}	29.53^{b}	31.55^{a}	29.57^{b}	29.18^{bc}	28.52^{c}	26.94^{d}		
Nitrogen sources								0.47
$(NH4)_2SO_4$		NH_4Cl		Yeast extract		Peptone	Urea	
29.82^{c}		31.71^{b}		33.97^{a}		31.21^{bc}	31.82^{b}	

Average in the same row having different superscripts are differ significantly (P<0.05) for a, b, c, d and e.

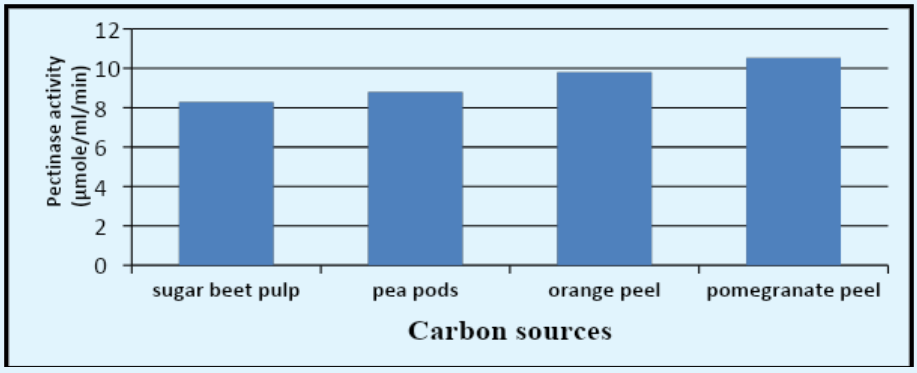

Figure 1: Impact of different carbon sources on pectinase production.

Effect of Different Carbon Sources on Pectinase Production

It is generally agreed that the optimum medium for the enhanced production of pectinase is that containing pectic materials as an inducer [28-30]. The selection of a suitable substrate for the fermentation process is a critical factor. As shown in Table 2 and Figure 1 Pomegranate peel as pectolytic substrate increased significantly (P<0.05) pectinase activity by *Penicillium chrysogenum* reached (10.53 µmole/ml/min), while sugar beet pulp gave the lowest pectinase activity (8.29 µmole/ml/min). From the previous data, pomegranate peel selected as a sole carbon source for conducting further studies on pectinase production by *Penicillium chrysogenum*.

Effect of Carbon Source Concentration on Pectinase Production

Data illustrated in Table 2 and Figure 2 showed effect of different concentration of pomegranate peel powder ranged from 2.5% to 20% (W/V) on pectinase production by *Penicillium chrysogenum.* Maximum pectinase activity (P<0.05) reached (29.53 µmole/ml/min) was obtained at 15% (w/v) pomegranate peel powder concentration, while the minimum activity reached (15.49 µmole/ml/min) was obtained at 2.5% of pomegranate peel powder concentration of *Penicillium chrysogenum* growth medium. This result is in line with that obtained by Castilho, et al. [31] who found that pectinase formation by *A. niger* decreases at high moisture levels. Moisture content is a critical factor on enzymes production processes because this variable has influence on growth and biosynthesis and secretion of different metabolites [32,33]. Higher moisture levels (as 2.5% pomegranate peel powder concentration can cause a reduction in enzyme yield due to steric hindrance of the growth of the producer strain by reduction in porosity (interparticle spaces) of the solid matrix, thus interfering oxygen transfer [34]. On the other hand, Mamma, et al. [35] reported that lower moisture content causes reduction in solubility of nutrients of the substrate, low degree of swelling and high water tension. In addition, *Acuna Arguelles*, et al. [36] reported that, in media with low water-availability fungi suffer modifications in their cell membrane, leading to transport limitations and affecting microbial metabolism. This may be the reason for reduction of pectinase enzyme activity at 17.5 and 20% of pomegranate peel powder concentration of fungal growth medium. Based on these data, pomegranate peel powder concentration at 15% (w/v) was chosen for further studies.

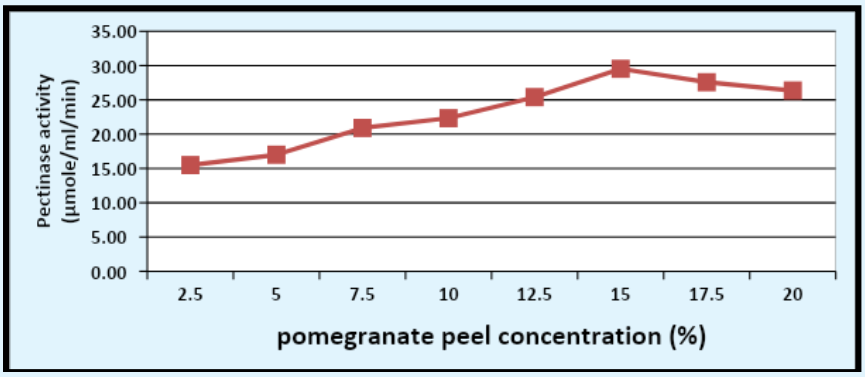

Figure 2: Impact of carbon source concentration on pectinase production.

Effect of Initial pH of Fungal Growth Medium on Pectinase Production

As shown in Table 2 and Figure 3 initial pH of the medium has profound effect on pectinase production. Pectinase production by *Penicillium chrysogenum* grown on deferent pH values of pomegranate peel powder showed its highest values (P<0.05) reached (28.44 µmole/ml/min), at pH 4.0. According to Dalagnol, et al. [37] extreme pH values may change the protein structure, leading to a partial or irreversible denaturation, resulting in a loss of activity. Moreover when the pH level increased, the enzyme production decreased. The initial pH of the medium has a great effect on the growth of the organism, permeability membrane, as well as on the biosynthesis and stability of the enzymes [38,39]. Optimum production of pectic enzymes from many molds

within the acidic pH range has been reported by previous studies [37,40-42]. While, Debing, et al. [43] found that the pH 6.5 was the optimal pH for pectinase production from *A. niger* by solid state fermentation. Also, Rasheedha, et al. [44] found that *P. chrysogenum* exhibited maximum polygalacturonase production at initial pH of 6.5. Based on the obtained results, the initial pH of the medium was adjusted to pH 4.0 in subsequent experiments.

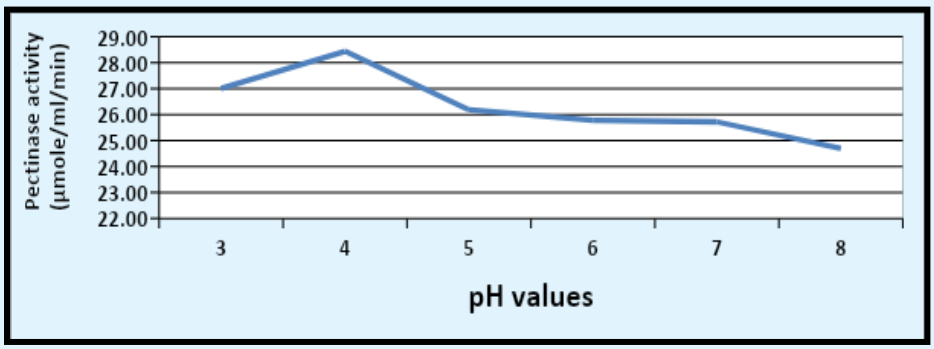

Figure 3: Effect of initial pH of fungal growth medium on pectinase production.

Effect of Incubation Period on Pectinase Production

Production of pectinase was monitored for a period of seven days as shown in Table 2 and Figure 4. The highest pectinase activity ($P<0.05$) reached (31.55 µmole/ml/min) was recorded after three days of incubation with *Penicillium chrysogenum.* In addition, when the incubation period increased, the enzyme production decreased. The time of incubation depends on the growth rate of the microorganism and its enzyme production pattern. Ghildyal, et al. [45] investigated that, maximum production of pectic enzyme from different molds varies from 1 to 6 days. Castilho, et al. [31] reported that, highest pectinase activity was obtained by *A. niger*, for fermentation time 22 h while, Rangarajan, et al. [46] found that, pectinase show maximum activity after 40 h of fermentation by *A. niger* grow on orange peel. In addition, Leda, et al. [47] reported that, the highest polygalacturonase activities were obtained by *A. niger* after 70 h of fermentation period. Moreover, Sarvamangala, et al. [48] observed a gradual increase in the production of pectinase from deseeded sunflower head by *A. niger* after 72 h of fermentation period in submerged and up to 96 h in solid-state conditions. From the previous data, 3 days incubation period was selected for conducting further studies on modified pomegranate peel powder medium.

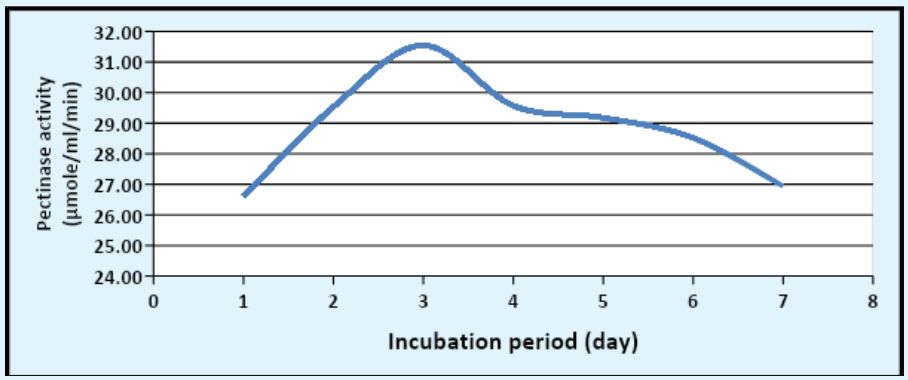

Figure 4: Effect of incubation period on pectinase production.

Effect of Nitrogen Sources on Pectinase Production

The effects of organic and inorganic nitrogen sources on the production of pectinase were extensively studied [49-51]. Data of Table 2 and Figure 5 showed that, among five nitrogen sources tested for screening their effect on pectinase production. Yeast extract was found to be the best nitrogen source producing the highest level (P<0.05) of pectinase activity (33.97 µmole/ml/min) by *Penicillium chrysogenum*. These data indicating that the source of nitrogen should be organic for the best level of pectinase production. Our results are in line with the work of Azzaz, et al. [6] who stated that, yeast extract was found to be the best nitrogen source for pectinase production by *A. niger* (3.78 U/ml) on beet pulp powder media. Also, Aguilar, et al. [49] who reported that yeast extract the best inducer of pectinases by *Aspergillus* sp. Moreover, Kashyap, et al. [50] found that, yeast extract, peptone and ammonium chloride were found to enhance pectinase production up to 24% and addition of glycine, urea and ammonium nitrate inhibited pectinase production. In addition, Vivek, et al. [51] found that, organic nitrogen sources showed higher endo, exo pectinases activities than inorganic nitrogen sources. Also the increasing trend in the enzymes activity with the increase in nitrogen source content was observed in the case of organic nitrogen sources while decreasing trend observed for inorganic nitrogen sources.

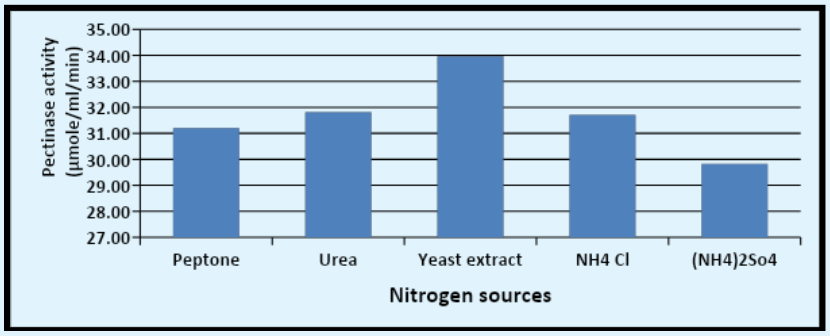

Figure 5: Effect of nitrogen sources on pectinase production.

In Vitro DMD, NDF and ADF Degradation of the Tested Rations

Results of Table 3 showed that, all the produced enzyme and SMIZYME® supplementation levels increased (P<0.05) the *in vitro* DMD, NDF and ADF degradation for the tested rations compared to control one. Increasing the produced enzyme and SMIZYME® supplementation levels up to 3g/kg DM gave the highest values of *in vitro* DMD degradation.

Table 3: Fibrolytic enzymes effect on DMD, NDF and ADF degradation of the tested rations (*in vitro*).

Enzymes Source	Enzyme level g/Kg DM	IVDMD%	±SE	IVNDF %	±SE	IVADF%	±SE
Control	0	45.16^{e}	0.58	26.74^{d}	0.6	20.22^{f}	0.19
The produced enzyme	1	47.45^{d}	0.55	30.39^{cd}	2.68	23.19^{d}	0.97
	2	48.8^{bc}	0.03	36.22^{a}	0.38	28.72^{ab}	0.61
	3	51.68^{a}	0.56	37.39^{a}	0.32	29.46^{ab}	0.15
	4	49.76^{b}	0.59	35.51^{a}	0.43	28.00^{bc}	0.68
	5	49.10^{bc}	0.09	34.45^{ab}	0.37	26.97^{c}	0.15
SMIZYME®	1	48.22^{cd}	0.3	28.68^{cd}	2.07	20.84^{ef}	0.13
	2	48.31^{cd}	0.24	31.40^{bc}	1.45	22.11^{de}	0.01
	3	52.19^{a}	0.35	34.42^{ab}	0.08	29.90^{a}	0.09
	4	51.59^{a}	0.12	38.19^{a}	1.03	29.71^{a}	0.29
	5	49.95^{b}	0.2	38.12^{a}	0.04	29.06^{ab}	0.53

Average in the same column having different superscripts are differ significantly (P<0.05) for a, b, c d, e and f.

Colombatto, et al. [52] mentioned that, using commercial enzyme product supplemented to alfalfa stems at six levels: 0, 0.51, 1.02, 2.55, 5.1 and 25.5 g/kg DM, increased *in vitro* DM, NDF, ADF, and hemicellulose degradation compared to control. Also Pinos, et al. [53] showed that, enzymes improved ruminal disappearance rates of DM and NDF *in situ* of total mixed ration (TMR) with three different forage: concentrate ratios (400:600, 500:500, 600:400 g/g) and two (0 or 2 g) levels of enzymes/kg DM. Moreover Azzaz, et al. [54] investigated that Asperozym and Bacillozym® addition to banana wastes increased IVDMD and IVOMD significantly compared with the untreated banana wastes (Control). From the result of the current study, increasing of DM, NDF, ADF, and hemicellulose degradation (*in vitro*) because of supplemented rations with fibrolytic enzymes may be increased numbers of total bacteria and *Fibrobacter succinogens* in the incubation medium with improving *in vitro* fermentation as mentioned by Mao, et al. [55]. Moreover Kholif, et al. [56] found that, the treatment with commercial cellulolytic enzymes (Veta-Zyme Plus®) increased DM digestibility (*in vitro)* further Aboul Fotouh, et al. [4] reported that, addition lactating goats with Asperozym and Phytabex plus® at levels up to 1000 unit of cellulolytic enzymes/kg DM increase (P≤0.05) values of IVDMD compared to control.

In Vitro PH Value, TGP, NH3-N and SCFA of the Tested Rations

Data of Table 4 showed that, all the produced enzyme and SMIZYME® supplemented levels decreased (P<0.05) *in vitro* pH value and increased (P<0.05) total gas production (TGP) compared to control ration. The highest values (P<0.05) of *in vitro* pH (6.7) was recorded by control ration, while the ration supplemented with SMIZYME® recorded the lowest values of *in vitro* pH (5.69) at levels up to 3g/kg DM .On the other hand, the highest values of *in vitro* TGP was recorded by the produced enzyme ration reached (295.26) at levels up to 4g enzyme /kg DM., while control ration recorded the lowest values of *in vitro* TGP reached (283.66).

Table 4: Fibrolytic enzymes effect on pH value andTGP of the tested rations (*in vitro*).

Enzymes Source	Enzyme level g/Kg DM	pH value	±SE	TGP/1g	±SE
Control	0	6.70^{a}	0.14	283.66^{d}	0.6
The produced enzyme	1	6.47^{b}	0.03	290.05^{bc}	0.93
	2	6.37^{bc}	0.05	291.62^{b}	0.44
	3	6.15^{d}	0.06	294.99^{a}	0.08
	4	6.24^{cd}	0.01	295.26^{a}	0.11
	5	6.38^{bc}	0.06	294.45^{a}	1.01
SMIZYME®	1	6.13^{d}	0.12	291.37^{b}	1.32
	2	5.89^{e}	0.01	295.02^{a}	0.73
	3	5.69^{e}	0.1	294.44^{a}	1.04
	4	5.74^{e}	0.07	288.07^{c}	0.18
	5	5.87^{e}	0.01	287.66^{c}	1.3

Average in the same column having different superscripts are differ significantly (P<0.05) for a, b, c, d and e.

No significantly difference was found among control and fibrolytic enzymes ration of *in vitro* NH3-N as reported in Table 5. Control ration recorded the highest value of *in vitro* NH3-N, being (2.78 µmol/ml) , while the lowest *in vitro* NH3-N, being (2.03 µmol/ml) recorded by ration supplemented with SMIZYME® at levels up to 1g/kg DM.

Moreover data of Table 5 showed that, all the produced enzyme and SMIZYME® supplementation levels increased (P<0.05) the *in vitro* short chain fatty acid (SCFA). Control ration recorded the lowest value of *in vitro* short chain fatty acid (SCFA) being (1.255 mmol/dl), while the highest value *in vitro* SCFA, being (1.310 mmol/dl) recorded by ration supplemented with the produced enzyme at levels up to 3-4 g per kg DM. Through the previous data, the increase of DM, NDF ADF degradation plus to increase production of TGP and SCFA of rations supplemented with fibrolytic enzymes may be due to increasing digestion and improving ruminal fermentation as mentioned by Nsereko, et al. [57]. Also Mohamed, et al. [58] found that, after 24 hr. of incubation, all enzymatic treatment decrease final pH, increased dry matter (DM), neutral detergent fiber (NDF) and acid detergent fiber (ADF) digestibility of substrate. Also, acetate and propionate productions were increased by all enzymatic treatments. Further Giraldo, et al. [59] indicated that, adding fibrolytic enzymes enhanced *in*

vitro fermentation by increasing substrate fiber degradation, Volatile Fatty Acids (VFA) production, and ruminal microbial growth. Moreover Elghandour, et al. [60] obtained decreased ruminal pH values and increased *in vitro* SCFA production when incubated 4 fibrous feeds, including corn stover, with different levels of exogenous fibrolytic enzyme. The effectiveness of enzymes depends upon substrate, enzyme specificity and enzyme dose causing variable responses with different enzyme preparations and doses [61]. Increased TGP indicated the increased fermentable material with enzyme addition [62]. Also Vallejo, et al. [63] investigate that, addition of cellulase and xylanase linearly decreased (P≤0.05) pH compared to control, this may be due to greater enzymatic hydrolysis of feeds into readily fermentable substrates that depress pH when fermented. Further Handique, et al. [64] reported that, the *in vitro* study by using complete feed containing bajra straw supplemented with exogenous fibrolytic enzyme increase in total volatile fatty acid and an unaltered rumen pH, total nitrogen and ammonia N compared to control. These support the hypothesis that a suitable enzyme dose could improve fermentation efficiency [65,66].

Table 5: Fibrolytic enzymes effect on NH_3-N and SCFA of the tested rations (*in vitro*).

Enzymes Source	Enzyme level g/Kg DM	NH3-N µmol/ml	±SE	SCFA (mmol/dl)	±SE
Control	0	2.78	0.09	1.255^{d}	0.005
The produced enzyme	1	2.75	0.2	1.284^{c}	0.005
	2	2.59	0.12	1.291^{bc}	0.001
	3	2.4	0.02	1.306^{a}	0.005
	4	2.66	0.07	1.307^{a}	0.003
	5	2.56	0.23	1.303^{ab}	0.004
SMIZYME®	1	2.03	0.21	1.289^{bc}	0.01
	2	2.6	0.32	1.306^{ab}	0.006
	3	2.29	0.51	1.303^{ab}	0.005
	4	2.33	0.14	1.275^{c}	0.004
	5	2.77	0.08	1.273^{c}	0.0039

Average in the same column having different superscripts are differ significantly (P<0.05) for a, b , c and d.

Conclusion

Fungal pectinase enzyme was locally produced (the produced enzyme) under the optimum conditions. This would contribute for reducing the cost of importation. The evaluated fibrolytic enzymes (the produced enzyme and SMIZYME®) were increased DM, NDF, ADF, TGP and SCFA (*in vitro)*.

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