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Effects of Adding Different Levels of Anaerobic Fungi on Cellulase Activity of Ostrich Digestive Tract's Microorganisms under *in Vitro* Condition

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***Abstract*—**the objective of this study is to measure the levels of cellulas activity of ostrich GI microorganisms, and comparing it with the levels of cellulas activity of rumen's microorganisms, and also to estimate the probability of increasing enzyme activity with injecting different dosages (30%, 50% and 70%) of pure anaerobic goat rumen fungi. The experiment was conducted in laboratory and under a complete anaerobic condition (*in vitro* condition). 40 ml of "CaldWell" medium and 1.4g wheat straw were placed in incubator for an hour. The cellulase activity of ostrich microorganisms was compared with other treatments, and then different dosages (30%, 50% and 70%) of pure anaerobic goat rumen fungi were injected to ostrich microorganism's media. Due to the results, cattle and goat with 2.13 and 2.08 I.U (international units) respectively showed the highest activity and ostrich with 0.91 (I.U) had the lowest cellulose activity ($p < 0.05$). Injecting 30% and 50% of anaerobic fungi had no significant incensement in enzyme activity, but with injecting 70% of rumen fungi to ostrich microorganisms culture a significant increase was observed 1.48 I.U. ($p < 0.05$).

***Keywords*—**Cellulase enzyme, Microorganisms, Ostrich, Ruminants

I. Introduction

LIGNOCELLULOSIC compounds are the most frequent agricultural residues in the world, which provide majority of tropical country's ruminant feed [11]. The ability of Herbivore's digestive tract, especially ruminants to consume such feed, depends on the fibrolytic digestion of the great variety of microorganism's living in the digestive tract, especially the fibrolytic bacteria and anaerobic fungi. The major cellulose degrading bacteria in the rumen are Fibrobacter –Succinogens, Colestridium lochheadii, Ruminococcus flavefaciens and Ruminococcus albus. These bacteria are the most colonizing bacterial of the plant's cell wall components, and secreted the most Fibrolytic enzyme especially Cellulase, to break down the compound's of cell walls [7]. Orpin separated anaerobic fungi from the rumen of sheep for the first time (1975) and since then it was found in other ruminant's and mammal's digestive tract too. Observations of electron microscopy has shown that anaerobic fungi mostly prefer to colonize the Ligninfied parts in plant's skeletal wall, by penetrating their rizoids into the plant's cuticle and with putting pressure on to the side walls, they cause a tissue collapse .on the other hand anaerobic fungal enzyme secretions also help the hydrolysis of plant's skeletal wall [1, 10]. The rumen's anaerobic fungal enzyme profile shows high levels of Fibrolytic enzymes secretion especially xylanase and Cellulase. Cellulase is a complex enzyme consisting the exocellulase, endocellulase and cellubiase which breaks down the cellulose in to β- units glucose. This complex of enzymes produce by coexist microorganisms in herbivore's digestive canal and so far there has been no reports on an internal secretion (Indogenuos) of Cellulase enzymes in the digestive tract of vertebrates (9). It must also be mentioned that according to recent researches, no presence trace of anaerobic fungi in the gastrointestinal tract of poultry and ostrich (as a herbivore bird) have been observed [4, 5]. Ostriches as a Hind-gut fermento, due to the anatomical and physiological structure of their digestive system's have a better ability to consume and digest fiber comparing to other birds. Their very long intestinal canal(Cecum and colon respectively 1 m and 12 m) and relatively slow passage rate and therefore long retention time for fiber feed in the colons (average 41 hrs) provide an appropriate circumstances for fibrolytic digestion and fermentation of microorganisms in their colon and cecum [2,4].

Since the anaerobic fungus are one of the major sources of discharging fibrolytic digestion especially for its fibrolytic enzymes, particularly Cellulase the absence of them in the gastrointestinal tract of ostrich will deprived this bird from an important enzyme for breaking down fiber compositions.

Now this question arises that whether by separation and purification of rumen's anaerobic fungi and injecting different levels of it into the media containing ostrich's gastrointestinal microorganisms under *in vitro* condition, is it possible to increase the secretion levels of Cellulase enzyme in ostrich microorganism's culture medium in order to improve the digestion of fibers by this bird.

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II. Materials and Methods

A. Providing Substrate for Growing the Microorganisms

1.3 g of wheat straw was grinded, sieved and was weighted by digital scale. Then respectively was put, in 200 serum bottles (50 ml). 40 ml of anaerobic microorganism's culture medium (Cald Well medium) was added under completely anaerobic conditions, and the door was immediately riveted by plastic ravels and aluminum seals [3].

For sterilization serum bottles were put in an autoclave for 20 minutes with a temperature of 120 ° C and pressure of 3 atmospheres.

B. Preparation and Injection of Digestive Tract Anaerobic Microorganisms Into Serum Bottles

For preparation of microbial samples, a slaughterhouse was visited for several times and from each of slaughtered animals (cattle, sheep and goat) five rumen were randomly selected. After opening the rumen, 20 ml of rumen fluid (as a microbial source) collected in plastic bottles after several stages of passing it through clean cloth, they were marked and immediately was transferred to the laboratory. It must be also mentioned that separately from five adult ostriches and in two shifts, the colonic liquid (as a microbial source) using the method mentioned before was collected from the proximal colon, , and was transferred to the laboratory. Under complete sterile conditions, 1 ml of rumen fluid and the collected colonic fluid was injected as a microbial source into 5 serum bottles separately. Serum bottles were put in an incubator for 48 hours with temperature of 39° C in order to reproduce and grow the microorganisms.

C. Purification of Rumen's Anaerobic Fungi and Injecting Them into the Medium Culture Containing Ostrich's Digestive Tract Microorganisms

Six days before slaughtering ostriches, rumen's anaerobic fungus was purified. Similar to previous attempts, in slaughterhouse, after opening the rumen of a slaughtered goat, microbial samples were taken and transported to the laboratory. Under complete sterile conditions, 1 ml samples for microbial sample were injected to multiple serum bottles for the anaerobic fungi growth. At this point, to prevent the growth of bacteria and anaerobic protozoa and providing a condition completely dedicated to the fungi growth, in three steps of subculture, three different doses of (0.5, 1 and 1.5 ml) 5% antibiotics solution[1] was injected into serum bottles and in each time of subculture bottles were put for 48 hours in an incubator (temperature 39 ° C).

[5] Antibiotics solution5%, including Chloramphenicol - Penicillin G- Tetracycline - Streptomycin and Ampicillin.

After three subculture, we were ensured that there was no sign of bacteria and protozoa growth inside the serum bottles and only rumen's anaerobic fungi were able to grow. Clearness and lucidity of the medium, and the rise of straw mass and their accumulation in the head bottles, were signs of fungi growth and finally the microscopic observations confirmed that. (Figure 1 and Figure 2)

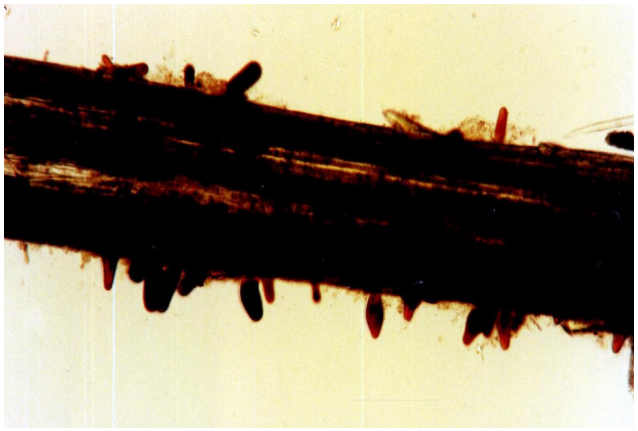

Fig. 1 Microscopic observation of pure anaerobic fungi in media (After three sub culture)

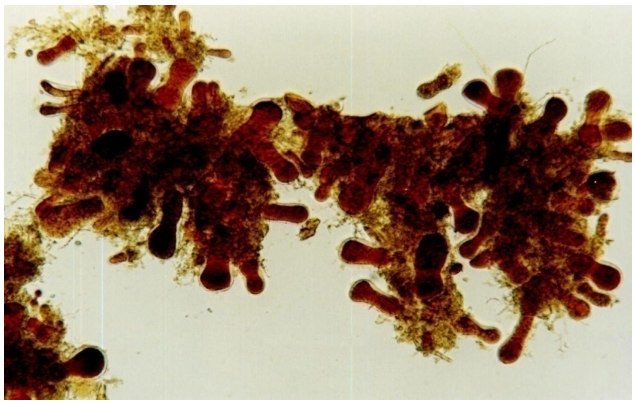

Fig. 2 Microscopic observation of pure anaerobic fungi in media (After three sub culture)

Three different levels (30 - 50 - 70%) of purified rumen anaerobic fungi according to the following formula, was injected to the serum bottles containing digestive tract microorganisms of ostrich.

0.3 ml of rumen anaerobic fungi + 0.7 ml of digestive tract microorganisms of ostrich
0.5 ml of rumen anaerobic fungi + 0.5 ml of digestive tract microorganisms of ostrich
0.7 ml of rumen anaerobic fungi + 0.3 ml of digestive tract microorganisms of ostrich

Serum bottles were put in an incubator for 48hours with temperature of 39° C. In order to be ensure of anaerobic fungi growth in mix culture media (rumen anaerobic fungi+ ostrich microorganisms), after opening the serum bottles, samples of wheat straw inside the bottles were separated and with microscopic observation, growth of anaerobic fungi was confirmed.

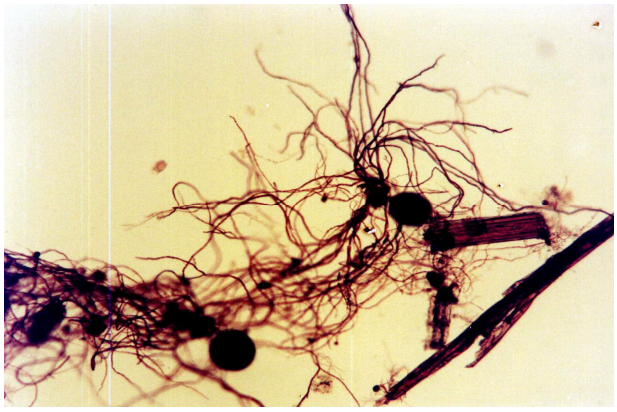

Fig. 3 Microscopic observation of anaerobic fungi in mix media

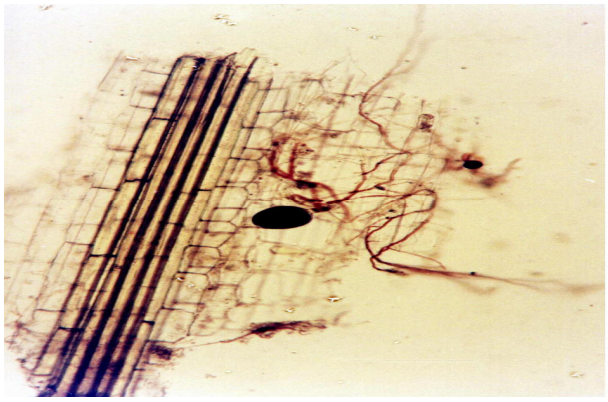

Fig. 4 Microscopic observation of anaerobic fungi in mix media

D.Separation of Straw from The Liquid inside The Serum Bottles

All serum bottles of different treatments (cattle, sheep, goat and ostrich and ostrich with 30, 50 and 70% goat's rumen anaerobic fungi), after 48 h of incubation was out of incubator and the existing straws in the culture media was separated by a thick nylon stocks (used as filters) from the liquid in the serum bottles.

E. Sample Preparation of Enzyme Samples for Spectrophotometer

The Liquid from different treatments in serum bottles were separately collected and were centrifuged for 7 minutes with the 10174g, obtained transparent brown liquid is the source of enzyme to be measured by spectrophotometry. Enzyme sample preparations were done according to TABLE (I).

Spectrophotometer was calibrated at wavelength of 550 nm. First, absorption of standard blank tubes and then absorption of each standard tube, and ultimately absorption of each treatment tube (testing tube) were recorded. Concentrations obtained from each of the test tubes were calculated by the regression equation y = a + bx using the following equation, and finally enzymatic activity of experimental treatments were determined.

(Cellulase activity I. U)[2] = [1 / k × (A - A0) × F÷ (30 × S) (1)

[1 / k × (A - A0) = concentration of glucose (micromoles per liter) produced in each test tube.

1 / k = converse of angle factor or slope of the regression equation of standard tubes.

F = final volume of each tube is equal to 10.5. This is the dilution factor and has no unit.

S = volume of used enzyme in treatment tube (testing tubes) which is equal to 0.1 ml's.

30= reaction time (minutes)

III. Statistics

Variance Analysis of obtained data were performed in a completely randomized design in seven treatments (sheep, cattle, goat, ostrich, and ostrich with 30, 50 and 70% goat's rumen anaerobic fungi) with five replication using software MS.TATC and the means were compared by Duncan multiple range test.

The statistical model is as follows:

$J_{ijk} = \mu + t_i + e_{ij}$ (2)

J_{ijk} = Average observations

μ = population mean

t_i = effect of treatments

e_{ij} = effect of test error

[6] A unit of enzyme activity in terms of international units or I. U is equal to the amount of enzyme that releases one micromole of glucose per minute.

TABLE I
PREPARATION OF ENZYME SOLUTES FOR SPECTROPHOTOMETRIC USAGE (A)

Tubes Materials (ml)	*Testing tubes (5times)*					*Testing blank*
	Tube 1	*Tube 2*	*Tube 3*	*Tube 4*	*Tube 5*	
Enzyme solution(ml)	0.1	0.1	0.1	0.1	0.1	0.1
Buffer solution(ml)	0.4	0.4	0.4	0.4	0.4	0.4
CMC solution(ml)	0.5	0.5	0.5	0.5	0.5	0.5
Glucose Standard solution(ml)	0.0	0.0	0.0	0.0	0.0	0.0
Vortex						
30 min in temperature 50° C						
10min in temperature 100° C						
DNS solution(ml)	1.5	1.5	1.5	1.5	1.5	1.5
Vortex						
15 min in temperature 100 ° C						
cooling in cold water for 5 min						
Distilled water	8	8	8	8	8	8
Vortex						
20 min waiting						
Final volume of each tube	10.5	10.5	10.5	10.5	10.5	10.5
Recording the absorption in wavelength (550 nm)						
Standard tube's concentration (µ/ml)	-	-	-	-	-	-

(B)

Tubes Materials (ml)	*Standard tubes*					*Standard blank*
	1	*2*	3	4	5	
Enzyme solution(ml)	0.0	0.0	0.0	0.0	**0.0**	0.0
Buffer solution(ml)	0.9	0.8	0.7	0.6	**0.5**	0.1
CMC solution(ml)	0.0	0.0	0.0	0.0	**0.0**	0.0
Glucose Standard solution(ml)	0.1	0.2	0.3	0.4	**0.5**	0.0
Vortex						
30 min in temperature 50° C						
10min in temperature 100° C						
DNS solution(ml)	1.5	1.5	1.5	1.5	1.5	1.5
Vortex						
15 min in temperature 100 ° C						
cooling in cold water for 5 min						
Distilled water	8	8	8	8	8	8
Vortex						
20 min waiting						
Final volume of each tube	10.5	10.5	10.5	10.5	10.5	10.5
Recording the absorption in wavelength (550 nm)						
Standard tube's concentratin (µ/ml)	0.158	0.317	0.476	0.634	0.793	0.000

IV. RESULTS AND DISCUSSIONS

Table II and Fig. 1 both show that cattle and goat's rumen microorganisms with 2.13 and 2.08 I.U, have the highest level of enzymatic activity respectively, and afterwards there is sheep with 1.63 I.U ($P < 0.05$). Microorganisms of the Ostrich ceca with 0.91 units (IU) show the lowest level of enzymatic activity and when 30 and 50% of purified anaerobic fungus of goat's rumen were added to the microorganism medium of the ostrich, no improvement in enzymatic activity was observed. The enzymatic activity of three treatments, ostrich and ostrich with 30 and 50 percent of goats anaerobic fungi were 0.91, 0.90 and 0.93 I.U respectively , and no significant differences was observed between them ($P<0.05$).

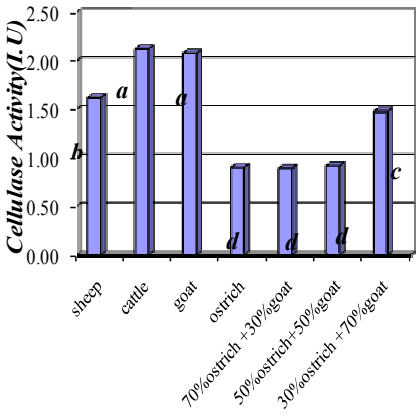

Fig. 5 Mean comparison of cellulase activity in experimental treatments (I. U)

TABLE II
LEVEL OF ENZYME ACTIVITY IN EXPERIMENTAL TREATMENTS (I.U)

Cellulase Activity Level(I.U)	*Treatment*
1.63 ^{b}	Sheep
2.13 ^{a}	Cattle
2.08 ^{a}	Goat
0.91 ^{d}	***Ostrich***
0.9 ^{d}	ostrich70%+goat30%
0.93 ^{d}	ostrich50%+goat50%
1.48 ^{c}	ostrich30%+goat70%
0.387	**SEM**

The mean with different letters in each column are significantly different ($p < 0.05$)

With increasing the level of anaerobic fungus and inserting 70% anaerobic fungi to ostrich's microorganism culture, Cellulase enzyme activity was seen to increased and improved to a significant level and reached 1.48 I.U. comparing to the treatment of ostrich microorganisms without anaerobic fungi an improvement equal to 0.57 I.U was observed.

Unfortunately, there is a limited and incomplete information source about Digestive enzyme activity level, particularly Cellulase in Ostrich and in general the whole Ratidia family. On 1990 Milton reported that digestive system of ostrich has no Cellulase secretion and ostrich like the other herbivore mammals depends on microbial Cellulase enzyme secretion in the cecum and colon to break down the fiber components. Lack of anaerobic fungi in digestion tract seems to decrease the Cellulase enzymes secretion level in ostrich digestive system.

Rezaeian et al (2005) did a research on capability of digesting fiber by adding anaerobic fungus of goat's rumen in *in vitro* condition and observed that on the first day, activity level of Xylanase enzyme of fungi was higher than Cellulase but from the second day activity level of Cellulase increased and played a significant role in the digestion of cellulose. [10].

Polland and Nilson (1985) did a survey on effect of increasing the Cellulase (fungi) on digestive and economic performance in meat goats which the results of this research showed that, adding the cellulase enzyme into the feed of under experiment animals, leads to improvement of digestion and increasing the products at all levels [6].

According to the obtained results from this project, it was clarified that addition of anaerobic fungus in high concentrations to the microorganism culture of ostrich digestive system leads to increasing of secretion and also improvement in Cellulase activity which can play a significant role in degradation of fibrous materials, especially poor-quality fibers existing in the diet.

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