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ORIGINAL ARTICLE

Tannic acid degradation by *Klebsiella* strains isolated from goat feces

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ABSTRACT

Background and Objectives: Tannins are toxic polyphenols that either bind and precipitate or condense proteins. The high tannin content of some plants is the preliminary limitation of using them as a ruminant feed. So, the aim of this study was the isolation and characterization of tannic acid degrading bacterial strains from goat feces before and after feeding on Pistachio-Soft Hulls as tannin rich diet (TRD).

Materials and Methods: Bacterial strains capable of utilizing tannic acid as sole carbon and energy source were isolated and characterized from goat feces before and after feeding on TRD. Tannase activity, maximum tolerable concentration and biodegradation potential were assessed.

Results: Four tannase positive isolates were identified as *Klebsiella pneumoniae.* Isolated strains showed the maximum tolerable concentration of 64g/L of tannin. The tannic acid degradation percentage at a concentration of 15.0 g/L reached a maximum of 68% after 24 h incubation, and more than 98% after 72 h incubation. The pH of the medium also decreased along with tannic acid utilization.

Conclusions: It is obvious that TRD induced adaptive responses. Thus, while the bacteria were able to degrade and detoxify the tannic acids, they had to adapt in the presence of high concentrations of tannic acid. So, these isolates have an amazing potential for application in bioremediation, waste water treatment, also reduction of tannins antinutritional effects in animal feeds.

Keywords: Biodegradation; Goat feces; *Klebsiella* strains; Tannic acid.

INTRODUCTION

Tannins are toxic, high molecular weight polyphenols that according to their structure are classified into hydrolyzable and condensed ones (1, 2). They are one of the most abundant plant components after cellulose, hemicellulose and lignin (3) which plays an important role in plant protection from ruminant (due to astringency and bitter taste) and microbial attacks (4-6).

Phenolic compounds toxicity in the environment has encouraged studies of bacteria with the ability of tolerating and/or metabolizing high levels of these compounds (7, 8). Tannins are also considered to be toxic to bacteria generally as a result of enzyme inhibition, substrate deprivation and its activity on membranes and metal ion deficiency (6). In spite of tannin antimicrobial properties, many microbes, especially bacteria can resist and develop different mechanisms for the tannin degradation in their habitats. So, Bacteria with the ability to grow in the presence of tan-

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nins as a sole source of carbon and energy are commonly considered tannin-degrading and degradation like resistance is not limited by species or geographical barriers (9). Different tannic acid degrading bacteria were isolated that belonged to genera *Bacillus, Staphylococcus, Klebsiella, Lactobacillus, Streptococcus, Pseudomonas, Pantoea* and *Serratia* (10-12).

As tannins are one of the most abundant plant components, the feces (13) and alimentary tract samples of animals feeding on a tannin rich diet can be good sources for isolation of tannin degrading bacteria. It is possible due to the presence of complex microbial population in the gastrointestinal tract, whose composition is mostly determined by the diet (14-16). Pistachios by products, which are produced by tones in many parts of Iran every year, are high tannin supply and can be used as a tannin rich diet.

The aim of this study was the isolation and characterization of tannic acid degrading bacterial strains (TDB) from goat feces before and after feeding on Pistachio-Soft Hulls as tannin rich diet.

MATERIALS AND METHODS

Sampling. Two male goats maintained at the Agricultural Sciences research farm of the Islamic Azad University, Khorasgan-Isfahan Branch, Isfahan Province, were used in this study. The goats were fed the same diets during September- October (Table 1). They were fed twice a day at 8:00 AM and 4:00 PM and had free access to water. Fecal samples were collected with fecal bags before and after feeding on mentioned diet. The samples were suspended in sterilized phosphate buffered saline, carefully mixed with a Homogenizer and a Vortex test-tube mixer in the biotechnology laboratory of Islamic Azad University Khorasgan (Isfahan) Branch.

Table 1. Goat's diet in adaptability and experimental period.

	Adaptability period (g/day) One week	Experimental period (g/day) Three weeks
Alfa- alfa	600	350
Silage	200	240
Concentrate*	200	200
Pistachio soft hulls	0	200

*The concentrate include: 24% Bran, 47% Barley, 24% Corn and a 5% mixture of vitamins (ADE) and mineral salts.

Enrichment cultures and TDB isolation. Aliquots of 1ml of fecal suspensions were added to 50 ml of liquid minimal salts medium (MSM) containing (g/L): Na_2HPO_4 $2H_2O$: 2.7; KH_2PO_4:1.4; $(NH_4)_2SO_4$:0.5; $MgSO_4 7H_2O$:0.2; yeast extract 0.02 and 10 ml trace element solution containing (g/l): Na_2EDTA $2H_2O$:12.0; NaOH:2.0; $MnSO_4$ $4H_2O$, 0.4; $ZnSO_4$ $7H_2O$, 0.4; H_2SO_4: 0.5 ml; Na_2SO_4: 10.0; Na_2MoO_4: $2H_2O$, 0.1; $FeSO_4$ $7H_2O$: 2.0; $CuSO_4$ $5H_2O$: 0.1; $CaCl_2$: 1.0, and incubated at 30 °C without shaking for 3-5 days. Tannic acid was supplied as the sole carbon and energy source, at concentrations of 0.5g/l (10). Chemical materials for MSM medium were purchased from Merck, Sigma and Aldrich Chemical Companies.

After three successful transfers, enrichment cultures which showed turbidity were selected for isolation. An aliquot from each culture was spread on MSM agar supplemented with a 2g/L tannic acid solution. After 48 h incubation (30 °C), colonies with different morphologies were selected as candidate tannic acid-degrading strains. The biochemical identification of Gram negative colonies was performed by catalase and oxidase activity, IMViC (Indole, MR, VP, and Citrate), TSI and motility tests.

16S rDNA gene analysis. The 16S rDNA gene amplification of selected isolates were done using extracted genomic DNA with each of the Forward and Reverse primers 27F (5′AGAGTTTGATCCTGGCTCAG-3′) and 1495R (5- GGTTACCTTGTTACGACTT-3). Partial sequence of 16S rDNA gene of each strain was determined. Nucleotide sequences were compared to those in the National Center for Biotechnology Information, Gene Bank database using BLAST program (17).

Tannase activity test. The tannase activity of the isolates was confirmed by the method of Kumar et al. (18). Briefly: Tannic acid (2%; filter sterilized) was added to nutrient agar plates. It forms a tannin–protein complex; that its cleavage by tannase producing bacteria forms a greenish brown zone around the colonies after 3–4 days of incubation. Then the plates were flooded with $FeCl_3$ solution (0.01 M $FeCl_3$ in 0.01 N HCl) and kept for 10 min at room temperature. FeCl3 reaction with tannic acid forms a brown

color; so, a clear zone is formed on a dark brown background.

Maximum tolerable concentration of tannins (MTC). MTC was selected as the highest concentration of tannic acid that allows growth after 24-48 hours (19). The increasing concentration of Tannic acid (1, 2, 4, 8, 16, 24, 32, 64 g/l) on MSM agar plates was used for testing the MTCs.

Biodegradation experiments. The biodegradation experiments were done in flasks containing 50 ml of MSM broth supplemented with 15g/l tannic acid (filter-sterilized). Optical density (wavelength 540 nm), pH readings and tannin concentration were determined periodically as biodegradation indices. The data are reported as the average of triplicate experiments.

Tannin concentrations were measured by bovine serum albumin (BSA) precipitation assay and the biodegradation percentage was calculated. For this purpose, the bacterial suspensions were centrifuged (3,000 g at 4 °C for 10 min) and the supernatant was taken for determination of tannins. For each sample, 1ml of a 1 mg BSA/ml acetate buffer stock solution were combined with 1ml of tannic acid solution and precipitation reactions were allowed to proceed under refrigeration for 18 hours, after all samples were centrifuged (3,000 g for 10 min), the precipitate were dissolved in SDS (sodium dodecil sulfate) solution (1% w/v). The aliquot of 1 ml of resulted solution was combined with 3ml TEA (triethanolamine) -SDS solution (7%TEA & 1% SDS). After several minutes 1ml of $FeCl_3$ was added and the optical density was measured after 60 min incubation at room temperature, then absorbance was measured at 520nm, zeroing the spectrophotometer with a tube containing all of the reagent plus water in place of the extract. Tannin contents were determined according to the calibration curve (R^2=0.971) which was prepared from commercial tannic acid ranging from 0-20 gr/l.

Statistical analysis. All data was analyzed using the statistical analysis system (2001). Multiple comparisons of means were done with the Duncan test method (at Sig<0.05).

Variation in the difference percentage between 16S ribosomal sequence of isolates (1 and 9) was determined by Pintail software version 1.

RESULTS

Isolation and molecular identification based on 16S rDNA gene sequence along with morphological and preliminary tests were carried out for identification of bacterial strains with the ability of growing in the presence of tannic acid as the sole carbon and energy source. The Gram negative, catalase positive and oxidase negative isolates with the colony diameter between 1.0 and 1.5 mm, were selected. The isolates were also indole negative, Voges-Proskauer positive, urease and lysine decarboxylase positive. All tests were compatible with *Klebsiella pneumoniae*.

Comparing the sequences of the 16S rDNA gene obtained from the strains 1, 5, 7, and 9 with the sequences in GenBank revealed that these strains exhibited the highest similarity (99 %) to different strains of *Klebsiella pneumoniae* and also *Klebsiella pneumoniae* subsp. pneumoniae HS11286 (ACCESSION NR_103936).

On the basis of 16S rDNA similarity, *Klebsiella* isolates of this study showed an obvious relationship with bacteria of the *Klebsiella* genus included into the phylum Proteobacteria, class Gamma proteobacteria, order Enterobacteriales, family Enterobacteriaceae.

Nucleotide sequences of the isolates were compared with standard strain and also each other using Pintail software version 1 for detecting any possible anomaly and variation in percentage of difference between 16S rDNA sequences. According to the results, no anomaly detected and it was also revealed that there is no variation in percentage of difference between 16S rDNA sequences of strains 1 and 9 (Fig. 1). So, the 16S rDNA gene sequences of the strain 1 were submitted to GenBank under accession numbers of KJ783439 (Table 2).

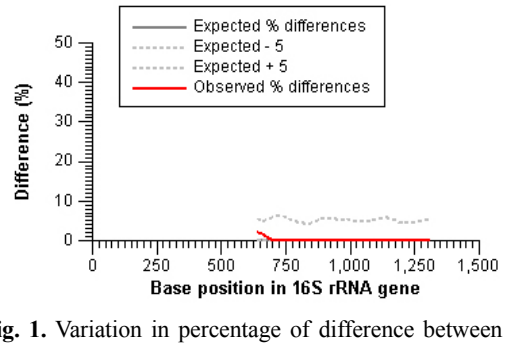

Fig. 1. Variation in percentage of difference between 16S rDNA sequences if starins 1 & 9 (resulted from pintail software)

Biodegradation experiments. Visual assessment of the enzymatic tests for tannase activity showed positive results for all of the four isolates, and a clear zone is formed on a dark brown background (Fig. 2)

As it is shown in the Table 3, strain 9 (isolated from goat feces before feeding on a tannin rich diet) showed the MTC of 16 g/l and strains of 1, 5 and 7 (isolated from goat feces after feeding on a tannin rich diet) showed the MTC of 64 g/l.

The bacterial isolates from goat feces were tested for the ability of tannic acid degradation in a concentration of 15 g/l. Visual turbidity increased (data not shown) and the pH of the medium decreased along with the tannic acid utilization as the sole carbon and energy source (Fig. 3).

Tannin contents were also determined according to the calibration curve (Fig. 4) and tannic acid degradation percentages were calculated. In relation to tannic acid degradation, the *Klebsiella* sp. strains 1 and 9 showed more than 98% utilization of tannic acid after 72h of incubation while the degradation percentages were 68 and 60 after 24 h of incubation, respectively (Fig. 5).

Table 2. Molecular identification of the most potent isolates in tannin degradation

Accession number	Similarity percentage	Bacterium	Isolates
KJ783439	99%	*Klebsiella pneumoniae subsp. pneumoniae*	1
		ATTA32	9
	99% (100% with strain1)	*Klebsiella pneumoniae subsp. pneumoniae*	

Fig. 2. Tannase activity test

Table 3. The Maximum tolerable concentration of tTannin (MTC)

Goat fecal samples	isolates	Tannin concentration of media (g/L)									
After		**0**	**0.5**	**1**	**2**	**4**	**8**	**2**	**16**	**32**	**64**
tannin	**1**	+	+	+	+	+	+	+	+	+	+
rich	**5**	+	+	+	+	+	+	+	+	+	+
diet	**7**	+	+	+	+	+	+	+	+	+	+
Before tannin rich diet	**9**	+	+	+	+	+	+	+	+	-	-

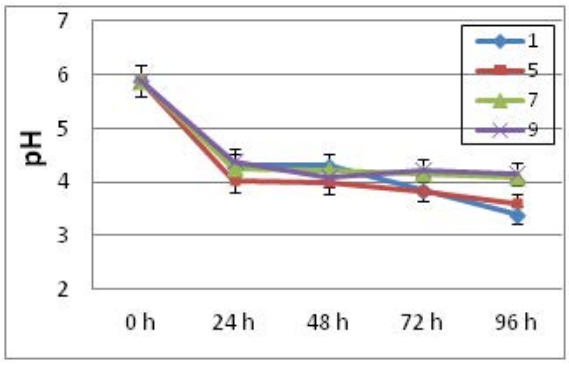

Fig. 3. The pH of MSM broth during growth and Tannic acid degradation

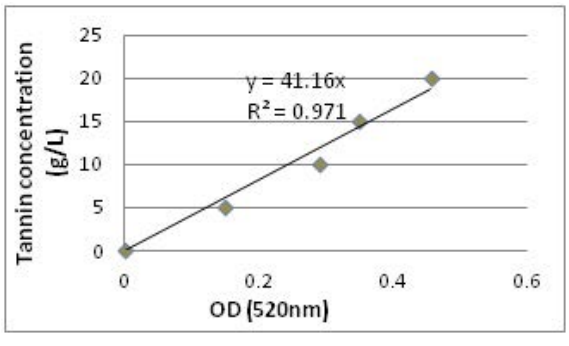

Fig. 4. Calibration curve constructed from commercial tannic acid ranging from 0-20 g/l.

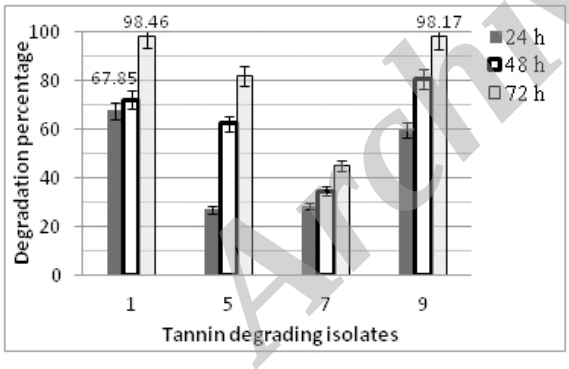

Fig. 5. Degradation percentage of *Klebsiella* strains

DISCUSSION

Biodegradation is a cost-effective technique which can be used for toxic compounds degradation into safe products (17). The use of bacteria for the transformation of tannins is gaining worldwide attention. The microorganisms' ability to degrade tannins has been related to tannase production. Tannase is an important enzyme capable of catalyzing gallotannins to gallic acid and glucose (20). Bacterial strains degrading tannic acid were isolated from different sources such as Oil Mill Wastes (6, 10), goat or cow feces (8, 16), mouse feces (21) and soil (22, 23). In this study *Klebsiella* spp with the ability of tannic acid degradation were isolated from goat feces before and after feeding on tannin rich diet.

Molecular identification method revealed that strains 1 and 9 identified as *Klebsiella pneumoniae* subsp. *pneumoniae*. Variation in the difference percentage between 16S ribosomal sequence of isolates 1 and 9 showed that the probability of two sequences with a DE (Deviations from Expectation) of 0.45 and differences percentage of 0.13 is estimated to be P> 0. 50. So, it is obvious that they are practically identical.

Klebsiella strains with the ability of tannic acid degradation were also isolated from Oil Mill Wastes (6). *Klebsiella pneumoniae* MTCC 7162 was isolated from tannery effluent in the presence of tannic acid (12), and a *Klebsiella* sp. strain with tannic acid degradation was isolated from garden soil (11).

The tannase activity of the *Klebsiella* isolates was confirmed by the method of Kumar et al. (18) because the method described by Osawa and Walsh (24) was not highly sensitive and the zone was not clearly visible. In this method additional staining with $FeCl_3$ provides a dark background allowing clear visibility of zones of tannin degradation (Fig. 1). Isolated *Klebsiella* strains showed the capability to tolerate high concentrations of tannic acid. Also, the number and tolerance ability of isolated *Klebsiella* strains against higher concentrations of tannin were significantly (P< 0.05) increased in goat feces after feeding on a tannin rich diet. As it is shown in the results the only strain 9 with the MTC of 16 g/l was isolated from goat feces before feeding on a tannin rich diet and strains 1, 5 and 7 with the MTC of 64 g/l were isolated from goat feces after feeding on a tannin rich diet. It is obvious that tannin rich diet induced adaptive responses. A same response showed by Rafii et al. (2009) during isolation of bacterial strains of bovine fecal microflora capable of degradation of ceftiofur (25). Elizendo et al. (2010) also showed such adaptive responses in the study of the effect of tannins on the in vitro growth of *Clostridium perfringens* (26).

Thus, while the bacteria were able to degrade and detoxify the tannic acids, they had to adapt in the presence of high concentrations of tannic acid. Adoptive

responses of Gram-negative bacteria to toxic compounds are carried out by a remarkable increase in saturation degree of fatty acids (6, 27). Pepi et al. (2013) also showed tannase activity for *Klebsiella* sp. strain C2A in the presence of tannic acid concentrations up to 50 g/l (6). The tannase-producing *K. pneumoniae* MTCC 7162 was able to grow in the presence of 10 g/l of tannic acid and also could produce tannase by adding 20 and 50 g/ L of tannic acid for tannase production (12).

According to the literature, the effect of polyphenols on bacterial growth and metabolism depends on the structure, the dosage and the type of strain (28). For example, Gram-negative bacteria are more resistant to polyphenols than Gram-positive bacteria; it is due to the differences between wall compositions (29). One of the potential mechanisms of polyphenols action on bacterial cells is that polyphenols can bind to bacterial cell membranes in a dose-dependent mode (30). In biodegradation experiment, tannic acid utilization as the sole carbon and energy source resulted in visual turbidity increase with a simultaneous production of acidic metabolites as indicated by an additional decrease in the pH of the medium. The primary pH of culture fluid was 5.9 that decreased to 3.39 – 4.15 after 96h of incubation; While, *Bacillus licheniformis* in the study of Ilori et al. (2007) decreased the pH from 4.5 to 4 (23). Also, the degradation percentage of tannic acid at a concentration of 15.0 g/l reached a maximum of 68%, for strain 1, after 24 h incubation, and reached more than 98% after 72 h incubation for *Klebsiella* strains of 1 and 9.

The high percentages of tannic acid degradation of *Klebsiella* isolates of this study were similar to those obtained by Pepi et al. (6). There was difference in tannic acid concentration in the medium so that degradation experiment of Pepi et al. was done in the presence of 5 g/l tannic acid. Chowdhury et al. (2004), who used strains isolated from tannery soil also showed similar results in the presence of 0.2% tannic acid (22). Other *Klebsiella* sp. strains showed similar tolerance and degradation behavior for tannic acid (11)

CONCLUSION

Our findings along with the fact these strains are able to grow on higher concentrations of tannic acid (up to 64 g/l) give them an amazing potential for its application in bioremediation, waste water treatment (especially tannery effluents), and also reduction of antinutritional effects of tannins in animal feeds. It could also be very valuable in industry for production of tannase. Furthermore, these tannase producing bacteria may find application in food (clarification of fruit juices) and pharmaceutical industries

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