
# Tannic Acid Biodegradation by *Pseudomonas* and *Pantoea* Isolates of Goat Feces with a Pistachio Soft Hull Diet

Arezoo Tahmourespour (PhD)[1]*, Nooroldin Tabatabaee (PhD)[2]

[1]*Associate Professor of Microbiology, Basic Medical Sciences Department, Islamic Azad University, Isfahan (Khorasgan) Branch, Isfahan, Iran*

[2]*Assistant professor, Faculty of Agriculture, Islamic Azad University, Isfahan (Khorasgan) Branch, Isfahan, Iran*

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***Corresponding Author:***

***Arezoo Tahmourespour***

**Email:** atahmoures@khuisf.ac.ir

**Tel:** +98-9133130052

## Abstract

**Introduction:** Pistachio soft hulls contain tannins that are polyphenolic compounds with some antinutritional effects. This research aims to isolate and characterize tannin-degrading bacteria from goat feces consuming soft pistachio hulls as a TRD (tannin rich diet) with high potential application in tannin biodegradation.

**Materials and Methods**: Enrichment culture was used in minimal media containing tannin to isolate degrading bacteria. The isolates were identified by morphological, biochemical, and molecular characterization. In addition, tannase production, the maximum tolerable concentration (MTC), pH, and the tannic acid amount of the media were determined during degradation.

**Results**: Four strains with tannin-degrading ability were isolated from feces of goat before (TA1) and after (TA2-4) consuming the TRD. In addition, 16S rDNA gene sequenceing, assigned TA1 and TA3 isolates to the genus *Pseudomonas* (ATTA 34), and TA2 to the genus *Pantoea* (ATTA33), and submitted to the GenBank (accession numbers KJ783441 and KJ783440). The isolates showed the MTC of 32 and 64g/L. The degradation percentage reached a maximum of more than 95% after 72h in the presence of 15g/L tannin. The pH as well as tannin utilization decreased except in the case of TA2, in which it increased again after 48h, which could be related to the gallate decarboxylase activity.

**Conclusion**: The TRD was able to induce adaptive responses. Although the bacteria were capable to detoxify and degrade the tannins, they needed to adapt to high concentrations of them (up to 64g/L). This study is the first report of *Pantoa stewartii subsp indologenes* with both tannase and gallate decarboxylase activities. These isolates possess wonderful potential for utilization in bioremediation, also minimization of tannins antinutritional effects in animal diets.

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## 1. Introduction

Pistachio soft hulls are the major pistachio waste material produced at the dehulling stage and are considered as pistachio side products. This organic waste accounts for about 50-80% of the whole wastes. It is rich in proteins, fats, minerals, vitamins, colorants, and terpenes. It also has a fibrous structure and is maybe suitable for utilizing in ruminant diet, although some antinutritional phenolic compounds can decrease their nutrient accessibility [1-3]. Tannins are a group of polyphenols with high molecular weight, which exist in the environment and are abundant in seeds, fruits, and leaves. Tannins are classified into hydrolysable and condensed groups, according to their properties and structures [4]. Tannins have toxicity effects on animals and can inhibit growth of microorganisms through enzyme inhibition, substrate deprivation, and their activity on cell membranes [5]. Protection of plants from ruminants, due to their astringency and bitter taste, and from microbial attacks is one of their important roles [5].

Toxicity of phenolic compounds in the nature has inspired studies on bacteria able to tolerate and/or metabolize high levels of these compounds [6, 7]. Despite antimicrobial properties of tannins, many microbes, including bacteria, can resist and develop different mechanisms for tannin degradation in their habitats. Biodegradation has been considered as an economical and environmentally friendly method, as against chemical and physical methods, for reducing adverse organic matters [8, 9]. So, bacteria with the ability of growing in the presence of tannins as the only source of energy and carbon are usually considered tannin-degrading bacteria; also, degradation like resistance is not restricted by the bacterial type or geographical barriers. In previous studies, different tannic acid degrading bacteria were isolated that belonged to different bacterial genera, such as Staphylococcus, Streptococcus, Bacillus, Pseudomonas, Klebsiella, Lactobacillus, and Serratia [10-12].

As tannic acid is one of the abundant plant components, feces and alimentary tract samples [6] of animals feeding on a tannin rich diet (TRD) could be good sources for isolation of tannin-degrading bacteria. This is possible due to the presence of a complex microbial population in the gastrointestinal system, which its composition is mostly ascertained by the diet [13, 14]. Pistachio hulls, which are annually produced about several tonnes in some parts of Iran, are rich sources of tannins and can be used as a TRD. The present study aims to isolate and characterize bacterial strains capable of using tannic acid as the only carbon and energy source from goat feces with and

without pistachio soft hulls as a TRD and also to investigate their potential in tannic acid degradation.

## 2. Materials and Methods

### 2.1. Sample preparation

In present study, two male goats were housed in Isfahan Branch, Islamic Azad University, Agricultural Sciences Research Farm, Isfahan Province). The goats were provided with the same diets from September to October as follows:

- The adaptability phase (g/day) for one week: Pistachio hulls (0), Alfa- alfa (600), Concentrate (200), and Silage (200),

- The experimental phase (g/day) for three weeks: Pistachio hulls (200), Alfa-alfa (350), Concentrate (200), and Silage (240);

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

The goats were fed twice a day (8:00 AM and 4:00 PM) and had free access to drinking water. Samples of goat feces were gathered with fecal bags before and after feeding on the diet. The fecal samples were suspended in sterilized phosphate buffered saline (PBS) and mixed with a vortex test-tube mixer.

### 2.2. TDB isolation and characterization

For enrichment culture, aliquots of fecal suspensions (1ml) were mixed with mineral salt medium (MSM) (50ml). The MSM contained (g/L) $KH_2PO_4$ (1.4), $Na_2HPO_4$ $2H_2O$ (2.7), $(NH_4)_2SO_4$ (0.5), $MgSO_4$ $7H_2O$ (0.2), yeast extract (0.02), and 10 ml of a trace element solution that contained (g/L) $Na_2EDTA$ $2H_2O$ (12.0), NaOH (2.0), MnSO4 $4H_2O$ (0.4), ZnSO4 $7H_2O$ (0.4), $Na_2SO_4$ (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 $H_2SO_4$ (0.5 ml), which was incubated (30°C) without shaking. Tannic acid (0.5g/L) was used as the only source of carbon and energy [11, 15].

After several successful transfers, enrichment cultures with visible turbidity were chosen for isolation. Next, 100µL of each culture was spread on MSM agar supplemented with the tannic acid solution (2g/L). After 48 h of incubation, colonies with different morphological features were chosen as tannic acid-degrading isolates. In addition, biochemical identification of gram-negative colonies was performed by catalase and oxidase activity, as well as IMViC, TSI, and motility tests.

16S rDNA gene amplification of most efficient isolates was done using extracted genomic DNA, with the primers of 27f (5′AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5-GGTTACCTTGTTACGACTT-3). Next, partial sequencing of each 16S rDNA gene was done. The resulting sequences were compared to those in the NCBI, GenBank database, with the help of the BLAST program.

### 2.3. Tannase activity test

Tannase activity of the isolates was confirmed according to Kumar et al.'s method [16]. In brief, tannic acid (2%; filter-sterilized) was added to nutrient agar plates. It can form a complex of tannin–protein; that its cleavage by tannase-producing bacteria will produce a greenish brown area around the colonies after 3–4 days of incubation. Then the plates were flooded with a solution of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01 N HCl) and placed at room temperature for 10 min. $FeCl_3$ reacted with tannic acid and made a brown color; thus, a halo zone was made on a dark brown background.

### 2.4. Tannins Maximum Tolerable Concentration (MTC)

MTC was considered as the maximum tannic acid concentration that bacteria can tolerate and grow after 24-48 h of incubation at 35°C. Tannic acid concentrations used on MSM agar plates included 1, 2, 4, 8, 16, 24, 32, and 64 g/L.

### 2.5. Biodegradation experiments

Biodegradation analyses were performed in flasks containing MSM broth (50ml) and 15g/L filter-sterilized tannic acid. Optical density (540nm), pH values, and tannin concentrations were examined at time intervals. The data were expressed as the mean of triplicate experiments.

Tannin concentrations were measured by the BSA precipitation assay, and the biodegradation percentage was calculated [17, 18]. Accordingly, centrifugation of bacterial suspensions was done (3,000 g; 4°C; 10 min), and the supernatant was used for determining tannins. In each test tube, 1ml of a 1mg BSA/ml acetate buffer stock solution was mixed with the tannic acid solution (1ml). Then, precipitation reactions were allowed to progress during refrigeration (18h). In the next step, samples were centrifuged at 3,000rpm for 10 min. The precipitate was then dissolved in a solution of 1% w/v SDS. Next, 1mL of the produced solution was mixed with the TEA-SDS solution (7% TEA & 1% SDS) (3ml). Some minutes later, 1ml of $FeCl_3$ was added, optical density was read after incubation at room temperature for 60 min, and absorbance was measured (520nm). To zero the spectrophotometer, a tube containing the entire reagent and water was used instead of the extract. Tannin contents were evaluated based on the calibration curve ($R^2$= 0.971) plotted from tannic acid solutions within the range of 0-20gr/L. The resulting equation was y= 41.16x.

## 3. Results

### 3.1. Isolation and identification of bacteria

After the sampling and enrichment procedures in the tannin-containing mineral medium, four strains were isolated from fecal samples before (strain TA1) and after (strains TA2, TA3, and TA4)

feeding on the TRD. All mentioned strains were gram-negative rods, aerobic, and catalase-positive. Table 1 shows biochemical characteristics and preliminary identification of the isolates.

### 3.2. Tannase activity and MTC determination

Tannase activity of all isolates was positive according to the visual assessment of enzymatic tests, i.e. the appearance of a halo zone on a dark brown background. The strains of TA1 (isolated from goat feces before feeding on a TRD) and TA4 showed the MTC of 32g/L and strains of TA2 and TA3 (isolated from goat feces after feeding on a TRD) showed the MTC of 64g/L. According to the results.

**Table 1-** Characteristics of isolated strains, Tannase activity (clear zone diameter), and Maximum Tolerable Tannin Concentration (MTC)

| Isolation Source | Strain ID | Macro & Microscopic char | Biochemical tests Lac Motility Indole MR/VP Citrate TSI | Preliminary identification | Clear zone (mm) | MTC (g/L) |
|---|---|---|---|---|---|---|
| **Feces before TRD** | TA1 | Circular; entire margins, flat elevation, mucoid gram-neg. rod | - + - -/- + - | Pseudomonas | 40 | 32 |
| **Feces after TRD** | TA2 | Creamy, pale, or beige; convex; entire margins; smooth gram-neg. rod | + - + +/- - A/A gas Tween40 + Tween80 + | Pantoea | 52 | 64 |
| | TA3 | Circular; entire margins, flat elevation, mucoid gram-neg. rod | - + - -/- + - | Pseudomonas | 41 | 64 |
| | TA4 | Circular; small, entire margins, convex, smooth gram-neg. rod | + + + +/- - A/A gas | Escherichia | 32 | 32 |

**TRD:** Tannin Rich Diet

### 3.3. Biodegradation experiments

Bacterial isolates from goat feces were examined for their ability to degrade tannic acid (15g/L). Accordingly, visual turbidity increased (data not shown), yet the pH of the medium decreased as the tannic acid was utilized. In the case of TA2, the pH of the medium decreased during 48h, and then it increased once more (Fig. 1). According to Fig. 2, the Pantoa isolate (TA2) was the most efficient strain in terms of tannin biodegradation on the first and second days of incubation with the degradation percentages of 81.6 and 94, respectively. A second-order polynomial was used to fit the data given in this figure. The Pantoea sp. (TA2) and the Pseudomonas sp. (TA3) showed more than 95% of utilization of tannic acid after 72h of incubation, while the degradation percentage of TA4 (*E. coli*) was less than 50% after 72 h of incubation.

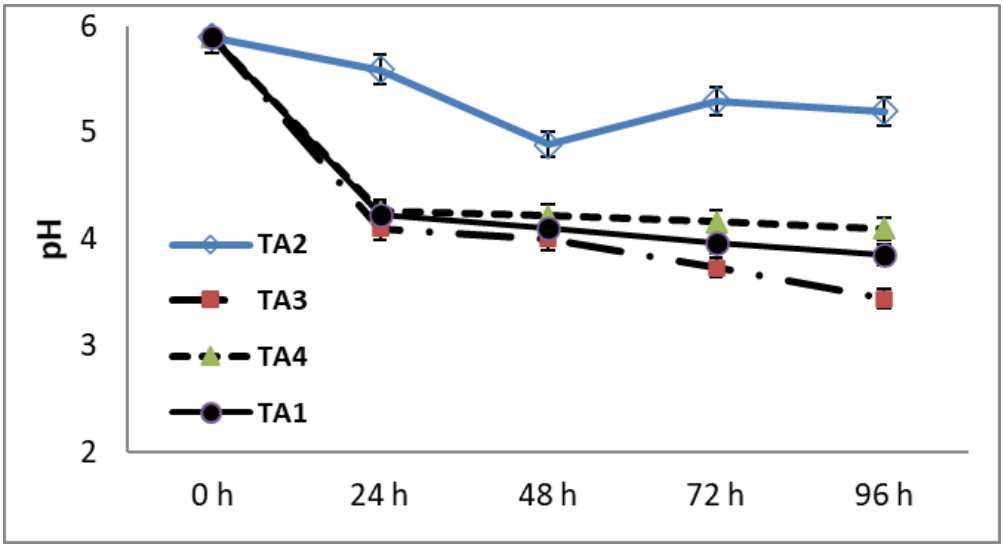


**Fig. 1**- Changes in the pH of MSM during bacterial growth and tannic acid biodegradation

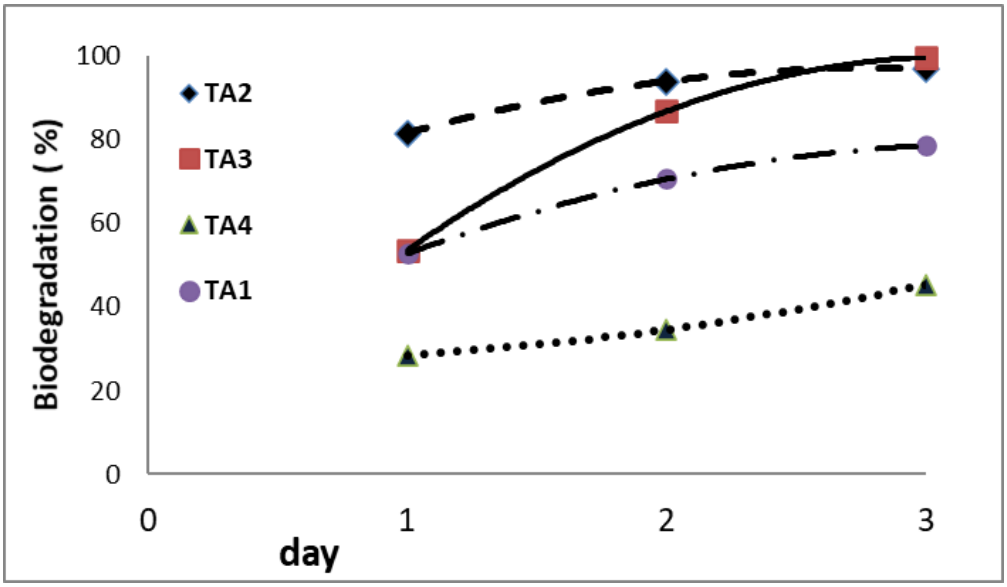


**Fig 2**- Tannic acid degradation percentages of isolated strains

### 3.4. Molecular identification of the most efficient strains

Table 2 presents data on the molecular identification of only those strains with the biodegradation percentage of more than 50% (TA1, TA2, and TA3). 16S rDNA gene sequences of TA1 and TA3 isolates showed the highest similarity (>99%) with the GenBank sequences of the *Pseudomonas oryzihabitans* strain AM 1568 and the *Pseudomonas oleovorans* strain AM 1508. Based on the results of comparing nucleotide sequences using Pintail version 1.0, no anomaly was detected. Variation in the percentage difference between the 16S ribosomal sequence of isolates TA1 and TA3 showed that the probability of the two sequences with a DE of 0.45 and the difference percentage of 0.13 was estimated to be P >0.50. Accordingly, it is obvious that they are practically identical (Fig. 3). Thus, strains TA1 and TA3 were identified as Pseudomonas sp. and submitted to the GenBank as Pseudomonas sp. ATTA34 under the accession number of KJ783441.

The 16S rDNA gene sequence of the TA2 strain compared to the sequences in the GenBank showed the highest similarity (99%) to different strains of *Pantoea brenneri* strain LMG 5343, *Pantoea stewartii* subsp., indologenes strain CIP 104006, and *Pantoea ananatis* LMG 20103.

According to the 16S rDNA similarity and biochemical tests (the indole positive and the ability to grow in the presence of tween 40 and tween 80), the Pantoea isolate of this study was identified as *P. stewartii subsp. indologenes.*

Nucleotide sequences of the isolates were also compared with the standard strain using Pintail version 1.0, and no anomaly was detected. So, the 16S rDNA gene sequence of strain TA2 was submitted to the GenBank as Pantoea sp. ATTA33 under the accession number KJ783440.

**Table 2**- Molecular identification of isolates based on the 16S rDNA gene sequences comparison

| Isolation Source | Strains | B Bacterial name | Percentage of similarity | Accession number |
|---|---|---|---|---|
| **Feces before TRD** | TA1 | Pseudomonas sp.ATTA34 | >99% with different strains of Pantoea | KJ783441 |
| **Feces after TRD** | TA2 | Pantoea sp.ATTA33 | | KJ783440 |
| | TA3 | Pseudomonas sp.ATTA34 | 100% with strain TA1 | KJ783441 |

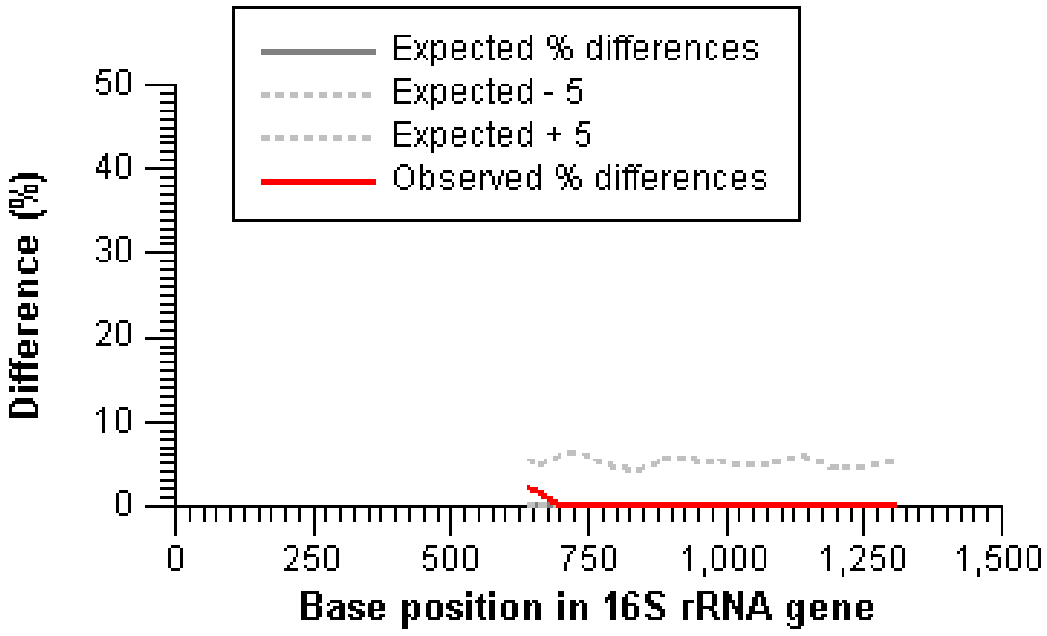


**Fig. 3**- Variations of the difference (in percentage) between 16s rDNA sequences of strains TA1 and TA3 (results from Pintail software)

## 4. Discussion

Bacterial isolation using the enrichment technique has been widely used to study biodegradation of different organic compounds. In this regard, bacterial tannin degradation is gaining worldwide attention. The tannin biodegradation ability of isolates has been attributed to tannase production, which is a key enzyme able to catalyze gallo tannins into glucose and gallic acid [19]. Bacterial strains degrading tannic acid were isolated from different sources, such as Oil Mill Wastes [5, 11], goat or cow feces [13, 6], and soil [20, 21]. In this study, tannin-degrading bacterial isolates were obtained from goat feces before and after receiving a TRD.

The morphological, biochemical, and molecular identification of the most efficient isolates (with more than 50% degradation) revealed that strains TA1 and TA3 were practically identical. So, they were identified as *Pseudomonas sp. (ATTA*34 with accession number KJ783441), and the TA2 strain was recognized as *Pantoea sp.* (ATTA33 with accession number KJ783440). According to complementary biochemical tests for Pantoea identification [22], it was revealed that the Pantoea isolate of this study could grow in the presence of tween 80 and tween 40, so it could be regarded as *P. stewartii subsp. indologenes.*

Pseudomonas strains with the ability of tannic acid degradation were isolated from different sources, such as sawdust [20], soil [21] koala feces [23], and tannery effluents [24]. Bacterial strains of the Pantoea genus were isolated from different sources, such as fecal samples, soil, and plants [25]. The isolate's tannase activity was confirmed by Kumar et al.'s method [15], in which additional $FeCl_3$ staining created a dark background enabled clear zones visibility of tannin degradation. Isolated strains showed the capability of tolerating high concentrations of tannic acid. Also, the number and tolerance ability of isolated strains increased against higher concentrations of tannin in goat feces after feeding on a TRD. Obviously, adaptive responses were induced by the TRD. Such responses were also reported by Rafii et al. (2009) during bacterial isolation from bovine fecal microflora with the ability of ceftiofur degradation [26]. Similarly, Elizendo et al. (2010) reported such adaptive responses in a study on tannin effects on *Clostridium perfringens* growth [27]. Accordingly, while the bacteria were able to detoxify and degrade tannins, they needed to adapt to high concentrations of them. Adaptive responses of gram-negative bacteria to toxic substances are accomplished with a remarkable increase in the degree of fatty acid saturation [5, 11]. Pepi et al. (2013) also showed tannase activity of Klebsiella, Serracia, and Pantoea strains in the existence of tannin concentrations (up to 50g/L) [5]. Furthermore, *Enterococcus faecalis* was reported as a tannase producer [28].

According to the literatures, polyphenols affect bacterial metabolism and growth. Such effects depend on the strain type, structure, and dosage [29]. For example, resistance to polyphenols in gram-negative bacteria is more than that in gram-positive bacteria, which is due to differences in peptidoglycan compositions [30]. One of the possible function mechanisms of polyphenols is their possible binding to cell membranes in a dose-related mode [31, 32]. In a biodegradation experiment, tannic acid utilization as the only source of carbon and energy led to an increase in visible turbidity along with the simultaneous production of acidic metabolites as evidenced by an additional reduction in the pH of the medium. During the first day of TA2 growth, the pH of the medium decreased, but it increased once more after 48h, which could be caused by depside and ester bonds of tannic acid hydrolysis in the degradation process. As a result of such hydrolysis reaction, gallic acid is created and decarboxylated by gallate decarboxylase to produce pyrogallol, which could increase the pH again. However, there was no evidence that pyrogallol was degraded further. The activity of gallic acid decarboxylase has been shown in few bacterial species [33]. In addition it has been shown that some species, such as *St. gallolyticus* [34], *Lonepinella koalarum* [35], *Lactobacillus plantarum, L. pentosus,* and *L. paraplantarum* have both gallic acid decarboxylase and tannase activity [36]. The evidence of tannase activity was confirmed in this study for *P. stewartii subsp. indologenes* for the first time.

In the biodegradation experiment, the tannic acid degradation percentage (at a concentration of 15.0g/L) reached a maximum of 81.6% for the TA2 isolate after 24h, also reached 97% after 72h of incubation. Thus, the strain of TA2 (*Pantoa stwartti* ATTA33) could be regarded as the most efficient strain in tannin biodegradation in the first and other days of incubation. The second-order polynomial equation fitted the data with the predicted $R^2$ of 1, being indicative of about 100% of variability in the response could be explained by the model. This implies that the prediction of experimental data is quite satisfactory. Therefore, the kinetics of the biodegradation percentage across the time was found to be logarithmic, with $R^2$= 1 (polynomial regression second order). The coefficient values of determination ($R^2$) also suggest that the model is a good fit.

The high tannic acid degradation percentages of the isolates were similar to those obtained by Pepi et al. (2010). The difference was in the concentration of tannic acid in the medium; accordingly, the degradation experiment of Pepi et al. was performed with 1g/L of tannic acid [11], but the biodegradation experiment of this study was performed with 15g/L of

tannic acid. Klebsiella sp. isolates of previous studies showed similar tolerance and degradation behavior for tannic acid [10, 18].

## 5. Conclusion

Characterization of Pseudomonas and Pantoea strains with the ability to grow on higher amounts of tannic acid (up to 64g/L) as well as their rapid degradation give them an amazing potential for biotechnological applications, such as bioremediation, wastewater treatment (especially tannery effluents), as also for reducing tannin antinutritional effects in animal diets. Such strains would be so precious for industrial tannase production. In addition, such tannase producing bacteria could be used in the food (clarification of fruit juices) and pharmaceutical industries.

## Conflict of interest

The authors of present research declare that there is no conflict of interest.

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