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Research Article

A New Native Source of *Tannase* Producer, *Penicillium* sp. EZ-ZH190: Characterization of the Enzyme

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Background: Tannase can be obtained from the various sources for example tannin rich plants; however microbial sources are preferred for industrial production. In microbial sources, the *Aspergillus* and *Penicillium* genus and *lactic acid* bacteria mostly produce tannase. However, it has been identified that this enzyme is produced by many fungi and bacteria, but researches are continuing to find new species.
Objectives: The aim of this study was to isolate a tannase-producing fungi from moldy tea leaves and to study some properties of its enzyme.
Materials and Methods: The present study was done via two steps. At first, industrially important tannaseproducing fungi were isolated from moldy tea leaves using the simple agar plate method followed by the screening of organisms capable of producing tannase using the enrichment culture technique in modified Czapek Dox's agar. Finally, tannase obtained from the best isolate was partially purified and characterized.
Results: Tannase produced by *Penicillium* sp. *EZ-ZH190* isolated from moldy tea leaves was partially purified and characterized. Maximum enzyme production (4.33 $U.mL^{-1}$) was recorded after 96 hours of incubation at 30 °C in submerged culture (100 rpm) utilizing 1 % (w/v) tannic acid as a sole carbon source. This tannase exhibited optimum activity at 35 °C and at pH of 5.5, and showed nearly 50 % of its maximal activity at 50 °C. In the present study, tannase from *Penicillium* sp. *EZ-ZH190* had KM and Vmax values of 1.24 mM and 17.09 $U.mL^{-1}$, respectively, and showed more than 50 % stability at salt (NaCl) concentration of 1 M for 24 hours.
Conclusions: Tannase productivity of *Penicillium* sp. *EZ-ZH190* (0.045 $U.mL^{-1}.h^{-1}$) is comparable with the maximum tannase productivity in the reported literatures, and the biochemical characteristics showed by *Penicillium* sp. *EZ-ZH190* tannase are considered favorable for tannin biodegradation in the industry. So, we concluded that *Penicillium* sp. *EZ-ZH190* is a good strain for use in the efficient production of tannase.

Keywords: Activity; Stability; Tannase; Tannic Acid

1. Background

Tannins are polyphenolic compounds with varying molecular weights that occur naturally in the plant kingdom. In these sources, tannins are found in leaves, bark, and wood. Tannins are considered as the secondary metabolic products of plants because they play no direct role in plant metabolism (1). Tannins are the second most abundant group of plant phenolics after lignin. The large amount of phenolic hydroxyl groups allows the tannins to form complexes with proteins and to a lesser amount with other macromolecules like pectin and cellulose (2). Some of the consequences of these interactions are the decline of the feed intake by livestock (3), clean-up requirements for effluents of the leather industry (4), and haze formation in chilled beverages (3). On the basis of tannins structure and properties they can be divided into condensed tannins and hydrolysable tannins.

Tannase (E.C. 3.1.1.20), also known as tannin acyl hydrolase, is an inducible enzyme produced in the presence of tannic acid by a number of fungi (5) and bacteria (6). It hydrolyzes the ester and depside linkages of tannic acid to produce glucose and gallic acid. The enzyme has wide applications in food, beverage, brewing, cosmetics, and chemical industries (7-9). It is mainly used for the preparation of gallic acid, coffee flavored soft drinks, instant tea, high grade leather tannin, clarification of fruit juice and bear, food detannification, and to clean up highly polluting tannin from the leather industry effluent (7, 10, 11). Beside this, gallic acid (3,4,5 tri-hydroxy benzoic acid), the enzymatic product of hydrolytic cleavage of

Implication for health policy / practice / research / medical education:
Food microbiologists or food biotechnologists may use the results of presented research.

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tannic acid, has several applications in chemical and pharmaceutical industries for the production of propyl gallate, pyrogallol, trimethoprim, semiconductor resin, etc. (12). Tannase is now known to be an ever-present enzyme of the microbial world (10) and has wide spread occurrence in various fungi. Most of the reported tannase producing organisms are fungi (13-15) and only a few are bacteria (16, 17). All the above known isolates are from the sources like plant material, soil, vegetable liquor, and from animal feces. Yet the search continues for organisms which are better sources of tannase.

2. Objectives

Due to the increasing industrial application of tannase, new researches are necessary to find new microbial sources with high production of tannase. According to this, the aim of this study was to isolate a new tannase producing fungi from moldy tea leaves and to study some properties of the enzyme.

3. Materials and Methods

3.1. Isolation of Tannase-Producing Fungal Strain

Moldy tea leave samples were taken from tea farms of Gilan, the north state of Iran. A series of dilutions (0.1, 0.01, 0.001, and 0.0001) was made by mixing the samples and sterile distilled water. From the dilutions, 0.5 mL was pipetted onto potato dextrose agar (PDA) and incubated at 25 °C for five days. The pure culture fungi were then made by the hyphal tip method (18). There were 16 potent tannase-producing fungi isolated on the basis of colony diameter (19). The fungi were grown on liquid isolation medium (Czapek Dox medium) containing 2.0 g.L $^{-1}$ of $NaNO_3$, 1.0 g.L $^{-1}$ of K_2HPO_4, 0.5 g.L $^{-1}$ of KCl, 5.0 g.L $^{-1}$ of $MgSO_4$, 0.01 g.L $^{-1}$ of $FeSO_4$, $ZnSO_4$ (trace), $CuSO_4$ (trace), and 10.0 g.L $^{-1}$ of tannic acid. The solution of tannic acid was sterilized separately by passage through a cellulose nitrate membrane filter (25mm diameter, 0.45 µm pore size). Fungi were isolated based on tannase production and kept at 4 °C in PDA slant for further work. Identification of the selected fungal member was made after studying cultural and morphological characteristics.

3.2. Analysis of 18S rRNA Gene Sequence

Genomic DNA of the fungal isolate was extracted using CTAB DNA extraction procedure and employed as templates in PCR reaction using the universal 18S rRNA gene primers 18S-1 (5´-CCTGGTTGATCCTGCCAGTA-3´) and 18S-2 (5´-GCTTGATCCTTCTGCAGGTT-3´). The amplified 18S rRNA genes of each strain were sequenced on an automated DNA sequencer with forward and reverse primers. For gaining the complete sequences of the 18s rRNA genes, EditSeq and SeqMan software were employed. Sequence similarities for the complete sequences for the 18s rRNA genes of different fungal strains were determined via Blast analysis available in the NCBI database. The phylogenetic tree was constructed by means of the MEGA4 software using neighbor joining (NJ) algorithm (20).

3.3. Inoculum Preparation

A spore suspension was prepared by addition of 5 mL of 0.01 % tween 80 to a 10-days old agar slope culture, and the spores were scrapped well into the solution. The spore suspension was shaken thoroughly by cyclomixer to breakup any aggregates. The spore count was determined microscopically using the above suspension (19). Appropriate dilutions were made with sterile normal saline (NaCl 0.9 %) such that the final concentration of the spores was 5×10^5 cfu.mL $^{-1}$.

3.4. Production of Enzyme

Cultivation of fungus was carried out in 250 mL shake flasks using the Czapek Dox medium supplemented with filter sterilized tannic acid at 1 % concentration as the sole source of carbon and inoculated with prepared inoculum (2 % v/v), incubated for 96 hours at 30 °C and at 100 rpm in a rotary shaker. After incubation for the desired period the fungal mycelia were removed by filtration through Whatman No.1 filter paper, and the supernatant was centrifuged (10,000 rpm for 15 min) at 4 °C, and the supernatant was treated as crude enzyme for tannase activity assays (21).

3.5. Partial Purification of Tannase

The crude enzyme was mixed with acetone (1 : 2 volumes) at 4 °C for 4 hours. Then the enzyme and acetone mixture were centrifuged at 10000 rpm for 10 min. The supernatant was discarded and acetone was allowed to evaporate from the uncapped tube at room temperature for 30 min. The precipitate was dissolved in minimum amount of citrate buffer to obtain partially purified tannase (21).

3.6. Tannase Activity Assay

Tannase activity was determined spectrophotometrically according to the method of Ibuchi et al. (22). Then 0.5 mL of culture supernatant (crude enzyme) or partially purified enzyme was added to 2.0 mL of 0.35 % (w/v) tannic acid solution in 0.05 M citrate buffer (pH 5.5) in a test tube. Then 20 µL of the reaction mixture was taken out and 2.0 mL of 95 % ethanol was added to the reaction mixture to stop the enzyme reaction. The absorbance at 310 nm (A1) was noted immediately. The test tube was then incubated in a water bath at 37 °C for 10 min, after which ethanol was added to the reaction

mixture to stop the enzyme reaction. Then absorbance (A2) was measured at 310 nm. Tannase activity was calculated by using the following equation :

Figure 1. Equation of Tannase Activity

$$U/ml = \frac{(A_1 - A_2) \times 20.3 \times 1.0(ml) \times df}{0.71 \times 0.5(ml) \times 10(\min)} = \Delta A \times 5.72 \times df$$

A1, absorbance of the blank; A2, absorbance of the sample; 20.3 µmol of tannic acid in 1.0 mL of substrate solution; 0.71, change in absorbance after complete hydrolysis of 20.3 µmol of tannic acid under the test conditions; df, dilution factor.

One unit of enzyme activity is defined as the amount of enzyme able to hydrolyze 1 µmol of ester in 1 min.

3.7. Effect of Temperature and pH on Tannase Activity and Stability

To determine optimum temperature of the enzyme, partially purified tannase activity was measured at different temperatures (25 – 55 °C) in pH 5.5 according to the Ibuchi et al. method. Thermal stability of the enzyme was determined by incubating it at different temperatures for different time intervals (1 / 2, 1, 6, 12, and 24 hours). After the desired incubation periods, enzyme aliquots were withdrawn and assayed at optimal assay conditions to determine the residual tannase activities (23). To find out the optimum pH, partially purified tannase activity was measured at different pH values (3.5 – 6.5) according to the Ibuchi et al. method. For pH stability, the enzyme solutions were kept at different pH values at 4 °C and ambient temperature (25 °C) for 1 / 2, 1, 6, 12, and 24 hours, and then residual activity was measured at optimum pH and temperature.

3.8. Salt Tolerance Test

The partially purified enzyme was incubated in 50 mM citrate buffer (pH = 5.5) containing various concentrations of NaCl (0 – 4 M) at 4 °C and ambient temperature (25 °C) for 1 / 2, 1, 6, 12, and 24 hours, and then residual activity was measured at optimum pH and temperature.

3.9. Kinetic Constants of Tannase

The effect of the tannic acid concentration on the activity of tannase in the partially purified enzyme was determined experimentally. Different concentrations of tannic acid solution (0.59 to 47 mM) were prepared in citrate buffer (50 mM, pH 5.5), and the effect of substrate concentration on tannase activity was determined. Then, the velocity of the reaction as a function of the substrate concentration was fitted with the Michaelis-Menten model, and the KM and Vmax values of tannase from *Penicillium* sp. *EZ-ZH190* was determined through a Lineweaver – Burk plot for the hydrolysis of tannic acid at 37 °C.

4. Results

4.1. Screening for Tannase Producing Fungi

The present article deals with the isolation and rapid screening of moldy tea leave isolates by a simple plate assay and determination of its quantitative production using submerged fermentation. The isolation technique yielded 16 isolates with different colony morphology and cultural characteristics. All the obtained isolates were subjected to screening on tannic acid agar medium. Among the 16 isolates, 8 isolates grew on tannic acid agar medium and had a colony diameter above 10 mm. This shows their utilization of tannic acid by producing the tannase enzyme. All these fungi were tested for their ability to produce tannase by submerged fermentation. The colony diameter and enzyme production of these isolates are presented in Table 1. Among the isolates the best tannase producer was *Penicillium* sp. *EZ-ZH190* which produced tannase at 4.33 $U.mL^{-1}$. This indigenous fungus was identified by using molecular method (18S rRNA sequencing) as mentioned in section 3.2, and then it was registered in EMBL databank under the accession number of HE648571.

Table 1. Colony Diameter and Enzyme Production of Moldy Tea Leave

Isolates Number	Isolates Name	Colony Diameter (mm)	Extracellular Enzyme ($U.mL^{-1}$)
1	*Penicillium* sp.	12	0.23 ± 0.05
2	*Penicillium* sp.	18	0.41 ± 0.03
3	*Penicillium* sp.	22	0.5 ± 0.02
4	*Trichothecium* sp.	24	0.62 ± 0.04
5	*Penicillium* sp. EZ-*ZH190*	48	4.33 ± 0.03
6	*Neurospora* sp.	21	0.5 ± 0.03
7	*Fusarium* sp.	22	0.51 ± 0.04
8	*Trichoderma* sp.	26	0.82 ± 0.05

4.2. Effect of Temperature on Tannase Activity and Stability

To evaluate the effect of different temperatures on activity and stability of partially purified *Penicillium* sp. *EZ-ZH190* tannase, the temperature range was varied from 25 to 55 °C. With a rise in temperature, the tannase activity increased and optimum activity was recorded at 35 °C (12.41 U.mL $^{-1}$) (Figure 2). With a further increase in temperature, a decrease in activity was observed.

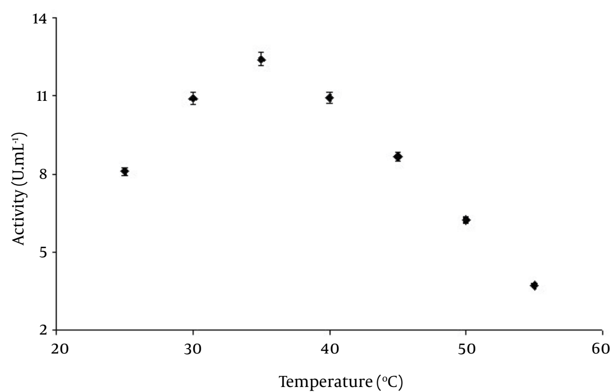

Figure 2. Tannase Activity Recorded at 35 °C

In the present study, *Penicillium* sp. *EZ-ZH190* tannase showed stability in the range of 25 – 40 °C for 24 hours retaining more than 79 % of its residual activity at 25 °C, 91 % at 35 °C, more than 70 % at 40 °C, and more than 30 % at 45 °C (Figure 3). At 55 °C, very low residual activity of 2.5 % in partially purified tannase was observed after 24 hours.

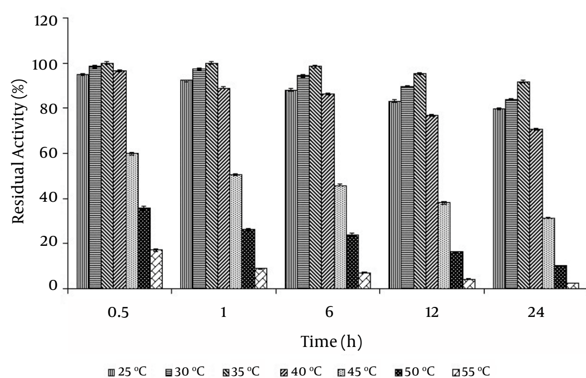

Figure 3. Penicillium sp. EZ-ZH190 Tannase Residual Activity at 25 °C

4.3. Effect of pH on Tannase Activity and Stability

To determine the effect of pH on partially purified *Penicillium* sp. *EZ-ZH190* tannase, the pH of acetate buffer was varied from 3.5 to 6.5. The enzyme was active at an acidic pH (pH < 7) and activity decreased as the pH approached the alkaline range and high acidic range (pH = 3.5 and 4). In the present study, the optimum activity of *Penicillium* sp. EZ-ZH190 tannase was recorded at pH 5.5 (Figure 4).

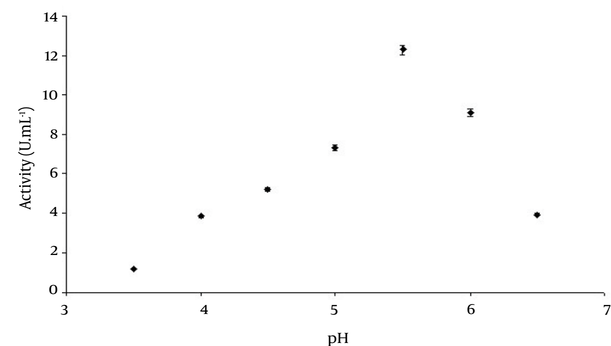

Figure 4. The optimum Activity of Penicillium sp. EZ-ZH190 Tannase Recorded at pH 5.5

Tannase from *Penicillium* sp. *EZ-ZH190* was stable in pH range of 4.5 – 6 for 24 hours at 4 °C and room temperature (Figure 5). It showed 3.62 % and 2.66 % residual activity at pH 3.5, more than 95 % and 81 % stability at pH 5.5, for 24 hours at 4 °C and room temperature, respectively. At pH 6.0 and temperature of 4 °C, partially purified tannase from *Penicillium* sp. *EZ-ZH190* retained more than 81 % of its residual activity for 24 hours. However, it retained 76.39 % of its residual activity at pH 6.0 and room temperature for 24 hours. At pH 6.5, tannase from *Penicillium* sp. *EZ-ZH190* retained more than 44 % and 34 % of its residual activity for 24 hours at 4 °C and room temperature, respectively.

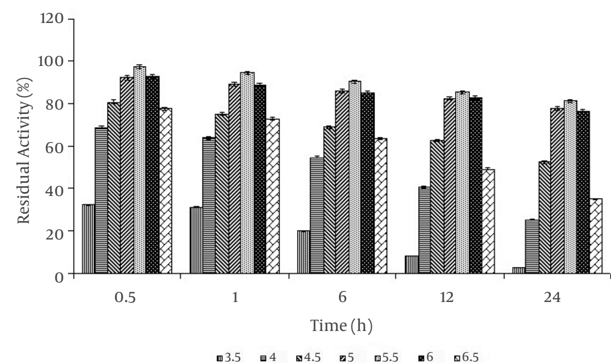

Figure 5. Stability of Tannase From Penicillium sp. EZ-ZH190 in pH Range of 4.5 – 6

4.4. Kinetic Constants of Tannase and its Salt Tolerance

For determination of Michaelis constant (KM) and maximum velocity (Vmax) values of the enzyme, tannase activity was measured with the substrate, tannic acid, at various

concentrations ranging from 0.59 to 47 mM (Figure 6). The KM and Vmax values of tannase, for tannic acid, were calculated from a plot of 1 / V vs. 1 / [S] (Lineweaver – Burk plot) and were found to be 1.24 mM and 17.09 U.mL $^{-1}$ respectively.

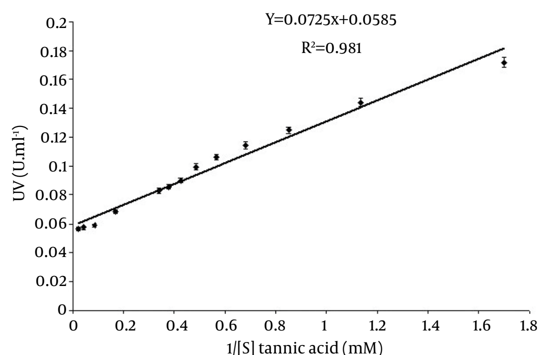

Figure 6. Values of the Enzyme, Tannase Activity

In the present study, partially purified tannase from *Penicillium* sp. *EZ-ZH190* showed more than 50 % stability at salt (NaCl) concentration of 1 M at 4°C for 24 hours (Figure 7). It showed no residual activity at concentration of 3 M and 4 M for 24 hours at 4 °C and ambient temperature, and had more than 95 % and 81 % stability without salt at 4 °C and ambient temperature, respectively. At concentration of 2 M, partially purified tannase retained 7.77 % of its residual activity after 12 hours at 4 °C. This observation shows that tannase is approximately a salt tolerant enzyme. The salt tolerant property of tannase will be very useful for pollution control and the bioprocess industry.

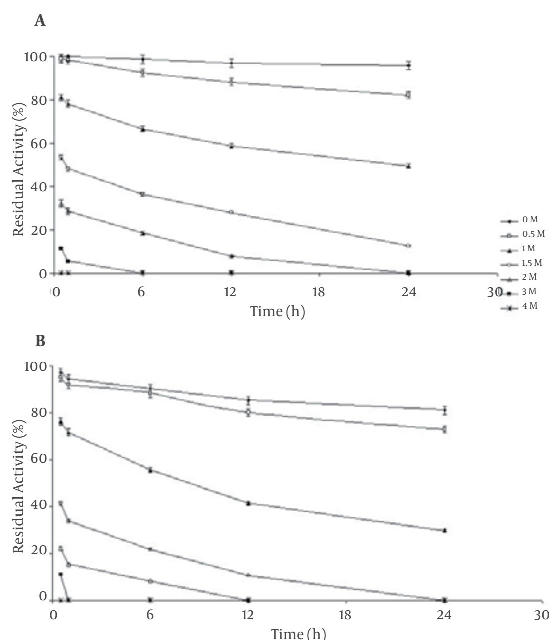

Figure 7. EZ-ZH190 Stability at Salt (NaCl) Concentration of 1 M at 4°C for 24 Hours

5. Discussion

Tannase can be obtained from various sources, such as tannin rich plants; however, microbial sources are preferred for industrial production, as microbial enzymes are usually more stable than their plant or animal counterparts and the fermentation process can be controlled more easily and can produce large amounts of enzymes (10). It is well known that tannins can inhibit the growth of many microorganisms, but some species have developed mechanisms like the production of tannase and other related enzymes to degrade tannins and use them as a carbon source (24). Previously, it has been claimed that tannase production is performed by only a few microorganisms. However, other studies have identified that this enzyme is produced by more than 70 species, and the number is increasing as a result of the continuing search for new sources of this enzyme (25). Fungi from the *Aspergillus* and *Penicillium* genus and *lactic acid* bacteria mostly produce tannase. The most common strategy for identification of sources of tannase, is to isolate microorganisms from tannin-rich environments and investigate their ability to produce tannase. For example, Murugan et al. (19) isolated 10 different morphological fungal strains from a tannery effluent. They isolated the microorganisms in PDA slants by serial dilution and investigated tannase production by a simple plate assay, and the selected microorganisms were tested for tannase production under SmF in a stirred tank bioreactor (19). Pepi et al. isolated 3 tannase producing bacterial strains from olive mill waste. The isolated bacteria, belonging to the *Pantoea* and *Serratia* genus, were grown in SmF with tannic acid as the sole carbon source and completely degraded a 1 % tannin solution within 24 hours (1).

Isolation of microorganisms from the environment is the microbiologists' first step in screening for natural products such as secondary metabolites and enzymes (26). In the present research, 16 colonies were isolated from moldy tea leaves and among them, 8 isolates grew in tannic acid agar medium and had a colony diameter above 10 mm, and among the 8 isolates, the best tannase producer was *Penicillium* sp. *EZ-ZH190* which produced 4.33 U.mL $^{-1}$ of tannase. In accordance with Zhong et al. (27), the tannase from *Aspergillus* oryzae produced from recombinant Pichia pastoris showed 7 U.mL $^{-1}$ activity after 96 hours of fed batch culture (productivity of 0.073 U.mL $^{-1}$.h $^{-1}$). Trevino-Cueto et al. (28) observed that the maximum tannase activity of *Aspergillus niger* Aa-20 (1.042 U.mL $^{-1}$) was obtained after 43.5 hours of fermentation at solid state culture of a tannin-rich desert plant (productivity of 0.024 U.mL.h $^{-1}$). Enemuor and Odibo (29) reported the maximum tannase production of 0.9 U.mL $^{-1}$ by *Aspergillus tamarii* IMI388810 after 144 hours of incubation (productivity of 0.006 U.mL.h $^{-1}$). From the present study it can be concluded that before optimization, tannase productivity of *Penicillium* sp. *EZ-ZH190* (0.045 U.mL.h $^{-1}$)

is comparable with the maximum tannase productivity in the reported literatures. Thus, we conclude that *Penicillium* sp. *EZ-ZH190* is a good strain for use in the efficient production of tannase.

Recently, several research groups have carried out searches for new sources of tannase. These researches are made for discovery of microorganisms with higher enzymatic production or enzymes with desirable properties, for example more stability at a broad range of temperature and pH. In many fungi, temperature optima for tannase activity have been reported to be in the range of 30 – 40 °C (30-32). However, tannases from *A. niger* var Tieghem (33) and *Bacillus* cereus KBR 9 (17) have been reported to have a temperature optima between 45 and 60 °C. Optimum temperatures of 60 – 70 °C and 20 – 25 °C have been reported for *A. Niger* and *Verticillium* sp. Tannase, respectively (15, 34). In the present work, the optimum activity of *Penicillium* sp. *EZ-ZH190* tannase was recorded at 35 °C, and it showed stability in the range of 25 – 40 °C for 24 hours retaining more than 79 % of its residual activity at 25 °C and 91 % at 35 °C.

Any change in pH affects the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme instability or its inactivation. From the results, it could be concluded that tannase from the novel isolate requires an acidic environment to be active, and in general fungal tannase is an acidic protein. There are reports describing the optimum pH to be 5.0 in case of tannase obtained from *A. awamori* (35), 5.5 in case of tannase obtained from *A. flavus* or *Aspergillus* oryzae, (36, 37), 2.0 and 8.0 in case from *Aspergillus* awamori (38), and 6.0 in case of tannase obtained from *P. chrysogenum* or *Aspergillus* niger (15, 39, 40). In this research, the optimum activity of tannase from *Penicillium* sp. *EZ-ZH190* was recorded at pH 5.5. This enzyme was stable in pH range of 4.5 – 6 for 24 hours, and it showed more than 95 % and 81 % stability at pH 5.5 for 24 hours at 4 °C and ambient temperature, respectively. More than 75 % of enzymes need the existence of metal ion activators to express their full catalytic activity. At low concentration, metal ions act as cofactors of many enzymes, so they increase the activity of the enzyme, but at high concentrations the catalytic activity is decreased. This may be due to the partial denaturation of the enzyme by the presence of excessive free ions in the enzyme extract (41, 42). Salt tolerance of *Penicillium* sp. *EZ-ZH190* tannase is shown in Figure 7. The *Penicillium* sp. *EZ-ZH190* tannase was stable in salt concentration range of 0 – 1 M.

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Authors' Contribution

All authors have participated equally in this study.

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There is no financial disclosure.

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