# Biosynthesis and Characterization of *Aspergillus Niger* AUMC 4301 Tannase.

**M. Z. El-Fouly[1]; Z. El-Awamry[2]; Azza A.M. Shahin[1]; Heba A. El-Bialy[1]; E. Naeem[2] and Ghadeer E. El-Saeed[1]***

[1] National Center for Radiation Research and Technology (NCRRT), Radiation Microbiology Department, Atomic Energy Authority, Cairo, Egypt. [2] Faculty of Science, Azhar University, Cairo, Egypt.

**Abstract:** A study on biosynthesis and characterization of an extracellular tannase from *Aspergillus niger* AUMC 4301 was carried out. *A. niger* AUMC 4301 was selected out of one hundred and thirty fungal isolates have the ability to grow in the presence of tannic acid. Maximum enzyme synthesis under solid state fermentation was attained in the presence of 3% tannic acid and 0.2% ammonium nitrate after five days incubation at 30°C. Effect of different carbon and nitrogen sources on tannase formation was also investigated. Crude tannase had maximum activity at pH 4.8, 60°C and 20 min as a function of reaction time. The catalytic action of biosynthesized tannase was directly proportional to the amount of enzyme in the reaction mixture. Using tannic acid as substrate, the $K_m$ value for tannase was 2.50 mM. Gallic acid was shown to be a competitive inhibitor to tannase and the inhibition constant ($K_i$) was 1.35 mM. Effect of EDTA and some metal salts on enzyme activity was also studied.
[M. Z. El-Fouly; Z. El-Awamry; Azza A.M. Shahin; Heba A. El-Bialy; E. Naeem and Ghadeer E. El-Saeed. **Biosynthesis and Characterization of *Aspergillus Niger* AUMC 4301 Tannase.** Journal of American Science 2010;6(12):709-721]. (ISSN: 1545-1003). http://www.americanscience.org.

**Keywords:** *Aspergillus niger*, tannase, tannins, gallic acid, solid state fermentation

## 1. Introduction:

Tannase (tannin acyl hydrolse, E.C.3.1.1.20) catalyses the hydrolysis of ester and depside bonds of hydrolysable tannins as tannic acid, methygallate, ethylgallate, n-propylgallate and isoamylgallate releasing glucose and gallic acid (Barthomeuf *et al*., 1994). Tannase can be obtained from plant, animal and microbial sources. Industrially, the most important source for tannase production is microbial way because of its stability (Bhat *et al*., 1998). Over the past decade, a few bacterial species have been reported to produce tannase (Ayed and Hamdi, 2002 and Manjit *et al*., 2009) whereas the most reports are fungal origin including *A. aculeatus*, *A. aureus*, *A. flavus*, *A. foetidus*, *A. japonicas*, *A. niger*, *A. oryzae* (Batra and Saxena, 2005 and Purohit *et al*., 2006), *Aureobasidium pullulans* (Banerjee and Pati, 2005), *Paecilomyces variotii* (Mahendran *et al*., 2006), *Penicillium chrysogenum* (Batra and Saxena, 2005), *Penicillium variable*, (Sharma *et al*., 2008) and *Rhizopus oryzae* (Purohit *et al*., 2006).

Nowadays, untraditional fruit juices (pomegranate, cranberry, raspberry, etc.) have been acclaimed for their health benefits, in particular, for its disease-fighting antioxidant potential. The presence of high tannin content in these fruits is responsible for haze results from protein–polyphenol interaction, tannase applied to remove haze and improve color, bitterness, and astringency of the juice upon storage (Rout and Banerjee, 2006). Tannase is extensively used in the production of instant tea by solubilization of tea cream and in the manufacture of coffee-flavored soft drinks (Lu *et al*., 2009). Tannase also participates in the preparation of animal feeding (Nuero and Reyes, 2002) and in leather industry (Orlita, 2004). Tannase can be potentially used for the degradation of tannins present in the effluents of tanneries, which represent serious environmental problems (Van de-Lagemaat and Pyle, 2001). As well as, tannase is used in the treatment of waste water containing polyphenolic compounds such as tannic acids and as an analytical probe for determining the structures of naturally occurring gallic acid esters (Mukherjee and Banerjee, 2006).

Other important application of tannase is the production of gallic acid and propylgallate (Kar *et al*., 2002). Propylgallate is considered as food antioxidant and used in the food industry and some dyestuffs (Sharma and Gupta, 2003). Gallic acid possesses a wide range of biological activities, such as antioxidant, antibacterial, antiviral, analgesic. It also shows cytotoxic activity against cancer cells, without harming normal cells (Beniwal and Chhokar, 2010). Gallic acid is used in the pharmaceutical industry for the synthesis of antibacterial drugs and in the food industry as substrate for the chemical synthesis of food preservatives such as pyrogallol and gallates. It is also used as an ingredient of developer in photography and printing inks.

Since, *Aspergillus niger* is an officially approved microorganism in France for enzyme

production in the food industry and is also classified as 'generally regarded as safe' (GRAS) by the US Food and Drug Administration (Barthomeuf *et al*., 1994), this study will concern on emphasis to produce tannase using local strain of *A. niger* AUMC 4301 through two strategies; optimization of cultivation conditions of *A. niger* AUMC 4301 and optimization of kinetic behavior of produced tannase by optimization of reaction mixture parameters.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals used were analytical grades (Adwic, Egypt); Tannic and gallic acids were purchased from Mumbai, India.

### 2.2. Microorganisms

One hundred and thirty fungal isolates were screened for their ability to grow in the presence of tannic acid in a preliminary experiment. Fungal isolates succeeded to grow in the presence of tannic acid were tested for tannase production individually (data not shown). The most active tannase producer was selected and identified as *Aspergillus niger* AUMC 4301 at Mycological Center, Faculty of Science, Assuit University, Egypt.

### 2.3. Fermentation process

*Aspergillus niger* was grown on Czapek-Doxs agar medium for one week at 30°C to enhance spore formation. Spores were collected under aseptic condition using Tween 80 (2.9%). The prepared spore suspension was adjusted to $10^7$ spores/ml. Three milliliters of prepared spore suspension were inoculated into 250 ml Erlenmeyer flasks containing 10 g of wheat bran, 3 g tannic acid and supplemented with 10 ml of mineral medium. Mineral medium containing (g/L) $K_2HPO_4$ 1.0, $NH_4NO_3$ 2.0, $MgSO_4.7H_2O$ 2.0 and $CaCl_2.2H_2O$ 0.002 was adjusted at pH 5.7. The inoculated flasks containing media were incubated at 30°C for 5 days.

### 2.4. Crude extracellular tannase extraction

Tannase was extracted from the fermented medium of the cultures cultivated under SSF conditions by adding 80 of 20 mM acetate buffer (pH 5.0). Flasks were shaken for 1 hr at 200 rpm to extract crude enyme. The buffer containing enzyme was filtered twice through cloth filter and Whatman filter paper. Then, the filtrate was used as crude extracellular tannase.

### 2.5. Tannase assay

Tannase activity was determined spectrophotometrically using tannic acid as a substrate according to the protocol of Mondal *et al.* (2001a) but with some modifications. The crude extracellular tannase (1ml) was incubated with 1ml of 4µmole standard tannic acid (substrate); in 0.2M acetate buffer (pH 5.4). After 20 min incubation in a water bath at 60°C, the reaction was terminated by icing. Tannase activity was determined spectro-photometrically at 530 nm using 1ml of ferric chloride reagent (0.13M). The enzyme activity was calculated from the difference in absorbance at zero time (beginning of the reaction) and after enzymatic reaction. The unknown amounts of tannic acid were determined from a standard curve prepared by the same procedure (Data not shown). One unit of tannase activity was defined as the amount of tannic acid hydrolyzed by 1ml of enzyme per minute of reaction. The protein concentration of crude extracellular tannase was determined by Lowry *et al*., (1951) after dialyzed against distilled water for 24 hr using dialysis bag (Medicell International Ltd.) to avoid interfered reactions with phenolic compounds. The specific activity of tannase was calculated according to amount of consumed tannic acid and protein content. Specific activity = Activity (U)/Protein concentration (mg).

### 2.6. Gallic acid assay

Gallic aid was determined spectrophotometrically according to the method of (Pinto *et al*., 2006).

### 2.7. Factors affecting tannase activity of A. niger AUMC 4301

Tannase was maximized through two stages of optimization; physiological optimization of tannase producer (*A. niger* AUMC 4301**)** and kinetic optimization of tannase itself. All experiments were studied separately by one way experimental design system (ANOVA) and the results were analyzed by Duncan test to detect significant differences among the treatments with a probability of 5% using SAS software package version 6.12.

To achieve physiological optimization; the effect of different incubation periods (2, 3. 4. 5, 6, 7) days, different incubation temperature degrees (5, 20, 30, 40) C° and various tannic acid concentrations (1.5, 3, 6, 9, 12) g/l were investigated on tannase activity of *A. niger*. As well as, the tannase activity of *A. niger* AUMC 4301 was determined at different fermentation states (solid state fermentation and submerged fermentation either under agitated or static conditions). For submerged fermentation, the wheat bran was removed from constituents of cultivation medium. In another experiment, tannic acid was removed from constituents of medium in individual experiment and replaced by different carbon sources (fructose, glucose, glycerol, mannitol,

starch, sucrose and xylose). In addition, ten different nitrogen sources were chosen to determine their efficiency to support tannase activity. They were L-alanine, L-cystein, L-lysine, L-ornithine and L-serine, as organic nitrogen sources and ammonium chloride, ammonium sulfate, potassium nitrate and sodium nitrate as inorganic nitrogen sources as well as ammonium nitrate was served as control. Ammonium nitrate concentration in cultivation medium was increased from 0 to 5 $gl^{-1}$ in order to determine the best nitrogen source concentration that achieved the maximum tannase activity.

To optimize kinetic parameters of tannase itself; the reaction was carried out as a function of time (5-60) min, the reaction mixture was incubated at different temperatures (30, 40, 50, 60, 70, 80)°C and the concentration of tannic acid in the reaction mixture was changed from one to ten micromole. The optimum pH that achieved that maximum tannase activity was estimated using sodium phosphate buffer with different pH values (3.6, 4.2, 4.8, 5.4, 5.6). The ratio between substrate (Tannic acid) and enzyme (Tannase) in the reaction mixture was studied and $K_m$ value of substrate was determined. Thermal stability of crude extracellular tannase at 60 and 70 °C and the effect of gallic acid, inhibitors as EDTA and metal ions such as calcium, cobalt, cupper, iron, magnesium, manganese and zinc addition into reaction mixture were also investigated.

### 3. 3. Results and Discussion

In general, the degrading enzymes of aromatic compounds are of considerable interest for bioremediation and biodegradation of organic waste products. Tannase hydrolyses ester bond of tannins to produce glucose and gallic acid. It is also utilized in a number of industrial applications including the manufacture of instant tea, beverages and gallic acid.

3.1. Optimization of cultivation conditions

3.1.1. Rate of gallic acid formation from tannic acid by tannase of A. niger AUMC 4301

The present experiment aims to demonstrate the rate of gallic acid formation from tannic acid by tannase of *A. niger* AUMC 4301. Figure (1) illustrated that tannic acid was hydrolyzed by the action of tannase yielding gallic acid and the increase in the amount of formed gallic acid was associated with a decrease in the amount of added tannic acid i.e the amount of consumed tannic acid after 30 minutes was almost equivalent to that of the formed gallic acid on equimolar basis.

3.1.2. Production of tannase by *A. niger* AUMC 4301 using different fermentation states.

Three states of fermentation; solid state, submerged states either shaking or static state were designed to compare their intensifying effect on tannase productivity by *A. niger*. Results recorded in table (1) indicated that tannase activity and accordingly specific activity of *A. niger* AUMC 4301 incubated under solid state fermentation (3.37±0.172 U/mg protein) was higher than those obtained with cultures grown under static (0.014±0.002 U/mg protein) and shaking condition (0.04±0.009 U/mg protein). In this concern, Lekha and Lonsane (1994) reported that titers of extracellular tannase produced by *A. niger* PKL 104 in solid state fermentation (SSF) were 2.5 and 4.8 times higher and required only about half of the fermentation time in comparison with those in the same medium in submerged and liquid surface fermentation, respectively.

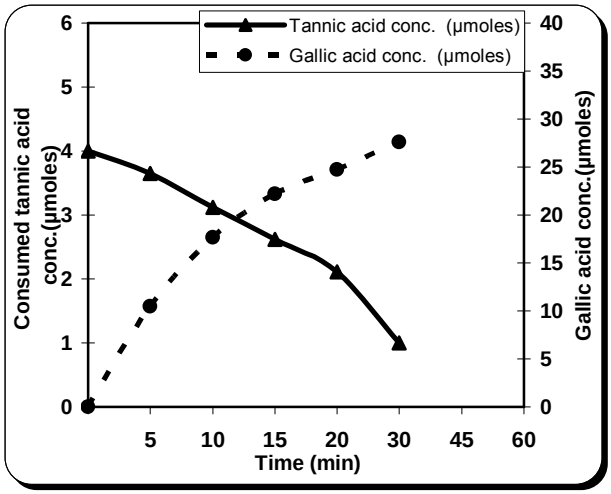



**Fig. (1): Rate of gallic acid formation from tannic acid by tannase of *A. niger* AUMC 4301**

Other studies were conducted to evaluate tannase production in submerged and solid state cultures (Belmares *et al*., 2004 and Marco *et al*., 2009). These studies proved that tannase yield by *Aspergillus* under SSF conditions was higher than that produced under submerged conditions. On the other hand, Srivastava and Kar (2009) stated that extracellular tannase of *A. niger* ITCC 6514.07 was produced optimally under submerged fermentation conditions. The favorability of tannase production using solid state fermentation in the present study could be explained by the positive effect of SSF on the production process itself and/or on the enzyme producer (*A. niger* AUMC 4301). The presence of support material as rice straw in SSF could be

considerable as additional source of carbon and energy (Sabu *et al*., 2005).

**Table (1): Production of tannase by *A. niger* AUMC 4301 using different fermentation states**.

| Fermentation state | Specific activity (U/mg protein) |
|---|---|
| **Solid state** | 3.37±0.172[B*] |
| **shaking state** | 0.038±0.009[B] |
| **Static state** | 0.014±0.002[A] |

*Different symbols means there is a significant difference

3.1.3. Effect of different incubation periods on the production of *A. niger* AUMC 4301

Tannase activity of the fungus under investigation was determined at different incubation periods ranging from 2-7 days. Figure (2) showed that the ability of *A. niger* AUMC 4301 to produce tannase didn't start before 2 days. The lag phase was followed by the exponential phase during third and fourth days where the specific activity of tannase was increased gradually (1.76±0.038 and 2.98±0.082 U/mg protein) and maximized at the fifth day of incubation (3.30±0.11 U/mg protein). Many authors mentioned different incubation periods ranged from (72hr to 120hr) that achieved maximum tannase, this variation depends on the specificity of producers and state of fermentation **(Sabu *et al.*, 2005; Banerjee and Pati, 2007; Rodrigues *et al.*, 2008** and **Enemuor and Odibo, 2009)**. After that, the specific activity of the produced tannase was significantly decreased ($R^2$=0.994). The decrease in tannase yield with prolonged incubation could be explained by shift in the reaction equilibrium due to accumulation of end product (gallic acid) in the fermentation media **(Kar *et al.*, 1999)** or accumulation of toxic metabolites in the fermentation medium due to fungal metabolism and tannic acid degradation that leads to fungal cell autolysis or enzyme denaturation **(Gautam *et al.*, 2002)**.

3.1.4. Influence of incubation temperatures on the production of *A. niger* AUMC 4301 tannase.

Biological processes generally only occur within a relatively narrow range of temperature (Sabu *et al.*, 2005). The present experiment aims to determine the optimal temperature for tannase formation by *A. niger* AUMC 4301 when grown at various degrees of temperatures ranging from 5 - 40°C. Figure (3) show the optimal temperature for enzyme production was 30°C and a slight decrease in enzyme specific activity was observed at 20 and 40°C where it recorded 2.69±0.062 and 2.34±0.09 U/mg protein in the same order. So, tannase production by local strain of *A. niger* AUMC 4301 can be evaluated at room temperature in our nation without additional efforts or costs. Similar temperature optima was reported for tannases of *A. niger* ATCC 16620 (Sabu *et al*., 2005) and *A. oryzae* (Rodrigues *et al*., 2008) while a somewhat different temperature optimum was recorded for tannase production by *A. fumigates* MA (25°C) (Manjit *et al*., 2008) and for *Trichoderma. viride* tannase (45°C) (Lokeswari *et al*., 2010).

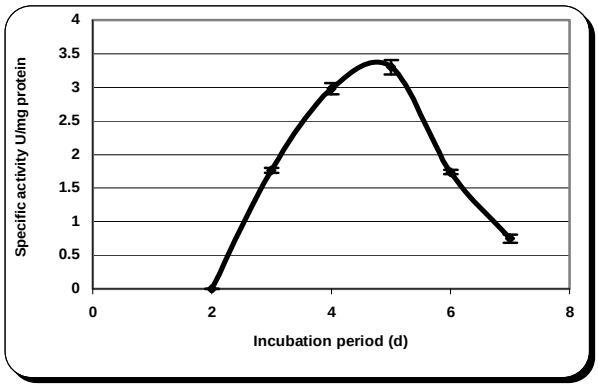



**Fig. (2): Effect of different incubation periods on the production of *A. niger* AUMC 4301**

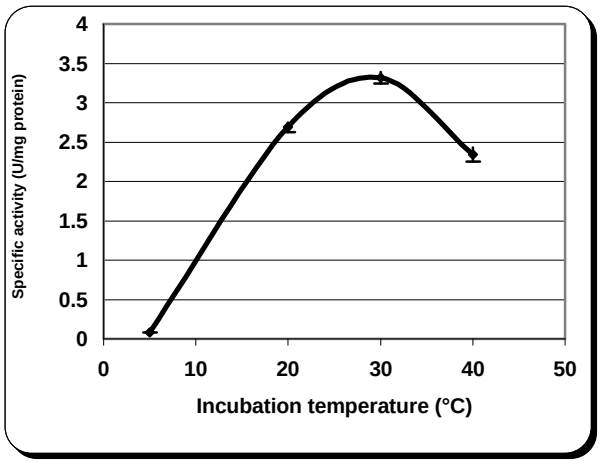



**Fig. (3): Influence of different incubation temperatures on the production of *A. niger* AUMC 4301 tannase.**

4.1.5. Effect of different carbon sources on the production of *A. niger* AUMC 4301 tannase.

All heterotrophic microorganisms need suitable carbon source to grow and positively react with surrounding environment to produce valuable compounds such as enzymes. Since tannase is an

inducible enzyme, in the following experiment, different carbon sources instead of tannic acid were tested for tannase enhancement by the selected fungal strain *A. niger* AUMC 4301. The tested carbon sources were added to the fermentation medium at the same concentration of tannic acid. Table (2) recorded that none of the tested carbon sources stimulated enzyme formation as compared with tannic acid-grown cultures (control). Glucose caused about 50% repression in enzyme production (Specific activity was 1.68±0.013 U/mg protein), whereas mannitol completely suppressed it. Fructose, glycerol, starch, sucrose and xylose resulted in great decrease in the enzyme activity in comparison with control; they supported specific activity about 0.5 U/mg protein. Comparable studies dealing with the induction of microbial tannase synthesis by tannic acid were recorded by many workers (Banerjee *et al*., 2001, Banerjee *et al*., 2007 and Paranthaman *et al*., 2009). It is worthy to mention that tannase of *A. japonicus* (Bradoo *et al*., 1996) was produced constitutively on simple and complex sugar substrates but activity was doubled in the presence of tannic acid as the sole carbon source. The suppression effect of readily metabolized sugars was also reported by (Lekha and Lonsane, 1997, Mondal *et al*., 2001b and Manjit *et al*. (2009). On the other hand, Van de Lagemaat and Pyle (2005) reported that if carbon source present in the media will be exhausted easily and rapidly, this may lead to the partial induction of tannase. Sabu *et al*. (2005) stated that glucose and other readily metabolized carbon source reduce the lag period required for tannase synthesis and production.

**Table (2): Effect of different carbon sources on the production of *A. niger* AUMC 4301 tannase.**

| Carbon sources | Specific activity (U/mg protein) |
|---|---|
| **Tannic acid(control)** | 3.35±0.081$^{A*}$ |
| **Fructose** | 0.56±0.022$^{C}$ |
| **Glucose** | 1.68±0.134$^{B}$ |
| **Glycerol** | 0.43±0.058$^{CD}$ |
| **Mannitol** | 0.03±0.044$^{E}$ |
| **Starch** | 0.32±0.022$^{D}$ |
| **Sucrose** | 0.38±0.020$^{CD}$ |
| **Xylose** | 0.52±0.032$^{CD}$ |

*Different symbols means there is a significant difference.

3.1.6. Synthesis of tannase as a function of tannic acid concentration in the culture medium of *A. niger* AUMC 4301.

From the preceding experiment, it is evident that tannic acid was the most suitable carbon source for tannase induction by the experimental fungus. It was then necessary to test the effect of tannic acid concentration (Ranging from 1.5 to 12 %) in the medium on the enzyme yield. The data illustrated in figure (4) show the optimum concentration of tannic acid for *A. niger* AUMC 4301 tannase was 3%, thereafter, the enzyme activity decreased by elevating tannic acid concentration in the medium and almost disappeared at 12%. Banerjee and Pati (2007) noticed the decrease of *A. pullulans* DBS66 tannase at higher tannic acid concentration, they explained this finding on the basis that tannic acid in higher concentration makes an irreversible reaction with surface proteins of the organism, thereby both growth and enzyme production may be reduced. Similarly, Seth and Chand, (2000) found that an over increase in tannic acid concentration induced decrease in tannase activity by deposition of gallic acid on the cell surface.

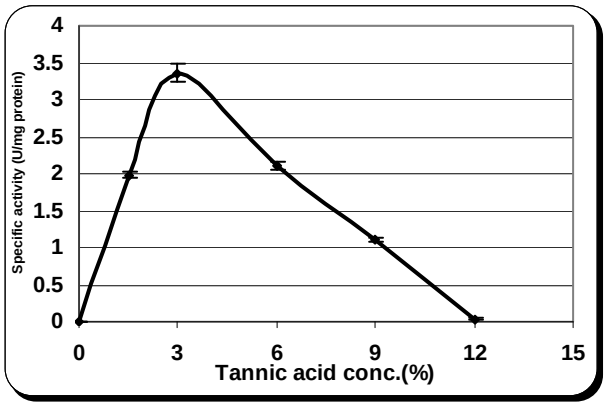



**Fig. (4): Effect of different tannic acid concentrations on the production of *A. niger* AUMC 4301 tannase**.

3.1.7. Effect of different nitrogen sources on tannase production by *A. niger* AUMC 4301.

Tannase or any other enzyme production depends mainly on the availability of both carbon and nitrogen sources in the medium. Both have regulatory effects on enzyme synthesis (Patel *et al.*, 2005). The nitrogen source ($NH_4NO_3$ 2gm/l) presented in the basal medium was replaced by equivalent amounts of nine different nitrogen sources in dry weight basis. It is obvious from the results cited in table (3) that none of the tested nitrogen sources enhanced the synthesis of *A. niger* AUMC 4301 tannase over that of ammonium nitrate–grown cultures (control). Tannase activity reduced significantly by nearly 90% when L-lysine, L-ornithine or L-serine were used as a nitrogen source

in the culture media ($R^2$=0.975), whereas ammonium sulfate suppressed it completely. These results suggested the production of tannase enzyme is highly affected by the nature of used nitrogen source. The inhibition of tannase by ammonium sulfate could be due to the toxicity of sulfate ion itself on fungal growth. In addition, organic nitrogen sources can be react with tannic acid forming a complex precipitate, this complex structure inhibit fungal consumption of both carbon and nitrogen source; so it affect greatly on the fungal growth (Kumar *et al*., 2007).

**Table (3): Effect of different nitrogen sources on the production of *A. niger* AUMC 4301 tannase.**

| Nitrogen sources | Specific activity (U/mg protein) |
|---|---|
| **Ammonium nitrate (control)** | 3.25±0.102$^{A*}$ |
| **L-Alanine** | 1.22±0.031$^{B}$ |
| **Ammonium chloride** | 1.25±0.078$^{B}$ |
| **Ammonium sulfate** | 0.02±0.009$^{F}$ |
| **L-Cysteine** | 0.70±0.102$^{D}$ |
| **L-Lysine** | 0.40±0.042$^{E}$ |
| **L-Ornithine** | 0.34±0.038$^{E}$ |
| **Potassium nitrate** | 0.99±0.061$^{C}$ |
| **L-Serine** | 0.28±0.015$^{E}$ |
| **Sodium nitrate** | 0.83±0.079$^{CD}$ |

*Different symbols means there is a significant difference.

3.1.8. Dependence of tannase formation by *A. niger* AUMC 4301 on ammonium nitrate concentration.

As shown in table (3) ammonium nitrate was the most favorable nitrogen source for production of *A. niger* AUMC 4301 tannase. So, it is interesting to study the effect of ammonium nitrate concentration on tannase activity. Data presented in figure (5) demonstrated that the specific activity of *A. niger* AUMC 4301 tannase was increased with increasing ammonium nitrate concentration up to 0.2% in culture medium. After that, tannase activity decreased rapidly with increasing ammonium nitrate concentration, the specific activity of tannase recorded 1.41±0.038, 1.14±0.033 and 0.56±0.035 U/mg protein at ammonium nitrate concentrations 3, 4, and 5 g/l respectively. In spite of ammonium nitrate is readily utilizable nitrogen that stimulates the synthesis of proteins (Djekrif-Dakhmouche *et al*., 2006), high concentration of ammonium nitrate caused tannase denaturation by changing the protein tertiary structure.

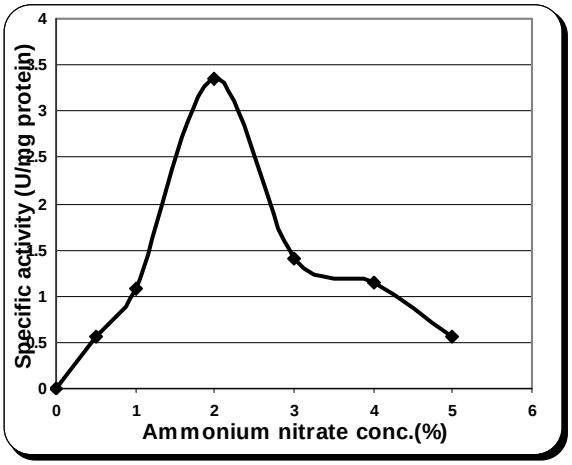



Fig. (5): Effect of different ammonium nitrate concentrations on the production of *A. niger* AUMC 4301 tannase.

3.2. Some kinetics and properties of A. niger AUMC 4301.

Any enzyme is a protein in nature that functions as a catalyst. The role of enzymes is to speed up the rate of chemical reactions without undergoing any permanent changes itself. In our case, extracellular tannase of selected *A. niger* AUMC 4301 strain was mixed with tannic acid (the substrate) and the conditions that maximize tannase activity and consequently tannic acid degradation in the reaction mixture were tested individually. They were reaction time, reaction pH, reaction temperature, enzyme concentration, substrate concentration and addition of metal ions, inhibitors and gallic acid.

3.2.1. Effect of reaction time on the activity of tannase from *A. niger* AUMC 4301.

An experiment was conducted to determine tannase activity as a function of reaction time. The reaction mixture was incubated at 60°C, and samples were withdrawn periodically for a period of 60 min and assayed for tannase activity. Results obtained are graphically presented in figure (6). The reaction was found to be more or less linear with time up to 30 min. Further increase in the reaction time resulted in a sharp decrease in enzyme activity; the percentage of decrease was nearly 70% after 60 min. So, it could be concluded that the rate of the reaction catalyzed by *A. niger* AUMC 4301 tannase couldn't proceed to completion. This may be attributed to the accumulation of gallic acid, one of the products of the reaction, which retard the reaction rate. Similarly,

Sabu *et al.* (2005) reported that the tannase activity by *Aspergillus niger* ATCC16620 (3.9 U/ml ) was maximized and stabilized at 15 and 20 min of reaction time and it decreased with further increase in reaction time. **Mukherjee and Banerjee (2006)** reported that an increase up to 5 min was observed in tannase activity of a co-culture of *R. oryzae* and *A. foetidus* followed by a decrease thereafter and the curve started leveling off. The difference in reported reaction time could be attributed to microorganisms employed.

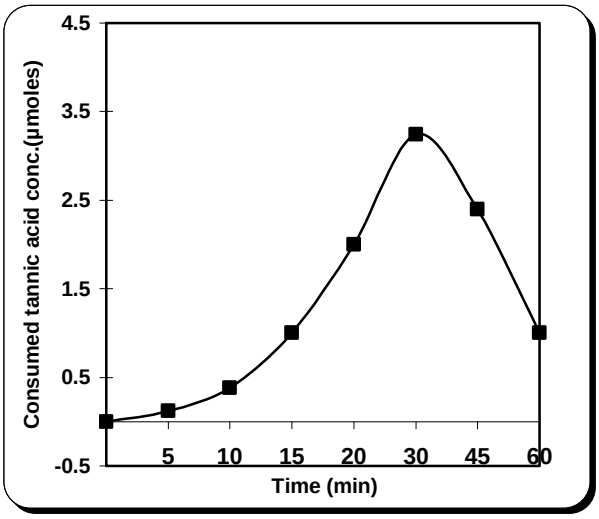



**Fig. (6): Effect of reaction time on the activity of *A. niger* AUMC 4301 tannase**.

3.2.2. Dependence of tannase activity of *A. niger* AUMC 4301on pH.

Different pH values (3.6, 4.2, 4.8, 5.4 and 5.6) were chosen to investigate the influence of pH on the catalytic activity of tannase. Figure (7) demonstrated the relationship, between pH and activity of *A. niger* AUMC 4301 tannase. Maximal enzyme activity was obtained at pH 4.8 where the amount of consumed tannic acid reached 2.93 µmol. By increasing the pH value above 4.8, a gradual decrease in enzyme activity was recorded. This pH optimum is more or less similar to that reported for the enzyme from *A. aculeatus* DBF6 (Banerjee *et al*., 2001), *A. awamori nakazawa* (Mahapatra *et al*., 2005), *A. foetidus* and *R. oryzae* (Mukherjee and Banerjee, 2006), and *P. variable* (Sharma *et al*., 2008). The pH value for optimal tannase activity of many strains of *A. niger* previously studied was about 6 (Anwar *et al*., 2009; Marco *et al*., 2009 and Srivastava and Kar, 2009). Tannase was active at acidic pH and activity decreased as the pH approached the alkaline range. Any change in pH affects the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or its instability (Mahapatra *et al*., 2005).

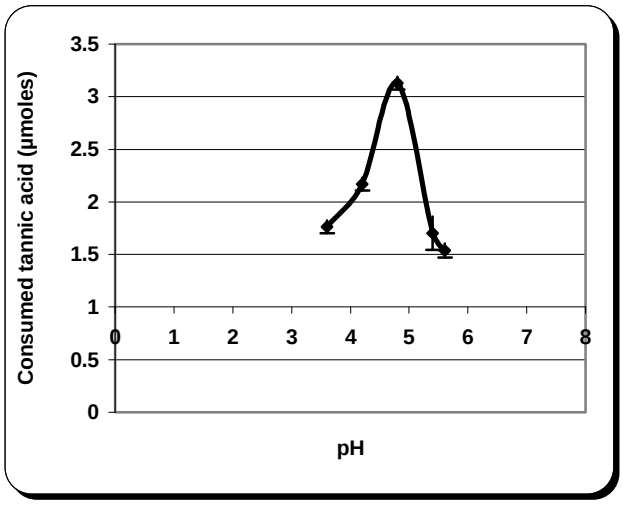



**Fig. (7): Effect of reaction pH on the activity of *A. niger* AUMC 4301tannase**.

3.2.3. Effect of temperature on tannase activity of *A. niger* AUMC 4301.

An experiment was conducted to find out the degree of temperature at which optimum activity of *A. niger* AUMC 4301 tannase could be achieved. A series of identical reaction mixtures were made and each was incubated at a different temperature. The range of temperatures used was from 30°C to 80°C. Figure (8) showed that maximal enzyme activity was achieved at 60°C (Consumed tannic acid was 3.3 µmol). The *A. niger* AUMC 4301 tannase activity at 70°C was about 50 % of that obtained at 60°C. Presumably the enzyme was denaturated at 70°C, an indication of its thermolability. The amount of consumed tannic acid declined to 1.68±0.13 and 0.98±0.074 at 70°C and 80°C respectively. Our data are in accordance with those reported for the tannase activity of *A. niger* van Teighem (Sharma *et al*., 1999) and *A. niger* GHI (Marco *et al*., 2009) in having an optimal temperature at 60°C whereas the activity of *A. niger* Aa-20 tannase was found to be maximal at 60 to 70°C (Ramirez-Coronel *et al*., 2003). These results indicated that elevating temperature to certain limit has positive effect on tannase activity; this could be attributed to increase in the kinetic energy of the substrate and enzyme molecules or/and increase the reaction rate with elevating temperatures. Beyond the optimum level of temperature, the internal energy of the molecules including translational, vibrational and rotational energy of the molecules increased, some of the weak bonds determining the three-dimensional shape of the active proteins break leading to thermal denaturation

of the tannase protein causing its inactivation. Temperatures above the optimum value also affect the protein ionization state, and the solubility of species in solution, which thus resulted in a reduction in enzyme activity **(Mukherjee and Banerjee, 2006)**.

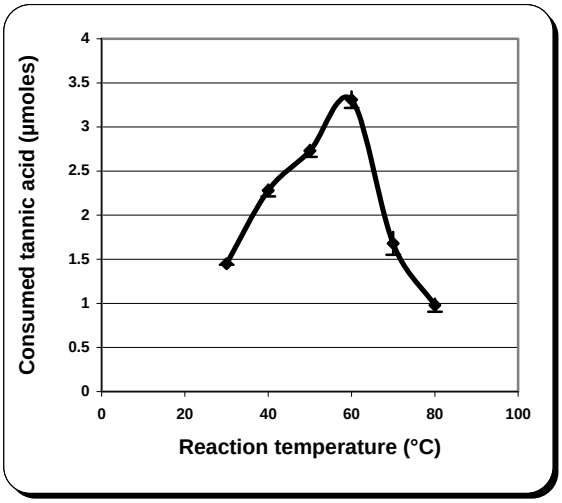



**Fig. (8): Effect of reaction temperature on the activity of *A. niger* AUMC 4301tannase.**

3.2.4. Heat inactivation kinetics of tannase from *A. niger* AUMC 4301.

Another experiment was designed to test the stability of tannase activity of *A. niger* AUMC 4301 in acetate buffer (pH 4.8) during incubation the enzyme (in absence of tannic acid) at either 60°C or 70°C for different time intervals. It is evident from the results represented in figure (9), a total loss of tannase activity occurred when the enzyme was incubated at 70°C for 5 min. However, exposing the enzyme to 60°C for 5 min and 20 min resulted in about 20% and 60% loss of its activity, respectively. These results indicate that *A. niger* AUMC 4301 tannase is thermolabile. Although the optimum temperature for the tannase activity was 60°C, yet it was inactivated when incubated at the same temperature, in absence of the substrate. This indicates that presence of the substrate in the reaction mixture protects the catalytic site from heat inactivation. Similarly, tannases of *A. niger* LCF8 (Barthomeuf *et al*., 1994) and *A. niger* GHI (Marco *et al*., 2009), were found to be thermolabile. Alternatively, Battestin and Macedo (2007) stated that the crude tannase of *P. variotii* was thermo-stable where it retained 96% and 99% residual activity at 20 and 70°C respectively.

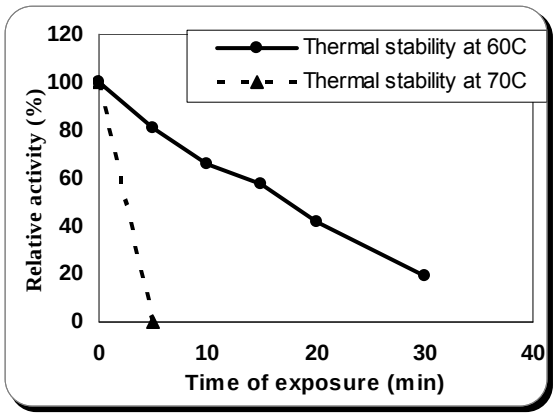



**Fig. (9): Heat inactivation kinetics of *A. niger* AUMC 4301tannase.**

3.2. 5. Tannase activity as a function of enzyme concentration

An experiment was designed to prove that the rate of catalytic activity of *A. niger* AUMC 4301 tannase is dependent on the amount of enzyme in the reaction mixture. Several reaction mixtures were set up which contained the same amount of tannic acid in acetate buffer at pH 4.8, but had varying amounts of the enzyme. Figure (10) revealed the relationship between tannase activity and protein concentration, the results indicate that the extent of catalytic action is directly proportional to the concentration of the enzyme (R= 0.9961). This could be explained by increasing enzyme concentration to certain limit, increase the availability of more active sites free on the enzyme and increase the incidence of substrate molecules to react with them.

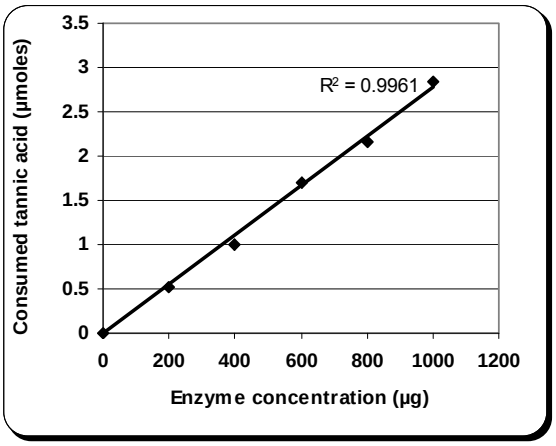



**Fig. (10): Tannase activity of *A. niger* AUMC 4301as a function of enzyme concentration**.

3.2.6. Determination of the apparent $K_m$ value of *A. niger* AUMC 4301 tannase for tannic acid.

Determination of the apparent $K_m$ (Michaelis constant) value of *A. niger* AUMC 4301 tannase was achieved through a study relating substrate concentration to the velocity of the reaction. Different concentrations of tannic acid were incubated with the same amounts of enzyme protein in acetate buffer (pH 4.8) at 60°C for 20 min. Figure (11) illustrates the effect of tannic acid concentrations on tannase activity of *A. niger* AUMC 4301. Figure (12) represents a Lineweaver Burk plot (Lineweaver and Burk, 1934) of the reciprocal of initial velocities and tannic acid concentrations. From this plot the apparent $K_m$ value of the enzyme was calculated and found to be 2.5 mM. It is clear also from the results seen in Fig. (11) that the enzyme activity was increased with increasing concentrations of tannic acid up to 2.5mM. Further increase in tannic acid concentrations resulted in a significant decrease in enzyme activity ($P>0.0001$). It could be suggested from such finding that gallic acid as a product of the reaction catalyzed by tannase may have an inhibitory effect on enzyme activity. However, tannase of *P. variable* exhibited a much higher $K_m$ value (32 mM) for tannic acid (Sharma *et al*., 2008). On the contrary, lower $K_m$ values for tannic acid were reported for tannases produced by both of *R. oryzae* and *A. foetidus* (Mukherjee and Banerjee, 2006), *A. niger* GHI (Marco *et al*., 2009) and *A. niger* HAYATI (Anwar *et al.,* 2009).

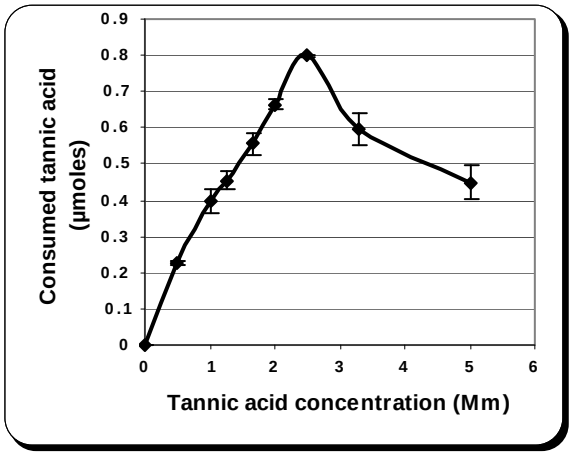
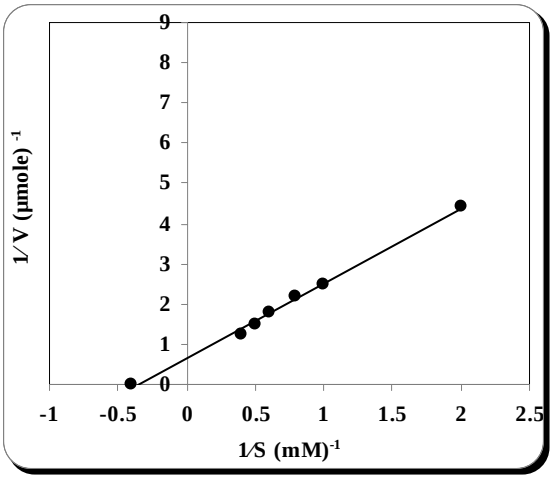



**Fig. (11): Determination of the apparent $K_m$ value of *A. niger* AUMC 4301 tannase for tannic acid.**




**-1/ $K_m$ = - 0.4 mM** **$K_m$ value = 2.5 mM**

**Fig. (12): Lineweaver-Burk plot of the reciprocals of initial velocities and tannic acid concentration.**

3.2.7. Influence of gallic acid on the activity of *A. niger* AUMC 4301 tannase.

Results of tannase activity as function of reaction time and substrate concentration revealed that gallic acid may be an inhibitor to the activity of *A. niger* AUMC 4301 tannase. Thus, another experiment was designed to investigate the effect of gallic acid on the tannase activity. This was carried out by adding gallic acid at a concentration of 5 mM to the reaction mixtures containing increasing concentrations of tannic acid (substrate) and measuring the rates of the reaction catalyzed by the enzyme. This was compared with analogous rates obtained without gallic acid addition. Data obtained are graphically presented in figures (13&14). These results indicated that gallic acid is a competitive inhibitor to *A. niger* AUMC 4301 tannase especially at high substrate concentrations. The $K_i$ (inhibition constant) was calculated and found to be 1.35 mM. Gallic acid itself acts as competitive inhibitor (Kar *et al*., 1999; Kar and Banerjee, 2000). Competitive inhibitors are substances; usually structurally related to substrate; that is able to combine with the enzyme at the same site as the substrate are competitive. Inhibitor and substrate therefore compete for the same site forming enzyme-substrate (ES) and enzyme-inhibitor (EI) complexes, respectively; ESI complexes are not produced. These unfavorable complexes (EI) greatly affect tannase activity. This was clearly appeared by decreasing Michaelis constant ($K_i$) in the presence of inhibitor compared to absence of it ($K_m$).

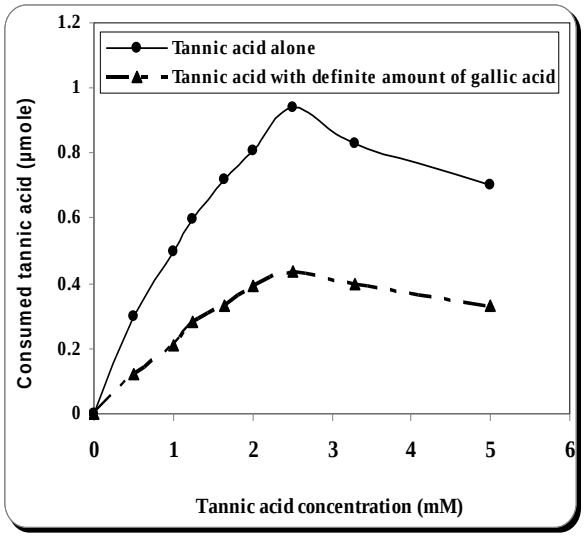
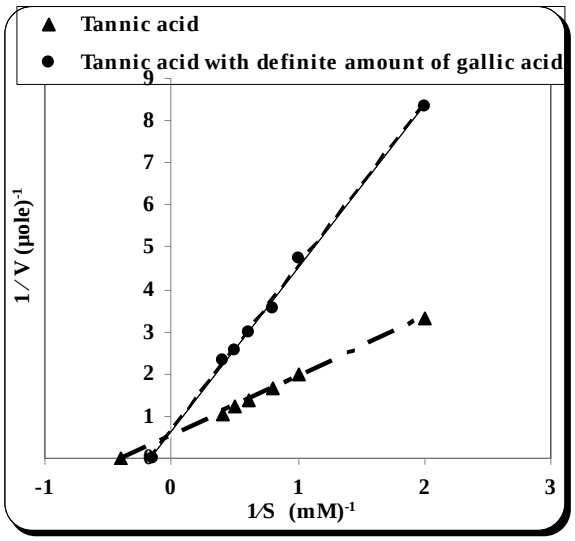



**Fig. (13): Influence of gallic acid on the activity of *A. niger* AUMC 4301 tannase**.




$K_m$ = 2.5 mM. $K_p$ = 1.35 mM .

**Fig. (14): Determination of the inhibition type exerted by gallic acid on tannase activity of *A. niger* AUMC 4301.**

3.2.9. Effect of EDTA and some metal salts on enzyme activity of *A. niger* AUMC 4301 tannase.

Many enzymes require metal ion activators for expressing their absolute catalytic activity. Since achieving maximum catalytic activity during enzymatic reactions is very important industrially, the effect of metal ion on tannase activity was studied in the following experiment. The results illustrated by figure (15) revealed that all the tested metal ions reduce tannase activity with different degrees. Hence, $Fe^{++}$, $Mn^{++}$, $Cu^{++}$, $Zn^{++}$ and $Ca^{++}$ showed inhibitory effect in a decreasing order on enzyme activity (Ranged from 90% to 20%), $Co^{++}$ and $Mn^{++}$ caused a least decrease in enzyme activity (About 6%). Since inhibition studies provide an insight into the nature of the enzyme and its cofactor requirements, in this experiment the effect of EDTA as a metal chelating agent, on the activity of *A. niger* AUMC 4301 tannase was also investigated. Addition of EDTA to the reaction mixture didn't inhibit tannase activity; this confirmed the negative effect of studied metal ions on the tannase under investigation. The effect of metal ions was studied in many previous reviews, some of them revealed the positive effect of metal ions on tannase activity while others attained the negative effect. Similar to our results, tannase obtained from *A. niger* MTCC 2425 was found to be inhibited by $Ca^{+2}$ (Bhardwaj *et al*., 2003). Sabu *et al*. (2005) also studied effect of metal ions on tannase from *A. niger* ATCC 16620 and found that the addition of metal ions like $Zn^{+2}$, $Mn^{+2}$, $Cu^{+2}$, $Ca^{+2}$, $Mg^{+2}$, and $Fe^{+2}$ inhibited the enzyme activity. At the same trend, Kasieczka-Burnecka *et al*. (2007) have reported inhibitory effect of $Zn^{+2}$, Cu, $^{+2}$ $K^{+}$, $Cd^{+2}$, $Ag^{+}$ , $Fe^{+3}$, $Mn^{+2}$, $Co^{+2}$, $Hg^{+2}$, $Pb^{+2}$, and $Sn^{+2}$ on tannase from *Verticillium sp*. Decrease in tannase activity in the presence of divalent cations could be due to nonspecific binding or aggregation of the enzyme.

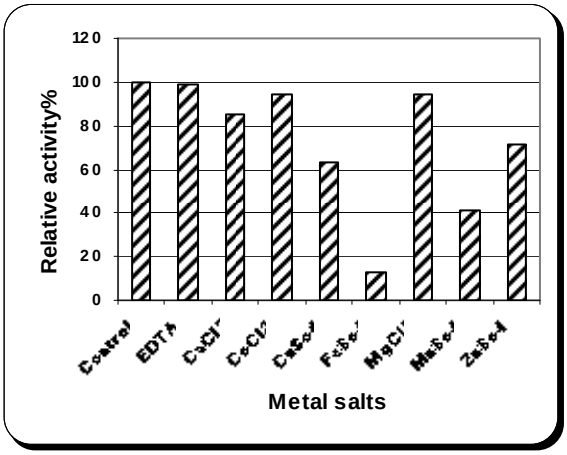



**Fig. (15): Effect of some metal salts and ethylene diamine tetra acetate (EDTA) on the activity of *A. niger* AUMC 4301 tannase.**

## 4. Conclusion

The results revealed in this study show that *A. niger* AUMC 4301 represents a valuable source of an economically attractive tannase with potential for application in various industries. The major

physicochemical and kinetic properties of the crude tannase were identified with special emphasis on the effect of substrate concentration, final product (Gallic acid) and metal ions on the enzyme activity. Other research work is in progress toward production of tannase utilizing agricultural wastes instead of tannic acid and improving the tannase productivity by *A. niger* AUMC 4301 using gamma radiation.

**Corresponding author**
Heba A. El-Bialy
National Center for Radiation Research and Technology (NCRRT), Radiation Microbiology Department, Atomic Energy Authority, Cairo, Egypt

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