

RESEARCH ARTICLE

Purification, Characterization and Application of Tannase Enzyme Isolated from Marine *Aspergillus nomius* GWA5

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Abstract

Tannase enzyme (EC 3.1.1.20) is an enzyme used in many biotechnological applications as in chemical, beverage, pharmaceutical and food industries. was isolated and purified from marine *Aspergillus nomius* GWA5 by 75% acetone fractional precipitation, followed by gel filtration in Sephadex G-100 and ion exchange chromatography on DEAE-Sephadex A-50 yielding 4.48-fold purification. Estimation of tannase molecular weight was carried out using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showing a molecular weight of 30 kDa. The highest activity (291 U/mg protein) were at pH 6.0 and 50 °C, respectively. Tannase stability was observed in acidic range (4-6) and was stable to heat treatment. In absence of its substrate it retained about 84.5% of its activity at 80 °C for 15 min. Effect of some metal ions and chelator on tannase activity was investigated. Mg²⁺ activated as activator of the pure enzyme while EDTA, Cd²⁺, Pb²⁺ and Hg²⁺ inhibited its activity and retained about 40.78, 51.55, 30.24 and 24.55% of its activity, respectively. Promising activity of Tannase was shown in removing tannin stains of tea from clothes.

Keywords: Tannase; *Aspergillus nomius* GWA 5; Purification; Characterization; Application.

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INTRODUCTION

Tannin is water-soluble polyphenolic compound found in plants as secondary metabolites¹. Many of phenolic hydroxyl groups present in tannins permit them to form complexes with proteins and to a lesser amount with other macromolecules like pectin and cellulose². Tannins also have toxic effects on some organisms, but they can resist tannins by degrading them into useful derivatives and oligomeric tannins such as gallic acid or pyrogallol using tannase enzyme³.

Tannase enzyme is tannin acyl hydrolase (EC 3.1.1.20) which can only hydrolyze the galloyl residues of galloyl esters of tannins and can be used in different industrial applications⁴. It finds a wide range of applications such as in the production of gallic acid, an intermediate for trimethoprim, pharmaceutical industry, food industry through chemical synthesis of pyrogallol or ester galates which are used as food preservatives, clarification of beer and beverages, manufacture of instant tea, manufacture of coffee flavored drinks, reduction of anti-nutritional effects of tannins in animal feed and high grade leather tanning⁵. Also, antioxidant in fats, oils and beverages (6) and in decontamination of tannery effluents⁷⁻¹⁰.

Tea preparation is one of the most promising application of this enzyme as it help in solubilization of polyphenolic complexes which are insoluble in cold water by hydrolyses the ester linkage between galloyl groups and various compounds present in unconverted tea leaves¹¹.

The enzyme can be produced by many microorganisms such as fungi, bacteria and yeast. Fungi are predominant tannase producers and are extensively studied¹²⁻¹⁶. *Aspergillus* sp. is the most abundant species of fungi which can grow in a wide types of environments with very little nutrients available and used for the tannase production¹⁷. Tannase usage is severally restricted due to high production costs, despite of its several important applications. Therefore, there is a continuous search for new sources of tannase^{18, 19} as well as improved methods for production, recovery, and application of the enzyme.

The present investigation was aimed to purification, characterization and investigation of valuable application of tannase produced by the locally isolated *Aspergillus nomius* GWA5 as a new source for tannase production.

MATERIALS AND METHODS

Organism and growth condition

Aspergillus nomius GWA 5 was isolated from marine sediment of Western Harbour, Alexandria, Egypt. The isolated fungus was identified as *Aspergillus* according to morphological and microscopically examination, Further identification to *A.nomius* GWA 5 was done using 18s rRNA sequence analysis (accession No. KU359224). The medium used for cultivation and production of enzyme was composed of (g/l) : $(\text{NH}_4)_2\text{SO}_4$, 1; K_2HPO_4 , 1; KH_2PO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.02; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; NaCl, 20. The pH of the medium was adjusted to 6.0 using 0.1M HCl or 0.1 N NaOH before sterilizing in an autoclave at 121°C for 20 min. After sterilization, one ml of a spore suspension (2×10^6 spore/ml) prepared from 5-day old slants of the test organism was used to inoculate 50 ml of sterilized medium dispensed in 250 ml Erlenmeyer flasks. Thereafter, the flasks were incubated for 72 hr at 30 °C under static condition²⁰.

Protein estimation

The protein content preparation was measured according to the method of Lowry *et al.*²¹ using bovine serum albumin as the standard.

Enzyme assay

Tannase activity was determined using the method of rhodanine²². The reaction mixture containing 0.25 ml enzyme preparation and 0.25 ml methyl gallate as a substrate (0.01M methyl gallate prepared in 0.05 M citrate buffer, pH 5.0). The mixture was incubated for 5 min at 30°C. Add 0.3 ml of methanolic rhodanine solution (0.667% w/v) for stopping the reaction and for formation of complex between gallate and rhodanine. The tubes were kept at 30°C for 5 min, followed by addition of 0.2 ml KOH solution (0.5M) and the tubes were again kept at 30°C for 5 min. All the tubes were diluted and kept again at 30°C for 10 min. The pink color developed was read at 520 nm using a spectrophotometer. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 µmol of gallic acid per minute under assay conditions.

Spectrophotometric estimation of gallic acid

Estimation of gallic acid was carried out by the method described by Sharma *et al.*²².

Partial purification

Tannase enzyme produced from *A. nomius* GWA5 cultures was purified by three steps purification involving precipitation (either by ammonium sulphate, ethanol or acetone), gel filtration and ion exchange.

Acetone or ethanol

Both acetone and ethanol were cooled at 4°C one day before the precipitation. A certain volume of acetone or ethanol was added slowly to a definite volume of crude enzyme with continuous stirring until the required concentration of acetone was reached. The obtained fractions at 25, 35, 50, 65, 75, 85 and 95% were dried over anhydrous calcium chloride under reduced pressure at room temperature, followed by precipitation and dialysis against distilled water in a refrigerator for 24h. The enzyme activity and protein content of each fraction were estimated²³.

Ammonium sulphate fractionation

Ammonium sulfate was added to definite volume of the culture filtrate at different concentrations to obtain various fractions at 25, 35, 50, 65, 75, 85 and 95% saturation. Each precipitate was dissolved in a certain amount of distilled water and dialyzed against distilled water in a refrigerator overnight after dialysis¹⁶.

Purification of tannase

Gel-filtration chromatography on Sephadex G-100

Sephadex G-100 (Sigma) was packed in a glass column (2.5 cm × 45.0 cm) and equilibrated with 400 ml of 0.02 M phosphate buffer at pH 8.0. 30 ml of 0.02 M phosphate buffer was used to dissolve the precipitate resulting from acetone fractionation 75% saturation and dialyzed for 24 h at 4 °C in 0.02 M phosphate buffer after dialysis. Elution of the enzyme protein was performed at a flow rate of about 30ml/h by using a peristaltic pump. The eluent was collected in 3ml fractions. The protein content and tannase activity of each fraction were estimated.

Ion-exchange chromatography DEAE Sephadex A-50

A slurry of diethylaminoethyl (DEAE) Sephadex A-50 was packed in a column (2.5 cm×45.0 cm). The sephadex bed (30 cm long) was equilibrated with 0.05M acetate buffer (pH 5.2). The fractions of highest specific activity obtained from gel filtration on Sephadex G-100 column pooled and applied to DEAE- Sephadex

A-50 column. Elution was performed with 0.05M acetate buffer, followed by 0.05M NaCl in 0.05M acetate buffer at pH 5.2, at a flow rate of 60 ml h⁻¹. Fractions (about five ml) were collected, tannase activity and protein content for each fraction were estimated. The fractions had higher specific activity were pooled.

Characterization of purified tannase enzyme

The effect of different temperatures, pH, pH and thermal stability of the enzyme fractions obtained after column chromatography was studied²⁴. Also, effect of some metal ions and chelators on the activity of purified tannase was studied.

Determination of molecular weight of pure tannase

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) was performed to determine the molecular weight of pure tannase according to the method described by Laemmli²⁵.

Effect of pH and temperature on the tannase activity

The effect of pH and temperature on the purified *A. nomius* GWA5 tannase enzyme was determined. Tannase activity was studied in the pH range of 3.0 to 9.0 using the following buffers: (0.05 M citrate buffer for pH 3-6, 0.05 M phosphate buffer for pH 7-8 and 0.05 M Tris-HCl for pH 9). The optimum temperature for tannase activity was determined by incubating enzyme and substrate at various reaction temperatures ranging from 20 to 70 °C. Tannase activity was performed as described before under the optimal conditions.

pH stability and thermal stability of tannase

The purified *A. nomius* GWA5 tannase was incubated at various pH values at 50°C for 30 min in absence of its substrate and then the residual activity was determined at the optimum pH (6). The thermal stability of the purified enzyme preparation was studied at the optimum pH. Identical enzyme solutions in acetate buffer were preheated separately (in absence of its substrate) at different temperatures (40- 80 °C) for (15, 30, 60 min). Tannase residual activity was assayed each time under the optimum conditions.

Effect of some metal ions and chelator on tannase activity

The purified enzyme solution was preincubated for 2 h at room temperature with the tested substance, MgSO₄·7H₂O, ZnSO₄, NaCl,

PbCl₂, HgCl₂, CdCl₂, BaCl₂, and EDTA (10 mM). The residual enzyme activity was measured by adding the substrate and carrying out the enzyme assay under the optimum conditions.

Application of tannase in removing tannin stains of tea

Two small square pieces (5x5 cm) of clean cotton cloth were treated with tea extract. The stained pieces were then subjected to the following treatment: First piece was washed with tap water while the second piece was immersed in 5ml of 0.05M citrate buffer pH 5.0 and treated with crude tannase and left under the observation²⁶.

RESULTS AND DISCUSSION

Partial purification of tannase

Tannases are enzymes used in different industrial applications as in food and pharmaceutical. In the last decade, there have been efforts looking for new sources for tannase enzyme production²⁷. Many researches on tannase synthesis showed clearly that almost all species of *Aspergillus* are the predominant source of tannase for industrial production and application²⁸. Most of the tannase producing fungi are of terrestrial origin and till date no reports are available on utilization of marine fungi for tannase production except the work done by Beena et al.²⁹ who isolated *Aspergillus awamori*, from marine environment as potential source for tannase. The present investigation aimed to purified and characterized the tannase produced from locally

isolated marine *A. nomius* GWA5²⁰ for used in many biotechnological applications.

Purification of *A. nomius* GWA5 tannase was efficient and effective. The partially purification was carried out by fractional precipitation with (NH₄)₂SO₄, acetone and ethanol, each used separately (Table 1). A total of 18 fractions were obtained and the highest recovered protein (13.82 %) was present in the fractions precipitated with acetone followed by (NH₄)₂SO₄ sulphate (11.972 %) and ethanol (11.517 %). Using of acetone (17.09 %) followed by (NH₄)₂SO₄ (12.331 %) and ethanol (11.611 %) recorded the highest tannase activity. Some of the obtained fractions showed a specific activity higher than that of the crude enzyme, indicating the presence of purification. Among all the obtained fractions, the 75% acetone fraction showed the highest recovered protein (3.175%). Also, 75% acetone fraction showed the highest tannase activity as it gave about 1.59-fold specific activity higher than that obtained from culture filtrate. Therefore, the fraction 75% acetone was selected for further purification and was considered as the partially purified enzyme. Many investigators have used acetone for precipitation of tannase^{12,30}. On the other hand, (NH₄)₂SO₄ with different concentrations was used by several researchers for precipitating tannase from the fermentation broth^{30-33,16}.

The concentrated partially purified tannase was exposed to dialysis and gel filtration on a SephadexG-100 column. The protein and

Table 1. Fractional precipitation of tannase from *A. nomius* GWA5 cultures using different agents

Agent concentration (%)	Acetone Ethanol Ammonium sulphate											
	PC	RP	TA	RA	PC	RP	TA	RA	PC	RP	TA	RA
Culture filtrate	844	100	41.8	100	844	100	41.8	100	844	100	41.8	100
25	9.22	1.092	22.94	0.599	8.11	0.961	12.62	0.29	8.98	1.064	20.09	0.511
35	11.65	1.38	40.86	1.347	10.23	1.212	42.91	1.243	12.55	1.487	43.77	1.555
50	18.88	2.237	44.65	2.386	15.65	1.854	49.85	2.208	18.34	2.173	45.28	2.35
65	23.76	2.815	52.23	3.512	23.99	2.842	51.73	3.512	22.11	2.62	52.15	3.264
75	26.8	3.175	66.49	5.043	22.78	2.699	42.85	2.763	20.11	2.383	48.54	2.763
85	14.9	1.765	62.89	2.652	16.44	1.948	34.3	1.596	18.95	2.245	35.22	1.889
95	11.45	1.357	47.86	1.551								
Total	116.66	13.822		17.09	97.2	11.517		11.611	101.04	11.972	20.09	12.331

PC: protein content (mg), RP: relative protein (%), TA: tannase activity (U/mg protein), RA: relative activity (%).

elution profiles for tannase from the Sephadex G-100 column are shown in Figure 1, which indicated that there are three peaks of protein. The first peak contains the highest protein content (16.65 mg) which represented about 82.2% of the total recovered protein from the column, and the highest specific activity (154.9 U/mg protein) showing about 2.3-fold purification. The most active fractions (6 to 20) obtained from the Sephadex G-75 column were collected and then purified by using DEAE-Sephadex A-50 column chromatography (Figure 2). The specific activity of the pooled fractions reached (187.315 U/mg protein), showing about 4.48-fold purification of the crude enzyme produced by *A. nomius* GWA5, 2.8-fold purification of the 75% acetone fraction (semi-purified) and 1.39-fold purification of gel filtration fractions.

Roushdy *et al.*³⁴ purified tannase from *Aspergillus flavus var. columnaris* using Sephadex G-200 column chromatography technique and achieved a purification with 5.8-fold. Beena *et al.*²⁸ observed that, purification of tannase from *A. awamori* when applied on Sephadex G-200

column chromatography raised the purification folds to 6.73 times, while Mahendran *et al.*³⁵ noted that, purification of tannase from *Paecilomyces variotii* by Sephadex G-200 column was 30.5-fold purification. Also, Paranthaman *et al.*³⁶ purified tannase from *A. niger* by using DEAE-Sephadex A-50. On the line with our results, Bagga *et al.*³⁷ used two chromatographic techniques DEAE-Cellulose column followed by Sephadex G-100 column for purification of tannase from *A. aculaetus* and a specific activity of purified enzyme was about 79.7 U/mg protein.

A summary of the purification of tannase from the culture medium of *A. nomius* GWA5 is presented in Table 2.

Characterization of purified tannase
Determination of molecular weight of pure tannase

The purity of the enzyme was analyzed by SDS-PAGE electrophoresis. SDS-PAGE revealed a single homogenous band with an approximate molecular mass of 30 kDa as illustrated in Figure 3. Many investigators have used SDS-PAGE for detection of tannase enzyme molecular mass^{28, 38, 39}

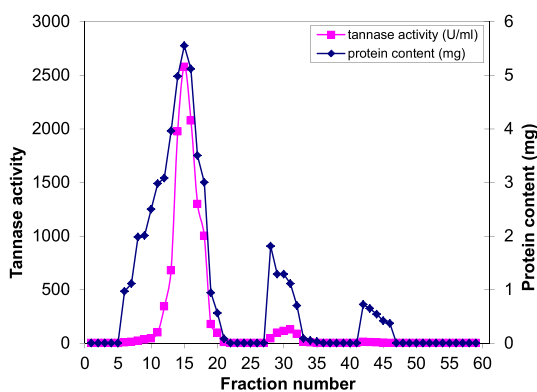


Fig. 1. Gel filtration in Sephadex-G100 of the semi purified tannase obtained from *A. nomius* GWA5 culture

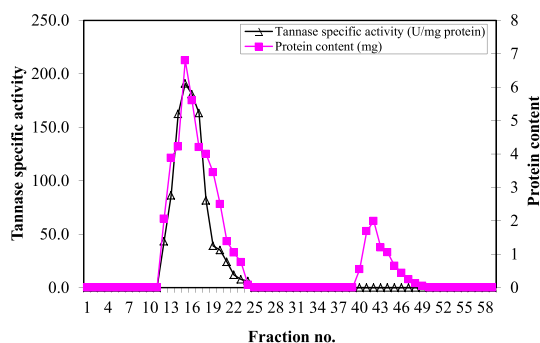


Fig. 2. Ion-exchange chromatography on DEAE-Sephadex A-50 of the major active tannase component obtained from gel filtration

Table 2. A summary of tannase from *A. nomius* GWA5 cultures

Purification step	Total protein (mg)	Total activity (U)	Specific tannase activity (U/mg protein)	Purification (Fold)	Yield (%)
Culture filtrate	844	35333	41.8	1	100
Acetone fraction (75%)	268	17820	66.49	1.59	50.04
Sephadex G-100	66.45	8933.37	134.44	3.21	25.28
DEAE-Sephadex A-50	20.82	3899.9	187.315	4.48	11.04

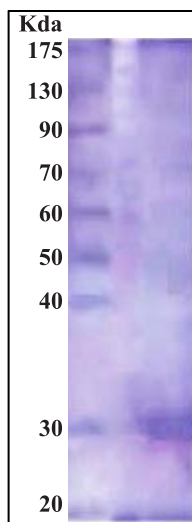


Fig. 3. SDS-PAGE of purified tannase from *A. nomius* GWA5. Lane A: molecular weight markers; Lane B: purified tannase

Effect of pH and temperature of the reaction mixture

The influence of pH value (ranged from 3 to 9) of the reaction on the purified enzyme activity (Figure 4) indicated that the optimum pH value for highest activity was 6.0 at which tannase activity reached its maximal value (242.5 U/mg protein). The lowest activity (107 U/mg) was obtained at the alkaline range and the enzyme lost about 49.7% of its activity at pH 9. The obtained results were in good agreement with those reported by Sabu et al.²⁴ and Renovato et al.⁴⁰. Many authors reported that the optimal pH values of tannase were ranged from 5.0 to 6.0^{41, 42, 33, 37}.

Enzyme showed an increase in its activity parallel to the increase in reaction temperature reaching a maximum value at 50°C (Figure 5).

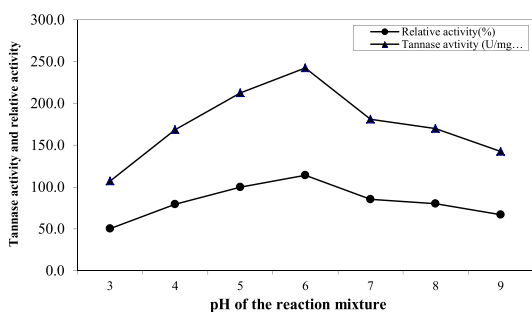


Fig. 4. Effect of pH of the reaction on the activity of the pure tannase produced by *A. nomius* GWA5

Further rise of temperature resulted in gradual loss in the activity. At high temperatures (60 and 70°C), the enzyme activity was decreased by about 72.3% and 51.4%, respectively, of that at 50°C. Also, the enzyme showed a stability in a broad temperature range of 40-70°C. These results are in comparable to the purified tannase preparation from *Paecilomyces variotii*³¹. However, this optimum temperature is much higher than that reported for purified tannase enzymes produced by *A. tamari*⁴³, *Penicillium* sp. EZ-ZH190³⁰, *A. flavus* var. *columnaris*³⁴ and *Kluyveromyces marxianus*¹⁶.

pH stability and thermal stability

pH stability of purified *A. nomius* GWA 5 tannase was studied using pH range of 3 – 9 (Figure 6). Also, the enzyme activity decrease with increasing in pH. However enzyme activity exhibited about 42% and 68.4% at pH 3 and 9, respectively of activity with respect to the activity of optimal pH. Our results are in agreement with

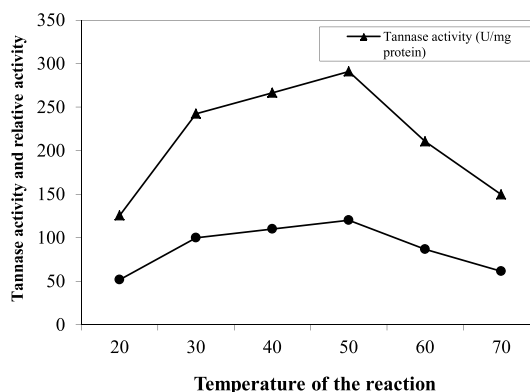


Fig. 5. Effect of the reaction temperature on the activity of pure tannase produced by *A. nomius* GWA5

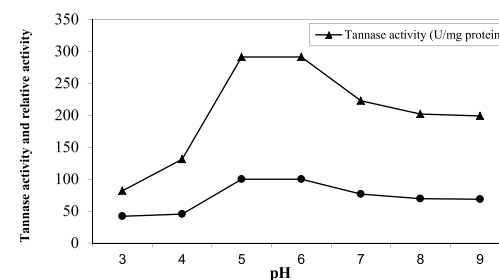


Fig. 6. pH stability of pure tannase produced by *A. nomius* GWA5

Andrea *et al.*⁴⁴ and Costa *et al.*⁴³. Also, Batra and Saxena⁴¹ reported that tannase from *A. fumigatus* and *A. flavus* was stable at pH 4.0 and doesn't show tannase activity at alkaline pH of 8.0. In contrast, *A. versicolor* tannase showed less stability at pH 3.0 with a maximum (100%) activity at pH 6.0.

Thermal stability of the pure enzyme in absence of its substrate depended upon temperature of heating and time of exposure (Figure 7). The enzyme retained about 96.2% and 66.2% of its activity when treated at 60 °C for 15 and 60 min, respectively. At higher temperature, the enzyme lost about 15.5% of its activity by heating at 80 °C for 15 min and about 54.8 % of its original activity by treatment for 60 min at the same temperature. The results of the present investigation showed also that the purified enzyme was fairly stable. The obtained results are in good with Bagga *et al.*³⁷ who reported that *Aspergillus aculaetus* tannase was stable up to 50 °C and lost only 5% activity at 60°C which was the optimum temperature of the enzyme. The thermal stability of *A. nomius* GWA5 tannase is comparable to the tannase isolated from *Aspergillus ochraceus*⁴⁵ which was stable in a wide range of temperatures from 40°C to 70°C.

Effect of some metal ions and chelator on tannase activity

The purified *A. nomius* GWA5 tannase was subjected to different metal ions to study their activation and inhibition effects on its activity. The results illustrated in Figure 8 indicated that only Mg^{2+} increased activity of tannase enzyme. Also,

the results showed that Zn^{2+} and Ba^{2+} partially inhibited tannase activity. On the other hand, the enzyme was strongly inhibited by Cd^{2+} , EDTA, Pb^{2+} and Hg^{2+} retained about 51.55%, 40.78%, 30.24% and 24.55% of its activity, respectively. The effect of some ions on the activity of the purified tannase preparation was studied. The results revealed that only Mg^{+2} activated the activity which in good agreement with many authors^{46-48, 4}. Also, metal ions Zn^{2+} , Cd^{2+} , Hg^{2+} , Pb^{2+} , Ba^{2+} and chelator EDTA were partially inhibited tannase activity. Many investigators reported that chelator EDTA disodium salt at a concentration 1 mM decreased tannase activity^{45, 48, 50, 51}. On the construct, Beniwal *et al.*⁴⁷ found that EDTA at a concentration of 1 mM did not affect tannase activity after 5 min of incubation period; however a slight loss was observed when the incubation period was extended to 30 min for thermophilic tannase from *Enterobacter cloacae*. The decrease detected in the presence of EDTA could be due to its influence on the interfacial area between the substrate and enzyme⁵².

Maximum inhibition of the tannase enzyme occurred in the presence of Hg^{2+} ^{45,47} because Hg^{2+} may interact with SH and S-S groups of proteins causing conformational changes in proteins. Also ions of mercury react with protein thiol groups as well as histidine and tryptophan residues. Moreover, the disulfide bond could also be hydrolytically degraded by the action of mercury. Also the decreased activity in the presence of divalent cations could be due to the non-specific binding or aggregation of the enzyme^{45,50}.

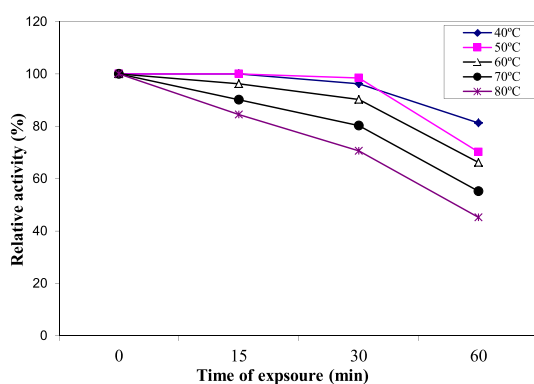


Fig. 7. Thermal stability of the pure tannase produced by *A. nomius* GWA5

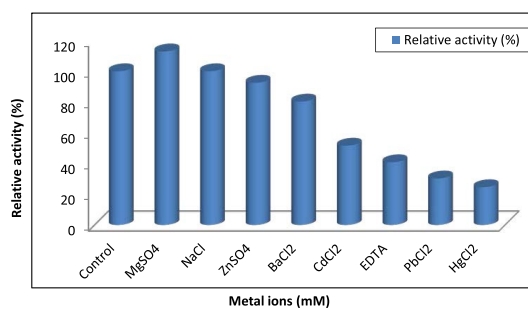


Fig. 8. Effect of some metal ions and EDTA on the activity of tannase obtained from *A. nomius* GWA5

Application of tannase in removing tannin stains of tea

Application of *A. nomius* GWA5 tannase in removing tannin stains of tea was investigated. It is obvious from Figure 9 that tea stains removal was better in cloth piece soaked in solution containing purified tannase when compared with control which was washed with tap water only. This is due to the activity tannase on tannins. The results were also indicated that the purified *A. nomius* GWA5 tannase can remove the tea stain and indicates that, tannase from *A. nomius* GWA5 can be used in formulation of tannin stain remover or detergents. These results in line with Sharma and Saxena²⁶ who worked on tannases from *A. niger* and *P. variable*.



Fig. 9. Removal of tea stains by crude tannase from *A. nomius* GWA5. (A) control; (B) Piece of cotton cloth treated with tap water; (C) Piece of cotton cloth treated with crude tannase.

CONCLUSION

The present study may append some new data on the production of tannase from *Aspergilli* and confirmed the capability of the marine *A. nomius* GWA5 to produce an active tannase for degradation of tannin. Moreover this enzyme can be used in different industrial and pharmaceutical uses. Good stability of pure tannase in wide range of pH and temperature was obtained. These properties collectively point that the tannase enzyme obtained from *A. nomius* GWA5 is a good candidate for removal of tannin stain of tea. Large scale production of tannase by using various recombinant engineering techniques will be tracked in the future study.

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