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Production of Tannase By Free and Immobilized Marine *Aspergillus nomius* Under Optimized Culture Conditions.

Sahar WM Hassan[1]*, Khaled M Ghanem[2], Aida F El-Shahat[3], and Asmaa MI El-Says[1].

[1]Marine Microbiology Laboratory, National Institute of Oceanography and Fisheries, Alexandria, Egypt
[2]Faculty of Science, Botany and Microbiology Department, Alexandria University, Alexandria, Egypt.
[3]Marine Biotechnology and Natural Products Extract Laboratory, National Institute of Oceanography and Fisheries, Alexandria, Egypt

ABSTRACT

Tannase is a hydrolytic enzyme that is involved in the biodegradation of tannins and it has biotechnological potential in pharmaceutical, chemical, food and beverage industries. Screening for tannase production indicated that *Aspergillus nomius* GWA5 identified using analysis of 18S rRNA gene was the most potent fungal isolate which yielded the highest hydrolysis of tannin (20 mm) using tannin agar medium. The highest activity was up on using minimal medium with 2% tannic acid after 72 h. Optimization of fermentation conditions was carried out using Plackett Burman experimental design. Under the predicted optimized medium composition for cultivation of *A. nomius* GWA5,tannase activity reached 46.6 U/ml with 1.5 fold increase. Immobilization of *A. nomius* GWA5 on luffa enhanced the enzyme activity and the yield of gallic acid by 1.1 and 1.4fold compared to free cells. Repeated use of the adsorbed cells retained high activity during the first three cycles.HPLC analysis confirmed the formation of gallic acid as the end product of tannin degradation by *A.nomius* GWA5 tannase enzyme.The present study indicated the potentiality of marine *Aspergillus nomius* GWA5 as new and economic source fortannase production which can be applied for large scale industries.

Keywords: Tannase; Marine *Aspergillus nomius* GWA5; Plackett Burman design; Immobilization.

Corresponding author

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INTRODUCTION

Tannins are the second most abundant group of plant phenolics after lignin [1] and the fourth most abundant plant constituents after cellulose, hemicellulose and lignin [2]. On the basis of structure and properties, tannins were traditionally classified into condensed tannins and hydrolysable tannins [3]. Currently, the most accepted classification divides the tannins into four groups: gallotannins, ellagitannins, condensed tannins, and complex tannins [4].

The large amount of phenolic hydroxyl groups present in tannins allows them to form complexes with proteins and to a lesser amount with other macromolecules like pectin and cellulose [5]. Some of the consequences of these interactions are the decline of the feed intake by livestock [6, 7], clean-up requirements for effluents of the leather industry [8] as it has been used for tanning for thousands of years[9,10], and haze formation in chilled beverages [6].

Tannin acyl hydrolase (EC 3.1.1.20) which is commonly referred as tannase is one of the hydrolytic microbial enzymes. Tannase is an industrially important enzyme because of its wide applications in different fields including biotechnology, food industry, ecology, pharmacology and medicine [11,12,13,14, 15, 16].It catalysis decomposition of hydrolysable tannins especially gallo-tannins such as: (tannic acid, methyl gallate, ethyl gallate,n-propylgallate, and isoamyl gallate) to glucose and gallic acid through the hydrolysesof ester and depside bonds [17].

Tannase is an extracellular inducible enzyme that can be obtained from different sources including fungi, bacteria, some yeasts, higher plants and animals[12, 14].These organisms produce tannase as developed mechanisms to degrade and use tannin as a sole carbon source.

The degradation of hydrolysable tannins particularly gallotannins is best known in fungal systems [4]. Earlier investigations on tannase production indicated very clearly that almost all species of *Aspergillus* are capable of synthesizing tannase on induction. *Aspergillus* sp. capable of growing on tannic acid medium containing it as sole carbon source might definitely producetannase for its survival. Among the various species, *A. niger, A. flavus andA. oryzae* were found to be the best tannase producers using tannic acid as a sole source of carbon [18].

The conditions for obtaining the maximal production of the enzyme depend on two factors: the system utilized and the source of the enzyme [19].Medium optimization by single dimensional search is laborious and time consuming, especially for a large number of variables and it does not ensure desirable conditions. Plackett-Burman design is widely used in screening experiment as the number of experiment run required are very few, leading to saving of time, chemicals and man power [20].

Another interesting approach is the production of tannase by immobilized cells. The immobilization of cells offers many advantages over the utilization of free cells, such as immobilized cell particles are more easily to handle and can be packed in fermenter system for industrial processes, the support materials provide a stabilizing effect on the cellular activities, the enzymes secreted are largely free of cells and cell debris which facilitates downstream processing [19].

According to the available literatures, studies on the tannase production using the local marine *A. nomius* GWA5have not been previously published. The present investigation deals with isolation of tannase producing marine fungi as a novel source of enzymes in addition to optimization of medium and some culture conditions for tannase production by *A.nomius* GWA5, and enhancement of production using different immobilization techniques.

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MATERIALS AND METHODS

Samples collection

Sediment samples were collected in a sterile screw capped bottles, transferred to laboratory in ice box, and stored at 4 ^{o}C till analysis.

Isolation and screening for tannase production by marine fungi

Different dilutions of each sample were prepared, 100 µl of each dilution was spread plated on to nutrient agar plates containing 1% tannic acid and incubated at 30 ^{o}C for 4 days. The nutrient agar plates supplemented with tannic acid were flooded with $FeCl_3$ solution (0.01 M $FeCl_3$ in 0.01 N HCl) and kept for (5–10) minutes at room temperature. $FeCl_3$ reacts with tannic acid and forms a brown color, thus a clear zone is formed on a dark brown background [21]. The diameter of the clear zone was determined and regarded as positive result [22].

Growth conditions for fungal culture

The isolated fungi were grown on Czapek- Dox agar slants supplemented with 1% (w/v) filter sterilized tannic acid and incubated at 30 ^{o}C for 4 days. After incubation, conidia were scraped with 5.0 ml of sterile saline solution and the spores were obtained and counted using Haemocytometer. Then, 1 ml ($2x10^5$spores/ml) aliquots were used to inoculate 50 ml of sterilized basal medium supplemented with 1% filter-sterilized tannic acid with initial pH of 6.0. The flasks were incubated for 4 days at 30 ^{o}C under static condition.

Preparation of supernatant

The fungal growth was separated by centrifugation at 6000 g for 15 min in a cooling centrifuge and the supernatant was used as the source of the crude enzyme.

Tannase activity assay

Tannase activity was estimated by the method of rhodanine [23]. The method is based on the formation of a chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2 thio-4- ketothiazolidine). The pink color developed was read at 520 nm using a spectrophotometer. Tannase activity was expressed in international units. One unit of tannase activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under the defined reaction conditions.

The reaction mixture containing 0.25 ml enzyme sample and 0.25 ml methyl gallate (0.01M methyl gallate prepared in 0.05 M citrate buffer, pH 5.0) was incubated for 5 min at 30°C. 0.3 ml of methanolic rhodanine solution (0.667%; w/v) was added for stopping the reaction and for formation of complex between gallate and rhodanine. The tubes were kept at 30°C for 5 min. This was 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. A set of blanks and controls were maintained and a standard curve was prepared using gallic acid.

Spectrophotometric estimation of gallic acid

Spectrophotometric estimation of gallic acid was carried out [23]. In a standard (gallic acid) or suitably diluted sample, 300 µl of methanolic rhodanine (0.667 % in methanol) was added followed by addition of 200 µl of 0.5 M potassium hydroxide. After incubation at 30^{o}C for 5 min, 4 ml distilled water was added. The absorbance was read at 520 nm after 5-10 min. Standard gallic acid stock (1mg/ml) was prepared in citrate buffer (0.05M, pH 5.0) and calibration curve (5µg- 50µg) was plotted.

Protein estimation

The protein content in the crude enzyme preparation was determined [24] and specific activity was calculated by dividing the enzyme units with protein content and was expressed as U/mg protein.

$$\text{Specific activity} = \frac{\text{Enzyme activity (U ml}^{-1})}{\text{Protein (mg ml}^{-1})}$$

Molecular characterization of isolate F5

For molecular identification, genomic DNA was isolated [25]and amplified by polymerase chain reaction (PCR). The primers ITS 1 5' (TCC GTA GGT GAA CCT GCG G) 3' and ITS 4 5' (TCC TCC GCT TAT TGA TAT GC) 3' were used for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30µl reaction mixture by using a EF-Taq polymerase (SolGent, Korea) as follows: activation of Taqpolymerase at 95°C for 2 min, 35 cycles of 95°C for 1 min, 55°C, and 72 °C for 1 min each was performed, finishing with a 10 min step at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA).Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at95 °C for 5 min, followed by 5 min on ice and then analysed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). All the previous mentioned work of DNA extraction, PCR reaction and sequencing of PCR-DNA product was carried out at Macrogen (Seoul, Korea).16SrDNA sequence obtained from the fungal isolate wascompared with known 16S ribosomal sequences in NCBIdatabase using BLASTn (www.ncbi.nlm.nih.gov/blast). Multiple sequence alignment and the phylogenetic tree were constructed by means of the MEGA6 software using neighbor joining (NJ) algorithm [3].

Optimization of tannase production (One factor at time)

Effect of different media

Culture of the selected fungus was grown on different 6 media (g/l): to each 10 g/l of tannic acid was added, medium I (Tannic acid medium): $NaNO_3$,3; KH_2PO_4, 1; $MgSO_4.7H_2O$,0.5, KCl, 0.5; $FeSO_4$. $7H_2O$, 0.5;medium II (Czapek'sDox medium): Sucrose, 2; K_2HPO_4, 0.1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4$. $7H_2O$, 0.01;medium III (Dimitri's medium): $NaH_2PO_4.2\ H_2O$, 1; KH_2PO_4, 2;$CaCl_2$, 0.025,$FeSO_4.7H_2O$, 005, $MnSO_4.7H_2O$, 0.015; $ZnSO_4.7H_2O$, 0.03; $(NH_4)_2SO_4$, 1; Fructose, 1; medium IV (Malt extract, 20; K_2HPO_4, 1; NH_4Cl, 1); medium V (Potato dextrose medium): Potato (peeled), 20; Dextrose, 20and medium VI (Modified basal medium) $(NH_4)_2SO_4$, 1; K_2HPO_4, 1; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.H_2O$, 0.02; $FeSO4.{}_7H_2O$, 0.001; NaCl, 20.

Effect of tannic acid concentration

Different concentrations (0.5, 1, 2, 3, 4 and 5%) of tannic acid were added to the production medium and incubated for 96 h at 30°C, then the cell-free extract was assayed for the extracellular tannase activity and gallic acid concentration [14].

Effect of incubation period

To study the effect of incubation period, the inoculated culture medium was incubated at 30°C for 7 days. The fungal biomass (dry weight), gallic acid concentration and tannase activity were estimated at time intervals [26].

Fungal biomass was isolated by centrifugation at 5000 g. The retained biomass was washed 3 times with distilled water and dried at 65 °C in an oven to a constant weight [27].

Optimization of nutritional factors using Plackett-Burman experimental design

Optimization process of different variables using statistical approach for maximal tannase production was carried out using Plackett-Burman experimental design [28]. Multifactorial design of 11 independent

variables was applied in order to identify which ingredients of the medium and culture conditions have significant effect on tannase enzyme production. High (+) and Low (-) levels of each independent factors were examined (Table 1). All trials were done in triplicates, and the mean of obtaining data was processed as the response of tannse activity and gallic acid concentration. The main effect of each variable was determined with the following equation:

$$Exi= (Mi+ -Mi-)/N$$

Where Exi is the variable main effect, and Mi+ and Mi- are the tannase activity (units), where the independent variable is present in high and low concentrations respectively, and N is the number of trials divided by 2. Statistical t-values of equal unpaired samples were calculated using Microsoft Excel to determine the variable significance.

Effect of Immobilization on tannase production

Entrapment in Na- alginate

Entrapment was done in 3% sodium alginate solution. A 20ml sodium alginate solution was prepared by dissolving 0.6g in 17ml distilled water and autoclaved at 121 °C for 10 minutes and left to cool. 3ml of fungal spore suspension was added to the sterilized alginate solution. 10ml of the mixture were transferred aseptically into a sterile syringe, and allowed to drop through a hypodermic needle into a cross linking solution (sterile 2% $CaCl_2$ solution) with continuous stirring to obtain spherical beads of calcium alginate gel entrapping the fungus. The beads were left in the calcium solution for about two hours to allow complete hardening, washed several times with sterile distilled water and then transferred to 50ml of sterile optimized cultivation medium.

Entrapment in K-carrageenan

3ml of fungal spore suspension was added to 17ml of sterile 3% K-carrageenan. The solution was mixed well and then 10ml fraction were introduced drop by drop with a sterile syringe into a sterilized solution of 2% KCl, left for 2 hours to harden, washed several times with sterile distilled water and then transferred aseptically to 50ml of sterile optimized cultivation medium.

Entrapment in agar-agar

For agar-agar entrapment, 0.6g agar-agar were dissolved in 16ml distilled water, and stirred well before sterilization. 1.5 ml of spore suspension was added to the sterilized solution and mixed well. The formed mixture was aseptically poured into sterile Petri- dish and left to solidify, then cut into cubes by a sterile glass cover and transfer into 50 ml of sterile cultivation medium.

Immobilization by adsorption

Spore of suspension (1.5 ml) was added to the 250 ml Erlenmeyer flask containing 50 ml sterilized culture medium and support materials such as sponge cubes, clay, ceramic, luffa pulp, pumice and art pumice.The used supports were in the form of particles (about 0.5cm diameter) in case of clay, ceramic and pumice, or in the form of small cutted cubic pieces of (about 0.5cm length) in case of luffa pulp and sponge.The flasks were incubated under static condition at 35 °C for 72 h [30].

Recycling of adsorbed *A. nomius* GWA5

Fractions of the production media (50 ml) were used. The experiment was carried out by batch wise reuse of the immobilized mycelia. The inoculated flasks were incubated at 35°C for 72hrs[31] At the end of each cycle the culture media were decanted and fresh media were added under aseptic conditions to the immobilized fungus and this was repeated for several times. Tannase activity and gallic acid concentration were estimated in each cycle.

Scanning electron microscopy

Morphological observation of immobilized cells preparations was performed using scanning electron microscope (SEM). After incubation period, sample was taken and fixed for SEM. Fixation, sample preparation and examinations were carried out at the Electron Microscope Unit at Faculty of Science, Alexandria University.

Characterization of hydrolytic products of tannic acid

Thin layer chromatography (TLC)

TLC was used to investigate the hydrolytic products of the tannic acid by the action of crude tannase. Gallic acid in the broth was extracted twice by ethyl acetate. Ethyl acetate was added in the ratio of 1:1 in separating funnel, mixed vigorously and left for 10 min to form two immiscible clear phase. The solvent was evaporated and the crude gallic acid obtained from evaporation was analyzed by TLC. Gallic acid standard was applied on the same silica gel plate [32].

The thin layer chromatographic plates were run in a solvent system comprising of ethyl acetate, chloroform and formic acid (4:4:1). The plates were air-dried and developed with ferric chloride reagent (0.81 g of $FeCl_3$ dissolved in 50 ml of distilled water). Identification of hydrolytic products was studied by comparing with the standard [33]. Retention factor (Rf) value was calculated according to the following equation from the chromatogram.

$$Rf = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent}}$$

Characterization with high performance liquid chromatography (HPLC)

The sample was further analyzed with HPLC. A sample was loaded to the HPLC (Ultimate 3000). Injection volume was 20µl. Absorbance was monitored at 280 nm and flow rate maintained at 0.5 ml/min. The sample was applied to Waters Spherisorb 5µm ODS2 (4.6*250 mm) column at 55°C temperature. The mobile phase consists of water with 1% glacial acetic acid (solvent A), water with 6% glacial acetic acid (solvent B), and water/acetonitrile (65:30 v/v) with 5% glacial acetic acid (solvent C). The sample and standard gallic acid were run identically.

RESULTS AND DISCUSSION

Isolation and screening of marine fungi for tannase production

Six fungal isolates (F1-F6) were isolated from the Western harbor, Alexandria, Egypt. The isolates were screened for their ability to hydrolyze 1% tannic acid. They showed different capabilities as indicated by zone of hydrolysis which ranged between (12-20 mm), F5 was the most potent isolate, which realized zone of hydrolysis (20 mm) So, it was choosen to compelete the study.

Molecular characterization of the most potent fungus exhibiting tannase activity

The extracted DNA was amplified using the primers ITS 1 and ITS 4. The produced amplicons were detected using agarose gel electrophoresis. The sequencing data obtained was 593 base pair. This sequence was compared with those which gave the highest homology using Blast search computer based program. The resulting data indicated that the isolate under study was identified as *Aspergillus nomius*. The obtained similarity was 98%. The nucleotide sequence was deposited to GenBank sequence database and has KU359224accession number. The phylogenetic relationships of this experimental isolate and the closely related relatives were analyzed as shown in Figure 1. Based on these results, the experimental marine fungal isolate has been identified in this work as *Aspergillus nomius* GWA5.

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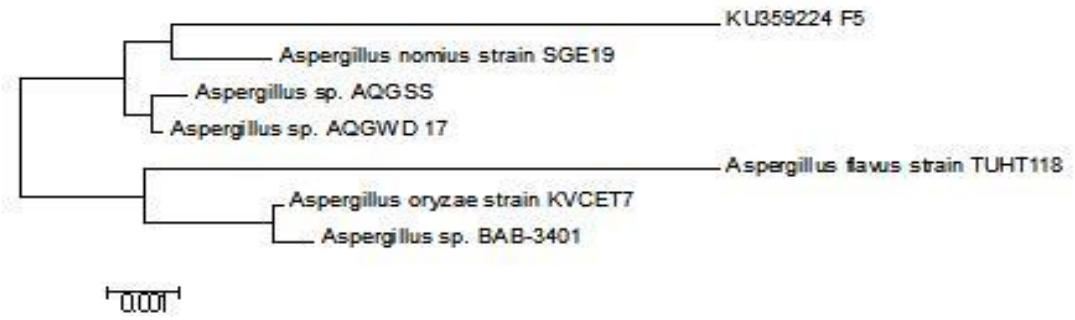
Figure 1: Phylogeneticrelationships among the representative experimental strain and the most closely related *Aspergillus* species. The dendogram was generated using MEGA6 program



Earlier researches on tannase synthesis showed that *Aspergillus* spp. were thepredominant source of tannase for industrial production and application [18]. Most of the tannase producing fungi are of terrestrial origin and too little about marine fungi.

Optimization of culture condition

Effect of different media on the production of tannase by *A. nomius* GWA5

Six media (I, II, III, IV, V and VI) were tested for higher tannase production. As illustrated in Figure 2A**,** medium (VI) showed the best tannaseactivity (27.8 U/ml) and gallic acid concentration (85.5µg/ml) followed by medium IIand medium III which realized less tannase activity (25.6 and 22.9 U/ml) respectively, and gallic acid concentration (83.6 and 76.6 µg/ml) respectively. On the other hand the lowest enzyme production was observed up on using the media IV and Vwith 15.5 and 15.7 U/ml respectivelyhowever; growth was appreciable in both the media.Some of these results go along with previous work [26] on *Trichoderma harzianum*. It was obvious in the present study that medium VI which contained tannin as the sole carbon source without addition of sugars realized the highest tannase activity as was previously documented [34, 35]. Concerning the effect of different nitrogen sources, ammonium sulphate supported tannase activity as in case of the medium III. The same finding was documented [36] as they stated that supplementation of the medium with ammonium sulphate as a nitrogen source had enhanced tannase formation by A. *fumigatus*.

Effect of tannic acid concentration

Effect of different concentrations (0.5, 1, 2, 3, 4 and 5% w/v) of tannic acid on tannase activity was tested. It was observed that 2% tannic acid was suitable for the highest tannaseactivity (31.9 U/ml) as illustrated in Figure 2B. 2% Tannic acid was optimum for enzyme production by *A. flavus* after 96 hour of incubation as was stated in parallel study [37]. It was indicated that the decrease in enzyme activity at high concentrations of tannic acid may be due to formation of complexes with membrane protein of the organism thereby both growth and enzyme production may be inhibited [38], also they stated that higher concentrations of substrate may lead to thickening (viscosity) of production medium that resulted in bad mixing of air which was essential for growth of the organism, and subsequently the production of enzyme. Excessive tannic acid also may act as repressor and prevents synthesis of mRNA. Moreover, an increase in tannic acid concentration induced an increase in tannase activity followed by a decrease because the enzyme synthesis was affected by deposition of gallic acid on the surface of the mycelia[39].

It can be concluded that the experimental organism in this work *A. nomius* GWA5 can tolerate high concentrations of tannic acid as it was able to show a suitable enzyme activity even at 5% tannic acid concentration of about (20.5 U/ml) and this is a significant feature which can be applied in future applications.

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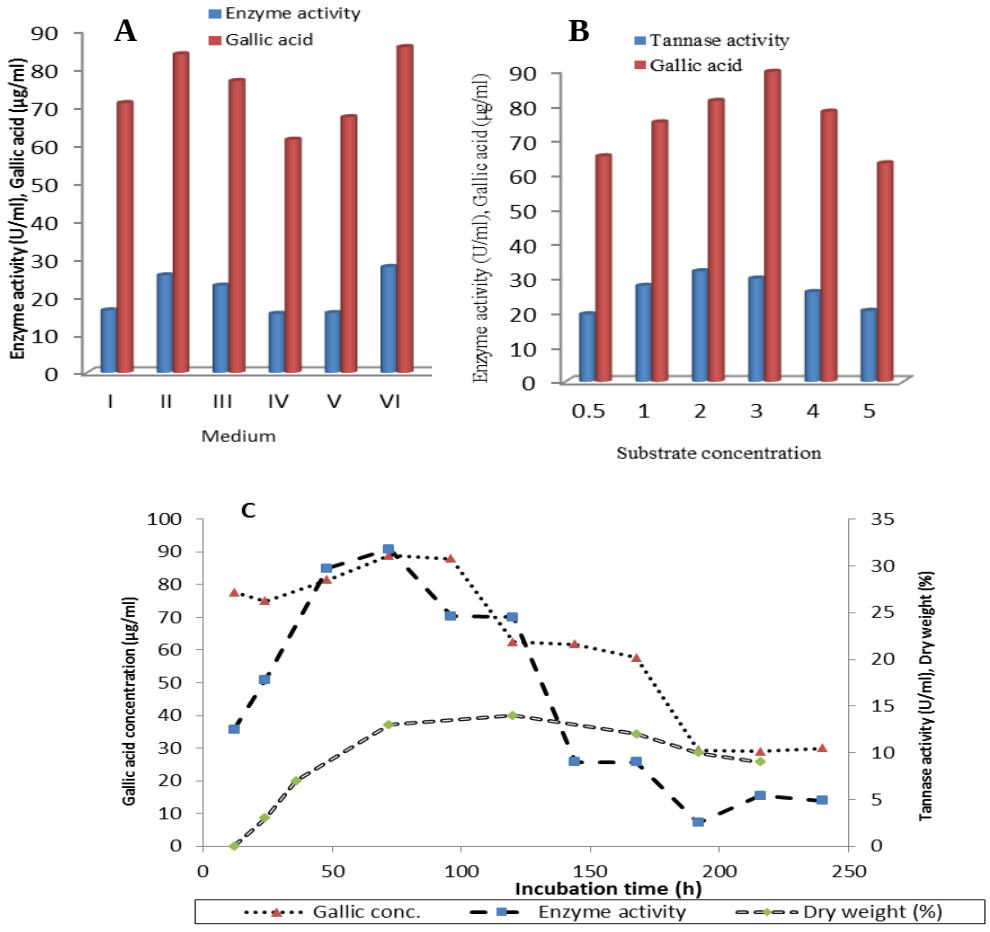
Effect of incubation period

In the present study the maximum tannase production, gallic acid concentration and dry weight were achieved in 72 hour of incubation with about 31.8 U/ml, 88.9and 13% respectively, they decreased gradually after that as showed in Figure 2C.

Time course of tannase production in association with growth of *A. nomius* GWA5 was studied at different time intervals. Results indicated that tannase of *A. nomius* GWA5was growth associated in which the maximum activity was detected at late exponential phase of growth after 72 h, similar results were reported for *A. terreus*[4]and *A. oryzae* [11]. Decrease in enzyme activity after 72 h may be due to reduction in nutrient level of the medium which affect the metabolic activity and enzyme synthesis causing inhibition and denaturation of the enzyme as mentioned in previous work [37]. Also may be due to inhibition of the enzyme by the end product (gallic acid) or substrate scarcity in the medium and secretion of toxic substances [11].

Figure 2: A) Screening of different media for maximum tannase production, B) Effect of substrate concentration on tannase activity, C) Effect of incubation period on tannase activity, gallic acid concentration and dry weight



Optimization of culture (fermentation) conditions using Plackett-Burman experimental design

The Plackett-Burman experimental design was used to evaluate the relative importance of various factors on the production of tannase enzyme in submerged cultures. Eleven culture variables were examined as shown in Table 1. All experiments were performed in duplicates and the averages of results (tannase activity) were presented as the response (Table1). The experiment was carried out with 2% tannic acid as a carbon source. The main effect of each variable on the production of tannase enzyme as well as t-values were estimated for each independent variable as shown in Table 1.

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Table 1: The applied Plackett–Burman experimental design for eleven cultural variables

	Factor symbol											Tannase activity (U/ml)
Trial	**N**	**K2**	**K**	**Mg**	**Ca**	**Fe**	**Na**	**pH**	**CA**	**IS**	**T**	
1	+ [1.5]	+[1. 5]	+[0.75]	+[0.75]	+[0.03]	+[0015]	+[25]	+[6.5]	+[5]	+[1.5]	+[35]	40.8
2	−[0.5]	+ [1.5]	−[0.25]	+[0.75]	+[0.03]	+[0.0015]	−[15]	−[4.5]	−[4]	+[1.5]	−[25]	38.9
3	−[0.5]	−[0.5]	+[0.75]	−[0.25]	+[0.03]	+[0.0015]	+[25]	−[4.5]	−[4]	−[0.5]	+[35]	39.6
4	+[1.5]	−[0.5]	−[0.25]	+[0.75]	−[0.01]	+[0.0015]	+[25]	+[6.5]	−[4]	−[0.5]	−[25]	35.8
5	−[0.5]	+[1.5]	−[0.25]	−[0.25]	+[0.03]	−[0.0005]	+[25]	+[6.5]	+[5]	−[0.5]	−[25]	40.2
6	−[0.5]	−[0.5]	+[0.75]	−[0.25]	−[0.01]	+[0.0015]	−[15]	+[6.5]	+[5]	+[1.5]	−[25]	36.3
7	−[0.5]	−[0.5]	−[0.25]	+[0.75]	−[0.01]	−[0.0005]	+[25]	−[4.5]	+[5]	+[1.5]	+[35]	40.6
8	+[1.5]	−[0.5]	−[0.25]	−[0.25]	+[0.03]	−[0.0005]	−[15]	+[6.5]	−[3]	+[1.5]	+[35]	34.7
9	+[1.5]	+[1.5]	−[0.25]	−[0.25]	−[0.01]	+[0015]	−[15]	−[4.5]	+[5]	−[0.5]	+[35]	34.8
10	+[1.5]	+[1.5]	+[0.75]	−[0.25]	−[0.01]	−[0005]	+[25]	−[4.5]	−[3]	+[1.5]	−[25]	35.3
11	−[0.5]	+[1.5]	+[0.75]	+[0.75]	−[0.01]	−[0005]	−[15]	+[6.5]	−[3]	−[0.5]	+[35]	37.6
12	+[1.5]	−[0.5]	+[.0.75]	+[0.75]	+[0.03]	−[0005]	−[15]	−[3.5]	+[5]	−[0.5]	−[25]	35.6
13	0[1]	0[1]	0[0.5]	0[0.5]	0[0.5]	0[001]	0[20]	0[4.5]	0[4]	0[1]	0[30]	31.2
Main effect	-2.7	0.83	0.003	1.4	1.6	0.37	2.4	0.1	1.07	0.5	1	
t- value	-1.3	0.59	0.02	1.02	1.16	0.74	**2.0**	0.07	0.76	0.35	0.71	

N: $(NH_4)_2SO_4$; K_2: K_2HPO_4; K: KH_2PO_4; Mg: $MgSO_4.7H_2O$; Ca: $CaCl_2.2H_2O$; Fe: $FeSO_4.7H_2O$; Na: NaCl; pH: pH; CA: Culture age; IS: Inoculum size; T: Temperature

Results indicated that ammonium sulphate has a negative main effect, while the other tested variables exhibited positive main effects on tannase enzyme activity. These results indicated that the use of lower concentration of ammonium sulphate will give maximum tannase activity and alsohigher concentration of all other medium components will give maximum activity [38].

$MgSO_4$ and $CaCl_2$ showed positive effect on tannase production as was documented in previous studies [5, 36]. Beneficial effect of $MgSO_4$ for tannase production by *Bacillus licheniformis* KBR6 and *Aspergillus* sp., respectively was confirmed [39, 40].$K_2HPO_4$and KH_2PO_4 had positive effect on tannase production where the process of tannin hydrolysis by tannase is an aerobic process, needs ATP(energy) that can be produced from tannin as the sole carbon source, phosphate was responsible for maintaining buffer environment in the production medium [41].It was noted with respect to marine microorganisms that potassium and phosphates in the medium are stimulatory towards enhancing growth and enzyme production [40].

Maximum enzyme activity was observed at highertemperature (35°C)as was reported for tannase from *Aspergillus* spp.[41]Other studies reported the fermentation temperature for optimum production of tannase to be 30°C [42, 43]. pH strongly influences many enzymatic processes and transport of various components across the cell membranes which in turn support the cell growth and product production [44]. In the present study, pH had positive effect on tannase production and the highest productivity was determined at pH 6.5, decrease in pH of the medium reduced tannase production which is consistent with previous study[26]which documented pH range of (4.5-6.5) as the most suitable for production.

Statistical analyses of the results (t-test) showed that variations in sodium chloride concentration in the tested ranges had the most significant effect on the activity of tannase produced by *A. nomius* GWA5. The influence of NaCl in addition to other factors on tannase production by marine isolated fungus *A. awamori* was studied [45]. It was illustrated that this observation could be attributed to the source of *A. awamori*, which is seawater, where the organism exists in high saline environment, and hence the tannase-encoding genes could be induced by the salt concentration. This fact goes along with the results obtained in the present work by marine *A. nomius* GWA5.

According to obtained results, the predicted optimized medium composition for cultivation of *A. nomius*, was as follows: (g/l): **(**$(NH_4)_2SO_4$, 0.5; K_2HPO_4, 1.5; KH_2Po_4, 0.75; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2o$, 0.03; $FeSO_4.7H_2O$, 0.0015; NaCl, 25; inoculum age, 5 days; Inoculum size, 1.5ml; Tannic acid, 20 adjusted to pH 6.5 and incubation period 72h at 35 ^oC.

In order to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was applied to compare between the predicted optimum levels of independent variables and the basal condition settings. Results in Figure 3 confirmed that tannase activity (U/ml) increased to 46.6 U/ml with 1.5 fold increase and the produced gallic acid concentration increased to102.1 µg/ml with 1.8 fold increase when compared with activity obtained under the basal conditions.

A verification experiment was applied also, to compare between the anti- optimum levels of independent variables and the basal condition settings. Results in Figure 3 confirmed that tannase activity decreased to 24.4 U/ml with 1.3 fold decrease when compared to cells grown under the basal conditions.

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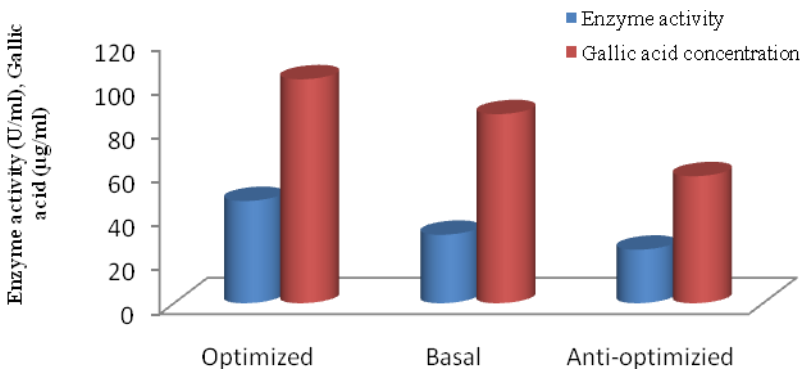
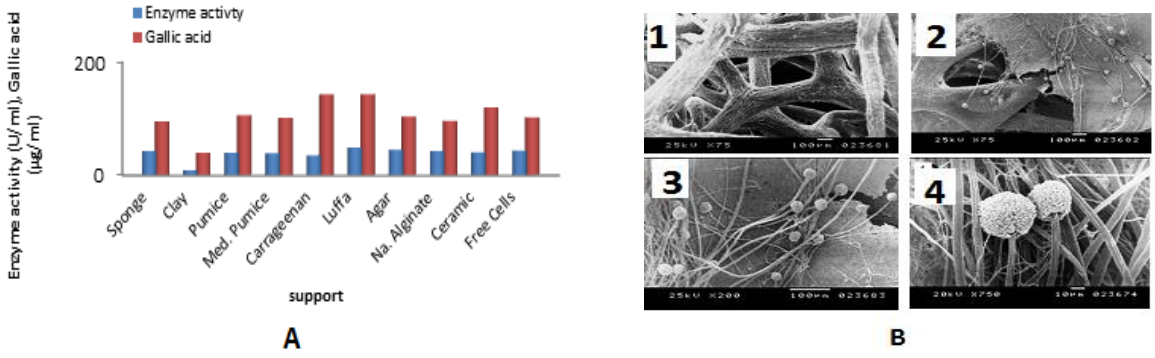
Figure 3: A verification of the Plackett-Burman experimental results

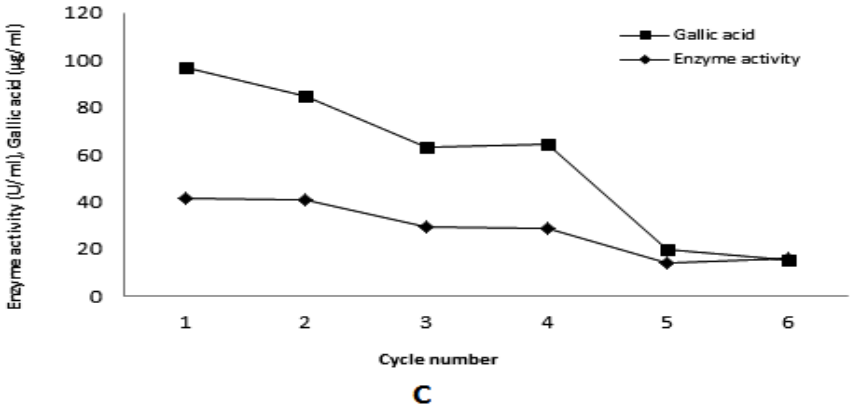


Effect of immobilization on tannase production by *A. nomius* GWA5

Immobilization by entrapment using different gel materials such as carrageenan, sodium alginate and agar in addition to immobilization on different support materials (sponge cubes, clay, ceramic, luffa pulp, pumice and art pumice (Fig.4A) were carried out aiming at enhancing tannase activity. Results indicated that adsorbed cells on luffa revealed the highest tannase activity and gallic acid concentration (49.2U/ml and 143.1µg/ml) with 1.1 and 1.4 fold increases, respectively when compared to free cells and also was superior than the entrapped cells. Thus cultures containing luffa realized the highest activity and was choosen to complete the study. Figure 4B(2) shows *A. nomius* GWA5 adsorbed on luffa pulp and Figure 4B(3,4) show free *A. nomius* GWA5.

Figure 4: A) Production of tannase by *A. nomius* GWA5 adsorbed on different solid supports; B) Scanning electron micrographs showing (1) control luffa pulp; (2) *A. nomius* GWA5 adsorbed on luffa pulp and (3,4) free *A. nomius* GWA5; C) Repeated batch culture for tannase production using immobilized cells of *A. nomius* GWA5.





The higher productivity of *A. nomius* GWA5 adsorbed on luffa pulp could be due to absence of toxicity problems, mechanical strength for essential support and open spaces within the matrix for growing cells therefore avoiding splitting and diffusion problems as was reported in previous studies [46, 47].

The semicontinuous production of tannase by *A. nomius* GWA5 adsorbed on luffa (Figure 4B.2) was performed for six successive cycles aiming for enhancing the enzyme activity and gallic acid productivity. The enzyme activity and yield of gallic acid was monitored each cycle. The graphically presented data in Figure 4C revealed that almost stable amounts of enzyme were produced during the first 3 cycles while the next cycles showed a drop in enzyme activity until reaching 30% of the enzyme activity at the first cycle.

Hamdy and Fawzy[31] studied the efficiency of the immobilized *A. niger* for tannase production in a repeated batch process, the results obtained by them were closely related to those of the present work. They explained the observed decrease in enzyme productivity after several cycles with the high growth of biomass, increasing cell density and/or the presence of dead cells that cause diffusional limitation of oxygen and substrate. Different studies reported the advantage of using immobilization in the enzyme production and reuse of the whole immobilized cells to increase the productivity [48, 49].

Characterization of hydrolytic products of tannic acid

Thin layer chromatography (TLC)

TLC was used to investigate the hydrolytic products (gallic acid) of the tannic acid by the action of crude tannase. As illustrated in Figure 5A,blue colored spots corresponding to the gallic acid with Rf value of 0.67 were observed indicating the production of gallic acid by *A. nomius* GWA5due to the hydrolysis of tannic acid, which correlated with the already reported value (0.69) in parallel study [18].

Characterization with high performance liquid chromatography (HPLC)

HPLC results indicated the purity of extracted sample of gallic acid and efficiency of the method in separating the gallic acid. Standard run of gallic acid had single peak near retention time of 4.3 minute (Figure 5B). A peak was observed at the same retention time in the sample run and indicated the presence of gallic acid in the crude extract (Figure 5C) as a result of tannic acid degradation by the action of tannase produced by *A.nomius* GWA5. Many researchers have used HPLC for detection of gallic acid as a product of tannin degradation by tannase enzyme [1, 31, 50].

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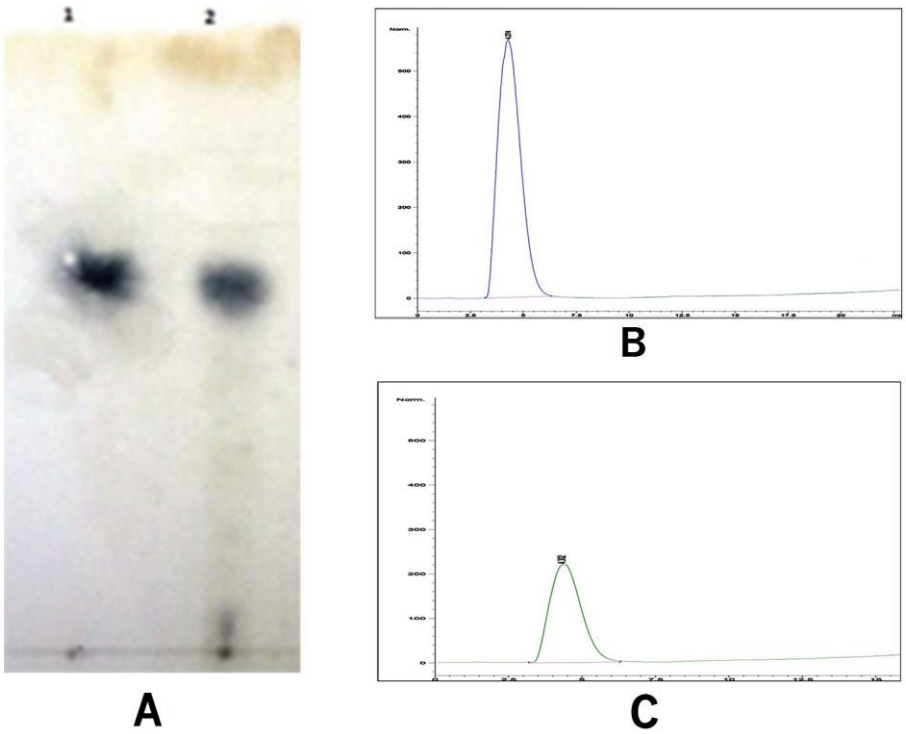
Figure 5: A) TLC analysis showing Lane (1) standard gallic acid; Lane (2) gallic acid produced by action of *A. nomius* GWA5 tannase; B) HPLC analysis showing standard gallic acid, C) HPLC analysis of gallic acid produced by action of *A. nomius* GWA5 tannase;



CONCLUSION

The marine *A. nomius* GWA5 appears to be good and economic candidate for tannase enzyme production. Successful immobilization of the whole cells and reuse of them led to increase the production of the enzyme which can be applied in different future industrial applications.

REFERENCES

[1] Pepi M, Lampariello LR, Altieri R, Esposito A, Perra G, Renzi M, Lobianco A, Feola A, Gasperini S, Focardi SE. Tannic acid degradation by bacterial strains *Serratia* sp. and *Pantoea* sp. isolated from olive mill waste mixtures. IntBiodeterBiodegr2010;64(1):73-80.

[2] Lekha PK, Lonsane BK. Production and application of tannin acyl hydrolase: State of the art. Adv ApplMicrobiol1997;44:215-60.

[3] Zakipour-Molkabadi E, Hamidi-Esfahani Z, Sahari MA, Azizi MH.A new native source of tannase producer, *Penicillium* sp. EZ-ZH190: Characterization of the enzyme. Iran J Biotechnol2013;11(4):244-50.

[4] Chávez-González M, Rodríguez-Durán LV, Balagurusamy N, Prado-Barragán A, Rodríguez A, Contreras JC, AguilarCN. Biotechnological advances and challenges of tannase: An Overview. Food Bioprocess Technol2012; 5:445–59.

[5] Bele AA, Jadhav VM, Kadam V. Potential of tannins: A Review. Asian J Plant Sci2010; 9(4):209-14.

[6] Belmares R, Contreras-Esquivel JC, Rodríguez-Herrera R, Coronel AR, Aguilar CN. Microbial production of tannase: an enzyme with potential use in food industry. LWT - Food Sci Technol2004;37(8):857-64.

[7] Kondo M, Jayanegara A, Uyeno Y, Matsui H. Variation of tannin contents in selected agro-industrial by-products and their biological activity in precipitating protein. AdvAnim Vet Sci 2016;4(2):66-70.

[8] MuruganK, Al-Sohaibani S. Biocompatible removal of tannin and associated color from tannery effluent using the biomass and tannin acyl hydrolase (E.C.3.1.1.20) enzymes of mango industry solid waste isolate *Aspergillus candidus* MTTC 9628. Res J Microbiol2010;5(4):262-71.

[9] Frutos P, Hervas G, Giraldez FJ, Mantecon AR. Tannins and ruminant nutrition: Review. Spanish J AgrRes2004;2(2):191–202.

[10] Aguilar CN, Rodríguez R, Gutiérrez-Sánchez G, Augur C, Favela-Torres E, Prado-BarragánLA, Ramírez-Coronel A, Contreras-Esquivel JC. Microbial tannases: Advances and perspectives. ApplMicrobiolBiotechnol 2007; 76(1):47–59.
[11] bdel-Nabey MA, Sherief AA, El-Tanash AB. Tannin biodegradation and some factors affecting tannase producing by two *Aspergillus* sp. Biotechnol2011;10(2):149-58.
[12] BelurPD, Mugeraya G. Microbial production of tannase: State of the art. Res J Microbiol2011;6(1):25-40.
[13] Beniwal V, Kumar A, Sharma J, Chhokar V. Recent Advances in industrial application of Tannases: A Review. Recent Patents on Biotechnology2013;7: 228-33.
[14] Brahmbhatt D, Modi HA, Jain NK. Preliminary isolation and screening of tannase producing bacteria and fungi. Int J Current MicrobiolApplSci 3(11):193-203.
[15] Girdhari, S.N., Peshwe,S.A., 2015. Isolation and screening of tannase producing fungi. Int J CurrMicrobiol App Sci2014;4(7):765-774.
[16] Zhang Y, YinJ, Chin J, Wang F, Du Q, Jiang Y, Xu Y.Improving the sweet aftertaste of green tea infusion with tannase. Food Chem2016;192(1): 470–476.
[17] Kapoor A, Iqbal H. Efficiency of tannase produced by Trichoderma *Harzianum* MTCC 10841 in pomegranate juice clarification and natural tannin degradation. Int J BiotechnolBioengin Res2013;4(6):641-650.
[18] Beena PS. Poduction, purification, genetic characterization and application studies of tannase enzyme from marine fungus *Aspergillus awamori*.Ph.Thesis, Facu. Sci 2010; Cochin Univ.
[19] Rodriguez-Duran VL, Valdivia-Urdiales B, Contreras-Esquivel JC, Rodriguez-Herrera R, Aguilar CN. Novel strategies for upstream and downstream processing of tannin acyl hydrolase: A Review. Enzyme Res2011; 1-20.
[20] Jamal P, Tompang MF, Alam MZ. Optimization of media composition for the production of bioprotein from pineapple skins by liquid-state bioconversion. J Appl Sci2009;9:3104-09.
[21] Kumar R, Kumar A, Nagpal R, Sharma J, Kumari A. A novel and sensitive plate assay for screening of tannase-producing bacteria. Ann Microbiol2010;60:177–179.
[22] Osawa R, Walsh TP. Visual reading method for detection of bacterial tannase. Appl. Environ Microbiol1993;59:1251-52.
[23] Sharma S, Bhat, Dawra RK. A spectrophotometric method for assay of tannase using rhodanine. AnalBiochem2000;279:85-89.
[24] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin-phenol reagent. J BiolChem1951;193:265-275.
[25] Loeffler J, Hebart H, Schumacher U, Reitze H, Einsele H. Comparison of different DNA extraction methods from blood and cultures. J ClinMicrobiol 1997; 35:3311-12.
[26] Iqbal H, Kapoor A. Culture conditions for the production of tannase from *Trichoderma harzianum* MTCC 10841. Int J SciTechnol2012;1(11): 584-95.
[27] Darah I, Sumathi G., Jain K, Lim SH.Tannase enzyme production by entrapped cells of Aspergillus niger FETL FT3 in submerged culture system. Bioprocess Biosyst. Eng2011a;34:795–801.
[28] Plackett RL, Burman JP. The design of optimum multifactorial experiments. Biometrika1946;33:305–25.
[29] Eikmeier H, Rhem HJ. Stability of calcium alginate during citric acid production of immobilized Aspergillus niger. ApplMicrobiolBiotechnol1987;26:105-11.
[30] Vassilev NB, Vassilev MC, Spassova DI. Production of gluconic acid by *Aspergillus niger* immobilized in polyurethane foam. ApplMicrobiolBiotechnol1993;39: 282–85.
[31] Hamdy HS, Fawzy EM. Economic production of tannase by *Aspergillus niger* van Tiegh adopting different fermentation protocols and possible applications. Romanian Biotechnol Let 2012;17(4): 7441-57.
[32] Belur PD, Pallabhanvi B. Investigation on production of gallic acid from Terminalia chebula extract using cell associated tannase of *Bacillus massiliensis*. In: International Conference on Advances in Biotechnology and Pharmaceutical Sciences (ICABPS'2011), Bangkok, 2011;222-25.
[33] Naidu RB, Saisubramanian N, Selvakumar D, JanardhananS, Puvanakrishnan R. Partial purification of tannase from *Aspergillus foetidus* by aqueous two phase extraction and its characterization. CurrTrends Biotechnol Pharm2008;2: 201-07.
[34] Sivashanmugam K, Jayaraman G.,. Production and partial purification of extracellular tannase by *Klebsiella pneumoniae* MTCC 7162 isolated from tannery effluent. Afr J Biotechnol2011;10(8):1364-74.
[35] Mondal KC, Banerjee R, Pati BR.Tannase production by *Bacillus licheniformis*. BiotechnolLet2000;22:767-69.

[36] Sena ARD, Santos ACB, Gouveia MJ, Mello MRF, Leite TCC, Moreira KA, Assis S. A. Production, characterization and application of a thermostable tannase from *Pestalotiopsisguepinii* URM 7114. Food TechnolBiotechnol2014;52(4): 459–67.
[37] El-Shora HM, Awadalla OA, Metwally SM. Optimization of tannase production by *Aspergillus flavus*.Egypt J ExpBiol2015;11(2): 121-27.
[38] Kumar R, Sharma J, Singh R. Production of tannase from *Aspergillus ruber* under solid-state fermentation using jamun (Syzygiumcumini) leaves. Microbiol Res2007;162(4): 384-90.
[39] Seth M, Chand S. Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori* - Optimization of process parameters. Process Biochem2000;36: 39-44.
[40] Reddy MN, Reddy LNG. Production of tannase by isolated *Aspergillus terreus* under submerged fermentation. Int J Sci Technol2015;4(2): 102-13.
[41] Jana A, Halder SK, Banerjee A, Paul T, Pati PR, Mondal KS, Das Mohapatra PK. Biosynthesis, structural architecture and biotechnological potential of bacterial tannase: A molecular advancement. BioresTechnol2014;327–40.
[42] Darah I, Sumathi G, Jain K, Hong LS. Involvement of physical parameters in medium improvement for tannase production by *Aspergillus niger* FETL FT3 in submerged fermentation. Biotechnol Res Int2011b;165:1682–90.
[43] Reddy BS, Rathod V. Gallic acid production and tannase activity of *Penicillium purpurogenum* employing agro based wastes through solid state fermentation: Influence of pH and temperature. Asian J BiochemPharmaceutRes2012;2(1): 59-62.
[44] Lata S, Rani KP. Production of tannase enzyme by *Penicillium duclauxii*, screened and isolated from soil. Res J Pharm BiolChem Sci2016;7(1):1471-85.
[45] Beena PS, Basheer SM, Bhat SG, Bahkali AH, Chandrasekaran M. Propyl gallate synthesis using acidophilic tannase and simultaneous production of tannase and gallic acid by marine *Aspergillus awamori* BTMFW032. ApplBiochemBiotechnol 2011; 164:612–28.
[46] Akhtar N, Iqbal J, Iqbal M. Removal and recovery of nickel (II) from aqueous solution by loofa sponge-immobilized biomass of *Chlorella sorokiniana*: Characterization studies. J Haza Mat B2004;108: 85–94.
[47] Iqbal M, Saeed A. Novel method for cell immobilization and its application for production of organic acid. Let ApplMicrobiol2005;40:178–82.
[48] Farag A ,Hassan S, Beltagy E, El-Shenawy M. Optimization of production of anti-tumor L-asparaginase by free and immobilized marine *Aspergillus terreus.* Egypt J Aqu Res 2015; 41(4): 295-302.
[49] Zuriana S, Sakinah A. Production of sorbitol by repeated batch fermentation using immobilized of *Lactobacillus plantarum* strain (BAA-793) via solid state fermentation. Food Res 2017; 1 (5): 176 – 182.
[50] Jimenez N, Reveron ., Esteban-Torres M, de Felipe FL, de las Rivas B. Munoz R. Genetic and biochemical approaches towards unravelling the degradation of gallotannins by *Streptococcus gallolyticus*. Microbial Cell Factories 2014; 13:1-11.