Production of Tannase from *Aspergillus niger* under solid state fermentation

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Abstract

Thirteen *A.niger* isolates were obtained from soil and food samples and screened on tannic acid agar for their ability to produce tannase. There isolates revealed large tannic acid hydrolysis zones, these isolates were cultured in liquid and solid substrate fermentation media to examine their production of tannase quantitatively .Solid substrate medium was more efficient than liquid medium ,and *A.niger* Ass19 gave the highest tannase productivity. Different kinds of SSF media and cultured conditions were performed to determine their effect on tannase production. The maximum yield of tannase was obtained in wheat bran with tea leaves hydrated with citrate buffer pH 5.5 at 1:3 (w/v) hydration ratio inoculated with 2×10^8 fungal spores and incubated at 28C for 72hr .the tannase productivity was 8200 U/mg at these conditions.

انتاج انزيم التانييز من فطر (*Aspergillus niger*) بتخمر الاوساط الصلبة

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الخلاصة

تم الحصول على ثلاثين عزلة للفطر *A.niger* من نماذج تربة وغذاء ، ثم غربلة هذه العزلات على وسط اكار حامض التانك لمعرفة قابليتها على انتاج التانييز .واظهرت ثلاث عزلات منطقة تحلل واسعة لحامض التانيك ،وزرعت هذه العزلات في اوساط تخمر سائلة وصلبة لاختبار انتاجيتها للتانييز كميا . لوحظ ان وسط التخمر الصلب يعطي انتاجية اعلى من الوسط السائل وان العزلة *A.niger Ass19* هي الاكثر انتاجا للانزيم من بقية العزلات .استخدمت تخمر الاوساط الصلبة وظروف زرعية مختلفة لمعرفة تأثيرها في انتاج التانييز وتبين ان اعلى انتاجية لأنزيم تكون في الوسط نخالة الحنطة مع ا وراق الشاي المرطب بمحلول السترات الدارئ برقم هيدروجيني 5.5 بنسبة ترطيب 1:3 (وزن :حجم) عند تلقيحه ب 2×10^8 ابواغ الفطر وحضنه بدرجة حرارة 28 درجة مئوية لمدة 72 ساعة وبلغت الانتاجية حوالي 8200 وحدة/ ملغم بروتين في هذه الظروف.

Introduction

Tannase or tannin acyl hydrolase (E.C.3.1.1.20) is an inducible enzyme that catalyses the breakdown of ester linkage in hydrolysable tannins such as tannic acid resulting in the production of gallic acid and glucose [1]. Hydrolysable tannins such as gallotannin and epigallotannin are widely distributed in the plant kingdomthese tannins bind readily with protein and other macromolecules to form indigestible or insoluble complexes[2], .*Aspergillus*sp. is one of the most important sources of tannase for industrial application but also tannase can be obtained from various sources such as

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animals, plants and microorganisms [3].The use of SSF presents advantages such as higher productivity ,lower capital investments ,low waste water output, and lower downstream processing cost[4].tannase is used in the manufacturing of instant tea and the production of gallic acid ,a substrate for propyle gallate production and trimethoprim synthesis [5]. ,Additionally, it is used to reduce the anti-nutritional effects of poultry and animal feed along with food detannification and industrial effluent treatment[6]. Several microorganisims are able to produce tannase such as:
Bacteria : *Bacillus licheniformis* [7], *Lactobacillus planetarium*[8], *Klebsiella pneumonia*[9] ,**Fungi** : *Penicillium variable*[10], *Aspergillus niger* [11], *Paelcilomyces variotii* [12] **Yeast**: *Candida sp*.[13], *Pichia sp*.[14] the aim of this research is to find the optimum conditions for tannase production.

Materials and Methods

Collection of samples

A total of 70 samples were collected from two sources : 20 samples from spoiled food (onion ,tomato,nuts,etc…) and 50 samples from soil and collected in sterile containers and transported to the laboratory until usage.

Isolation of *Aspergillus niger*

One gm of each soil sample was added to 9ml of sterile water and mixed vigorously .Serial dilutions were made for each sample by using sterile water .0.1ml of each dilution was spreaded on Potato dextrose plate agar containing 250Mg/1000ml, and incubated aerobically at 28C for 72hr.fungal isolated were obtained and identified as *Aspergillus niger* according to the morphological and microscopic examination[15].

Microscopic and Morphological Haracteristics

The morphology and shape of the fungal isolate on PDA agar plate were studied. a loop full of the fungal spore suspension was mixed on a slide with a drop of lactophenol.

Screening of tannase production

Semi-quantitative method

Twenty isolates of *A.niger* was investigated on tannic acid plate agar that contains the follow contents(gm/L):$NaNO_3$ 3gm, KCl and $MgSO_4.7H_2O$ 0.5gm ,KH_2 PO_4 1gm, $FeSO_4.7H_2O$ 0.01gm ,Tannic acid 10gm and Agar –agar 30gm .pH was adjusted to 4.5 then inoculated at 28C for 72hr [19]**.**

Quantitative method

1-Production of crude enzyme under liquid state fermentation

Liquid state fermentation media contained (gm/L):3 gm $NaNO_3$,0.5gm KCl ,0.5gm $MgSO_4$ .7H_2 O, 1gm KH_2PO_4 ,0.01 gm $FeSO_4.7H_2O$ and 10 gm of tannic acid .(pH 5.5) then autoclaved at 121 C and inoculated with $2*10^8$ of spore suspension then incubated at 28C for 72hr .after that culture media was centrifuge in 8000 rpm and supernatant was assayed for tannase activity and protein concentration[16].

2-Production of tannase under solid state fermentation

A solid state fermentation culture was prepared by addition of 10 gm of each solid materials and mixed with 40 ml of tap water in 100 ml conical flasks and autoclaved at 121C for 30 min.the media were inoculated with 1 ml of spore suspension contained $2*10^8$ spores and incubated at 28C for 72 hr. The enzyme was extracted from the medium by addition of 40ml tap water,mixed well and filterated through many layers of gauze. The filterate was centerifugated at 5000 rpm in a cooling centrifuge.The activity of tannase and protein were assayed in the supernatant [20].

Tannase assay.

A volume of 0.5 ml of crude enzyme was added to 2.0 ml of 0.35% (w/v) tannic acid solution in 0.05M of citrate buffer pH 5.5 and incubated for 10 min at 30C after that the reaction mixture was stopped by the addition of 2.0 ml of ethanol then centrifuge the mixture for 10 min at 5000rpm in cooling centrifuge and measuring the absorbance of supernatant at 310 nm by spectrophotometer .the enzyme activity unit is defined as the amount of enzyme required to hydrolyzed 1 Mmol of ester linkage of tannic acid in 1 min under assay condition and it is express as U/ml/min [17].Protein concentration was measured by Bradford method[18] .

Enzyme activity (U/ml) =O.D (310 nm) / (slope x volume of enzyme x incubation period

Protein concentration (mg/ml) =O.D (600 nm) / (slope x 1000)

Calculation of specific activity
The specific activity of the enzyme was calculated as following

$$\textbf{Specific activity (U/mg protein)} = \frac{\textbf{Enzyme activity (U/ml)}}{\textbf{Protein concentration (mg/ml)}}$$

Determination of optimum condition for tannase production under solid state fermentation

1.Tannase production media under SSF using different solid agricultural materials and plant containing tannins:
Different plant wastes were used for preparation of solid media included (wheat bran, corn bran, wheat straw , soy bean bran) these compounds were prepared either alone ,with and without tannic acid and with tannin containing –plants (tea leaves, *Punicca granatum* peals ,crushed bark of *Acacia nilotica*) in 100 ml of erylen mayer flasks containing :
A) Ten grams of solid agricultural residues+0.5 g of tannic acid +40ml of citrate buffer 0.05M pH 5.5
B) Ten grams of solid agricultural residues+40ml of citrate buffer 0.05M pH 5.5
C) Ten grams of optimum solid agricultural residues +0.5 g of each of tannin-containing plants (tea leaves, *Punica granatum* peals ,bark of *Acatia nilotica*)+40 ml of citrate buffer 0.05M pH 5.5
D) Ten grams of these tannin-containing plants were experimented for tannase production (10 g of each one) without plant wastes + citrate buffer 0.05M pH 5.5

2.Effect of different moisture ratio
Wheat bran media with tea leaves (as optimum) were prepared with different moisture ratio of citrate buffer 0.05M (pH 5.5) started with 1:2 ,1:3,1:4,1:5,1:6 ended with 1:7(solid state : citrate buffer) then autoclaved for 25 min at 121C and inoculated with 1 ml of spore suspension containing $2*10^8$ of fungal spore suspension incubated at 28 C for 72hr .extraction was done with different volumes until reached 80 ml with started.

3.Effect of incubation time
Wheat bran media was prepared with tea leaves moisted with 1:3 ratio and pH 5.5 then autoclaved and inoculated with 1 ml of spore suspension containing $2*10^8$ of fungal spores and incubated at 28C for 24hr-169hr, the enzyme was extracted after each incubation period and tannase activity and protein concentration were assayed until the day seven.

Results and Discussion

Isolation and identification of *Aspergillus niger* :
Sixty fungal isolates of *Aspergillus spp.* were obtained from spoiled food and soil of Iraq. thirty fungal isolates were identified as *A. niger* producing tannase according to morphological feature of fungal colonies on PDA medium and microscopical examination ,hyphae of *A. niger* were septate and hyaline. Conidial heads were radiate initially, splitting into columns at maturity. The isolate was biseriate (vesicles produced sterile cells known as metulae that support the conidiogenous phialides). Conidiophores were long , smooth, and hyaline, becoming darker at the apex and terminating in a globose vesicle, Metulae and phialides covered the entire vesicle, Conidia were brown to black, very rough and globose. The growing hyphae were purified by sub culturing on PDA media for many times until pure culture was obtained then stored at 4ºC as stock culture and recultured every two weeks .

Screening of tannase producing *A.niger*:

Semi-quantitative Screening
Tannic acid agar media (TAA) containing 0.1% tannic acid as carbone source was used for screening of tannase . The results showed that 8 isolates from 30 isolates were able to produce tannase with different diameter of tannic acid hydrolysis zones at 28˚C incubation. The ratio of tannic acid hydrolysis zones * (diameter of the clear zones/diameter of the colony) ranged between 18-60mm, as shown in table-1.
Aspergillus species are important in commercial microbial fermentations. For example, the production of hydrolytic enzymes (amylase ,lipase, xylanase, etc…) [2].

Table 1- Production of tannase (semi-quantitative method) by *Aspergillus niger* isolates expressed by their hydrolysis ratio on T.T.A. medium after incubation at 28C^{o} for 72hr

No.of isolates	Hydrolysis zone of Tannase activity After 24hr *	Hydrolysis zone of tannase activity After 48hr *	Hydrolysis zone of tannase activity after 72hr *
Ass17	18mm	25mm	25mm
Ass18	23mm	30mm	30mm
Ass19	28mm	33mm	38mm
Ass21	22mm	27mm	29mm
Ass24	30mm	38mm	40mm
Ass27	38mm	50mm	60mm
Ass28	27mm	30mm	30mm

*Diameter of the clear zones/ diameter of the colony

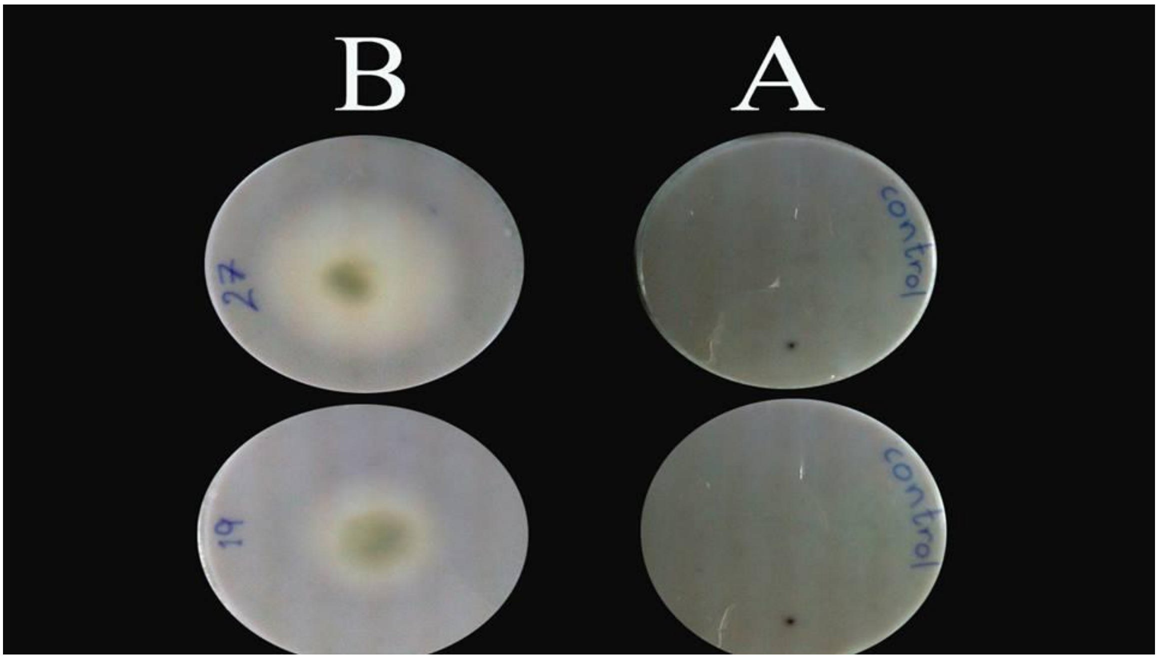

Figure 1- Detection of tannase producing fungi (A)TAA medium as control B) represents tannic acid hydrolysis by *Aspergillus niger* Ass19 and Ass27 tannase

Quantitative screening of *A.niger* producing tannase:

Three fungal isolates were experimented for tannase production on solid state fermentation (SSF) (wheat bran with tannic acid) and Liquid state fermentation (LSF) .The results showed that SSF had the highest specific activity, compared with LSF, for tannase production and the most active isolate on SSF was Ass19 (figure2). It was cleared that tannase production under SSF compared with LSF had advantages such as higher productivity ,lower capital investments, low waste water out, and lower downstream processing cost [22]

Because of the low availability of water reduces the possibilities of contamination by bacteria and yeast.higher levels of aeration ,the low moisture availability may facilitate the production of tannase that may not likely be produced or poorly produced in LSF also tannase production through SSF are more stable and produced in higher quantities than LSF.

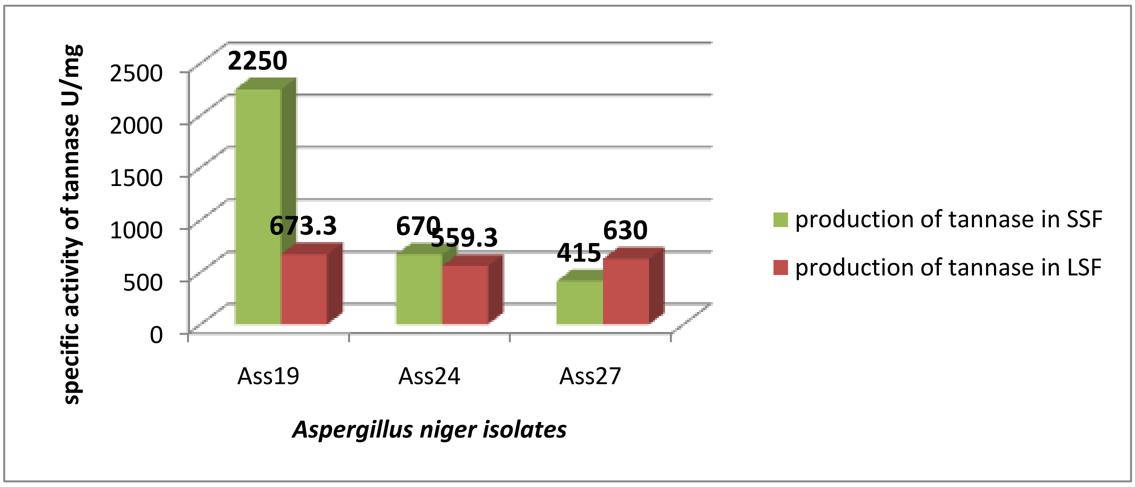

Figure 2- Tannase production under solid state fermentation and liquid state fermentation after incubation at 28C for 72hr

Tannase production media using different solid agricultural residues

Different agricultural residues with and without tannic acid were examined for tannase production . Wheat bran mixed with tannic acid gave the highest activity for tannase production (figure 3) because of its high quality of nutrient that present in wheat bran which fungi need to grow such as nitrogen ,carbone and substrate that present in tannic acid.

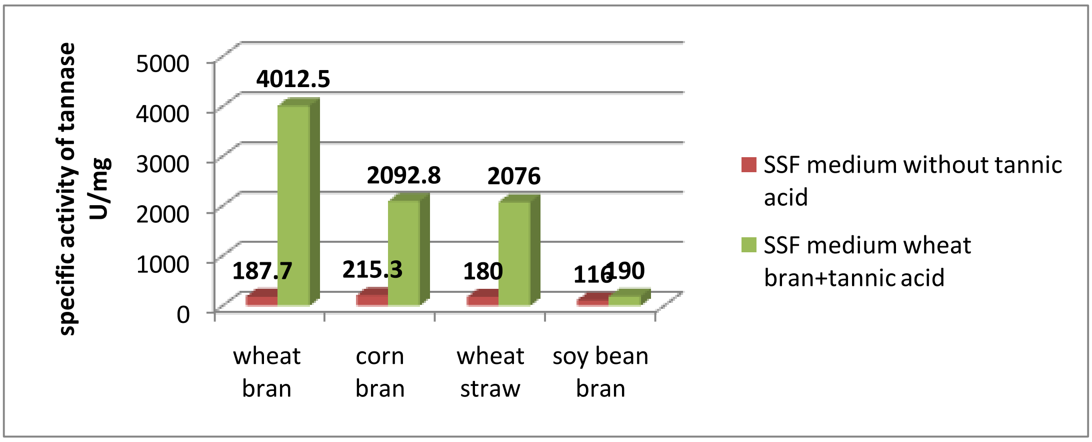

Figure 3-Tannase production from *A.niger* Ass19 in different SSF medium at 28C for 72hr.

By replacing agricultural residues containing tannins(tea leaves, Punica granatum peals,Acacia nilotica barks) with tannic acid , the result showed that tea leaves had the higest levels of tannase production reached to 3382.5 U/mg (figure 4).

Pomegranate rind, jamun bark and amaltash leaves also supported tannase production they were the optimum substrates for tannase production under SSF from *Trichoderma harzianum* MTCC 10841 [22] . *Rhizophora apiculata* bark , a tannin-rich waste material, was used for *Aspergillus niger* tannase production under SSF[23].

While the selection of a substrate for enzyme production by fermentation depends on several factors:i.e: cost,availability and stability of the substrate for obtained the desired product of fermentation therefore tannase production was examined for suitable substrate with the optimum SSF (which was wheat bran)and without Solid state as in figure 4.

Others explained that maximum tannase production was reported at 65% moisture level in Aspergillus niger cultures. The effect of hydration ratio was studied by others ,some results showed that the most active hydration ratio was 60% for *Aspergillus flavas* tannase [28] while 1:1 was the most active hydration ratio for tannase production under solid state fermentation from *Bacillus subtilis* [29].

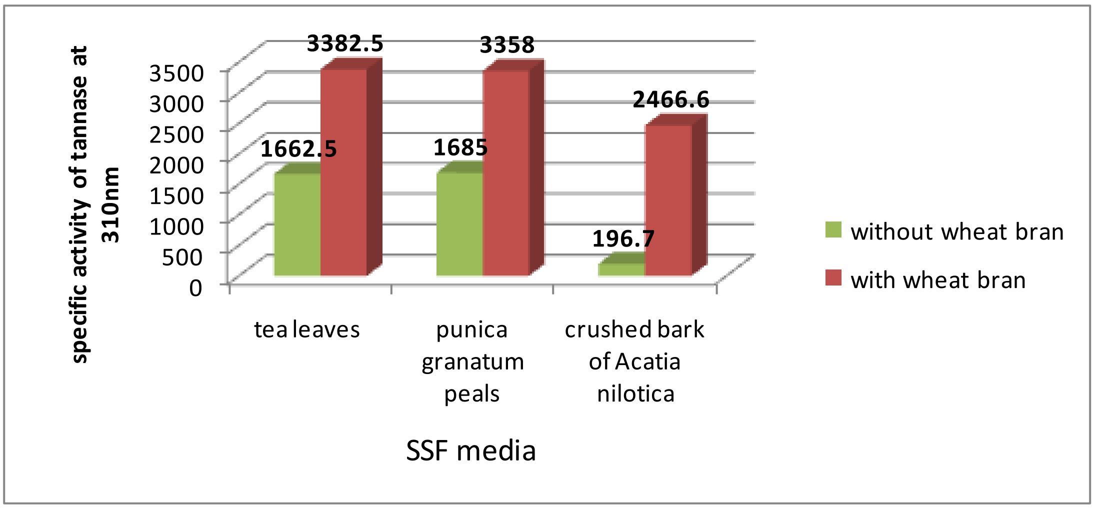

Figure 4- Production of tannase from *A.niger* Ass19 cultured onto solid state fermentation media incubated at 28C for 72hr

Effect of moisture ratio (solid state/buffer contents) on tannase production:

The enzyme production gradually increased with increase in moisture level and then it suddenly showed decrease in the production of enzyme.The maximum activity was recorded 1:3(6145U/mg) fig4 .At lower and higher initial moisture levels ,the metabolic activities of the culture and consequently product synthesis were variously affected .This could be explained by the fact that lower moisture levels leads to reduce solubility of the nutrients in the solid substrates, a lower degree of substrates swelling,and higher water tension .Similarity ,higher moisture contents were reported to cause decreased porosity ,loss of particulate structure ,development of stickness ,reduction in gas volume,decrease gas exchange ,and enhanced formation of aerial mycelium[10]. Among the several factor that are important for microbial growth and enzyme production under SSF (wheat bran) using a particular substrate (tea leaves residues),moisture level (content)/water activity is one of the most critical factors. Because ,solid state fermentation processes are different from submerged fermentation culturing ,since microbial growth and product formation occurs at or near the surface particle having low moisture content thus ,it is crucial to provide optimized water level that controls the water activity (aW)[24].

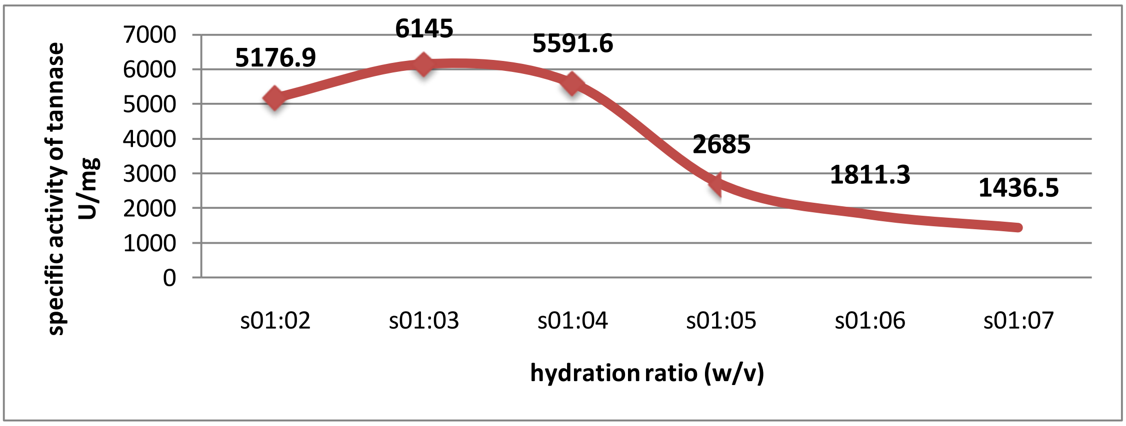

Figure 5- Tannase production in wheat bran with tea leaves (SSF) hydrated with different ratios of citrate buffer 0.05M pH 5.5 and incubated at 28C for 72hr

Effect of duration on tannase production

[As in figure 6]Best selected combination was incubated for 24hr to 168 hr and enzyme activities were measured to optimize the incubation time.The activity of enzyme increased upto 3rd day i.e 11026.6U/mg, but further incubation showed decrease in the activity of enzyme. The reduction in enzyme yield after optimum peroid was probably due to depletion of nutrients available to *A.niger*.

other repotes showed that Some reports showed that maximum tannase production appeared after 120hr [25] others explained that maximum tannase production reached its maximum peak after 24hr[26]. while another report observed that maximum tannase activity was at 7th day (168hr) of incubation [27].

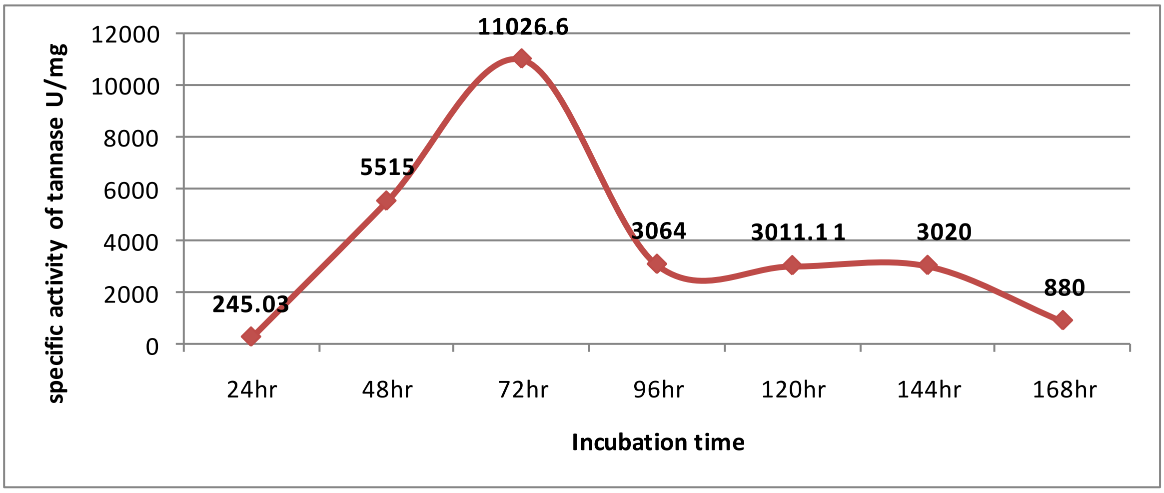

Figure 6- Tannase production under solid state fermentation at different incubation peroids,the medium was wheat bran with tea leaves moistured with 1:3 (w/v) with citrate buffer 0.05M pH 5.5 and inoculated with $2*10^8$ fungal spore suspension then incubated at 28C for 72hr.

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