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PRODUCTION AND CHARACTERIZATION OF TANNASE ENZYME BY ASPERGILLUS NIGER UNDER SOLID STATE FERMENTATION USING FRUIT PEELINGS (ORANGE AND BANANA PEELS)

Anum Shahbaz[*1], Aneela Tabassum[2], Sara Mahmood[3], Dr Afsheen Aqeel[4], Qaiser Ali Sultan[5], Yasmin Khanam[6], Rida Sattar[7], Kiran Ghafoor[8]

*[*1]MS environmental Science, Lahore college for women university, Lahore, Pakistan*
[2]Department of Zoology, University of Education Bank Road Campus Lahore, Panjab, Pakistan
[3]Instutite of Microbiology, Government college university Faisalabad, Pakistan
[4]Department of microbiology, University of Karachi, Pakistan
[5]Pakistan Standards and Quality Control Authority, Peshawar, KPK, Pakistan
[6]Assistant Director, Standards Development Centre, Pakistan Standards and Quality Control Authority (PSQCA) - Ministry of Science and Technology.
[7]Department of Pathobiology, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan
[8]Department: Microbiology and Molecular Genetics, University of Okara, Pakistan

[*1]anamrehman492@gmail.com; [2]aneela.tabassum.vunwl@gmail.com; [3]saramahmood@gcuf.edu.pk; [4]afsheenaqeel@gmail.com; [5]nakhuda@aup.edu.pk; [5]nakhudasolo@gmail.com; [6]yasmin_khanam@yahoo.com; [7]ridasattarabdulsattar@gmail.com; [8]kiran.ghafoor.microbiologist@gmail.com

[5]ORCID 0000-0001-6479-176X

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Corresponding Author: *
Anum Shahbaz

Abstract

Tannase (tannin acyl hydrolase) is a hydrolytic enzyme widely used in food and medical industries. Pure tannic acid is currently used for industrial scale production of tannase which is expensive and adds on to the cost of the final product. The present study focuses on utilization of fruit peelings (banana and orange peels) as a cheap substrate for producing tannase through solid state fermentation by using Aspergillus niger. Fermentation parameters like temperature, pH and fermentation period etc were optimized for enzyme production. The maximum yield (23.3U/ml/min) of tannase was obtained by using banana peels substrate at 35°C, pH 5 and an incubation period of 96 hrs by using Aspergillus niger. After optimization tannase was partially purified by ammonium sulphate fractionation. Partially purified enzyme was characterized by considering the effects of different parameters on enzyme activity. Highest activity of partially purified tannase was found at temperature (35°C), incubation time (25min),pH (5) and tannic acid concentration (0.7%w/v) with substrate of banana peels .The purified enzyme showed maximum activity at the Vmax of 0.83U/ml and Km of 0.155 g/ml when orange peels was used as a substrate using Aspergillus niger.

INTRODUCTION

Tannase (tannin acyl hydrolase) is a hydrolytic enzyme widely used in food and medical industries. Tannase is an inducible enzyme sequentially acts on hydrolysable tannins (e.g. tannic acid) to cleave depside bonds followed by hydrolysis of ester bonds which liberates glucose and gallic acid (Beniwal *et al*, 2013).

Microbial tannase is ubiquitous (Lekha and Lonsane, 1997), having widespread occurrence in fungi (Aguilar *et al*., 2007) and is less common in bacteria (Belur *et al*. 2010) and yeasts (Belmares *et al.* 2004). Microbial tannase is favored also because of the microbes can be subjected to genetic manipulation more readily than plants and animals, resulting in an increase in tannase production (Aguilar and Gutierrez-Sanchez, 2001; Purohit *et al*., 2006).

Fungi are the most studied microorganisms for tannase production. Fungi have the ability to degrade tannins as a sole carbon source (Aguilar and Gutierrez-Sanchez, 2001). Among fungi, Aspergillii and Penicillium are reported to be the major producers of tannase through submerged and solid state fermentation (Kumar *et al*., 2007; Paranthaman *et al*., 2008).

Industrially the production of tannase is usually performed via Submerged Fermentation (SF), however this method can be quite costly for the industry, especially the demand for large quantities of water. Alternatively, to minimize production costs, there is the solid-state fermentation (SSF) (Pandey, 2000).

Solid state fermentation (SSF) is a batch process used for its many advantages such secreted enzymes in the culture filtrate are more concentrated and of higher quality, with a less effluent generated and lower a lower cost of recovery than in the liquid state cultures (Pandey, 1994).

In addition to gallic acid production, tannases have potential for applications in the food, feed, beverage and brewing, pharmaceutical and chemical industries. Tannase has been widely used in the clarification of beer and fruit juices, manufacture of coffee flavored soft drinks, and improvement in flavor of grape wine, and as an analytical probe for determining the structure of naturally occurring gallic acid esters (Seth, Chand *et al.*, 2000). 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).

The present day trend is the utilization of waste material for production of byproducts which boosts up high economic returns in many industries. With the advent of biotechnology, attempts have increasingly been made globally to make potential use of fruit peelings and agricultural residues for value addition by production of enzymes, The present study is proposed to assess potential use of banana and orange peelings for the production of tannase, which are very cheap and readily available substrate in Pakistan.

Materials and Methods:

Microorganisms and Maintenance of Culture

The present strain of *Aspergillus niger* was collected from Institute of Agriculture Sciences (IAGS), University of Punjab, Lahore.

Aspergillus niger and *Aspergillus flavus* culture was maintained on potato dextrose agar media (PDA) slants.4g of potato-dextrose agar (PDA) was measured in a flask and dissolved it in 100ml of distilled water. After mixing it with distilled water, the medium was kept in autoclave for 15 minutes at 121 and 1.05kg/cm^2 pressure for sterilization.

PREPARATION OF SLANTS

The culture of *Aspergillus niger* and *Aspergillus flavus* was propagated on potato dextrose agar (PDA) slants separately. Sporulation medium was poured into culture test tubes and autoclaved for 15 minutes at 121 and 1.05kg/cm^2 pressure for sterilization. Then tubes were laid in the slanting position for solidification of the medium. The slants were inoculated with spores of *Aspergillus niger* and finally placed in incubator at 25 - 30 for 5- 6 days. Fully mature slants were stored at 4and re- cultured once a month.

Inoculum Preparation

Fungal spore inoculum was prepared by using Vogel's medium.Vogel's salts were weighed and mixed in distilled water one by one to prevent possible precipitation in a conical flask. The pH of

the media was adjusted with 1M HCl/1M NaOH and volume was made (50ml/flask) with distilled water. Glass beads (6-8 in numbers) were added in 250 ml flasks to break the mycelial pellets to form uniform suspension. The flasks were plugged with and autoclaved at 121 and 1.05 kg/cm^2 pressure for 15 minutes. After cooling to ambient temperature, 2ml of sterilized 50% (w/v) stock solution of glucose were aseptically added to the autoclaved Vogel's medium as carbon source.The test flasks containing autoclaved Vogel's media were inoculated with a loop full of *Aspergillus niger* under aseptic conditions. They were allowed to grow at 30±2 on a rotary shaker (120 rpm) for 42 hr.

Substrate Preparation

Fruit peels of banana and orange and leaves of neem and dharek were spread on the roof and sun dried for 48 hr. After sun drying the substrate were collected and oven dried at 70°C for 24 hr. Oven dried substrate was grounded to powder form in electrical grinder and sieved. After that finely grounded powder of substrate was stored in polyethylene bags separately at room temperature (30±5°C).

Production of Tannase under Solid State Fermentation

A ten 10g substrate of orange peel powder and banana peel powder was taken in 250 ml Erlenmeyer flask separately and both substrates were moistened with 10 ml of salt solution.After adding 10 ml of salt solution in each flask and these flasks were plugged with cotton and sterilized by autoclaving at 121°C and 1.05 kg/cm^2 for 20 min. After cooling to room temperature each flask was inoculated with 1 ml of prepared spore inoculum, mixed well and incubated statistically for 120 hr at 30 °C.

Determination of Optimum Conditions for Tannase Production under Solid State Fermentation

Three factors (temperature, pH and incubation period) were considered in order to determine the optimum conditions for tannase under solid state fermentation at different concentrations by using peels of banana and orange as a source of raw tannin.

Extraction of Crude Enzyme

Add 50ml of distilled water containing 0.01% Tween 80 into fermented substrate. Contents were mixed well for 30 min on rotatory shaker .Mash was filtered through muslin cloth. Crude enzyme was separated from fermented matter by centrifugation at 5000 rpm at 4°C for 20 min in a refrigerated centrifuge. After centrifugation filtrate was collected in capped bottles and preserved for further studies.

Assay of Tannase Activity

Tannase was assayed following the method of Mondel *et al* using tannic acid as substrate at concentration of 0.2 M acetate buffer (pH 5.5).0.3 ml of 0. 5%(w/v) tannic acid was prepared in 0.2 M acetate buffer pH 5.5 was taken in a test tube.0.05 ml of enzyme extract was also added in same test tube and incubated at 60°C for 20 min.In order to stop the enzymatic reaction 3ml BSA solution was added which also precipitated the remaining tannic acid. In the same way reference tube was prepared with heat denatured enzyme. Tubes were centrifuged at 5000 rpm for 20 min at 4°C.After centrifugation resultant precipitate was dissolved in 3 ml SDS-triethanolamine solution. Then 1ml of $FeCl_3$ reagent was added and kept for 15 min for stabilization of color. The absorbance of both tubes was measured at 530nm against the blank (without tannic acid). One Unit of the tannase enzyme was defined as "the amount of enzyme that hydrolyzed 1 ml of substrate tannic acid in 1 minute under the assay condition"

Protein Estimation

Proteins were estimated using Bradford Method. Bovine serum albumin (BSA) was used as standard.

Protein assay for enzyme solution:

Proteins in unknown sample were estimated by adding 100µL of enzyme in test tube containing 1ml of Bradford reagent. The absorbance was measured at 595 nm under the same conditions as done for the standard.

Protein concentration (mg/ml) = O.D (595nm)/ (slope)

Partial purification of tannase

15 ml of crude tannase was precipitated with different concentration of ammonium sulphate and vortimixed tubes for saturation under cooling condition. Then these tubes were centrifuged at 5000 rpm for 20 min. The supernants were assayed for tannase activity .After optimization of ammonium sulpahte precipitation, the remaining enzymatic concentrates were placed in ice and solid ammonium sulfate was dissolved bit by bit to attain initially 60% saturation in case of banana and orange peels substrate at O°C.

After gentle vortimixing the pallet of precipitated protein was discarded .In supernants more crystal of ammonium sulphate were added to attain 80% saturation in case of peels substrate and 75 % saturation in case of leaves substrate at 0°C.It was again kept for a night at 4°C and centrifuged as previously. This time pallet was collected and supernants was discarded. The pallet was dissolved in acetate minimum amount of 0.2 M acetate buffer and assayed.

Dialysis of Precipitated Protein

Precipitated proteins were transferred into dialysis tube using micropipette and dialyzed against 0.2 M acetate buffer (pH 5) for 1 day. The buffer was stirred through magnetic stirrer to enhance solute exchange .Dialysis was conducted over night and the buffer was changed several times to increase the efficiency of dialysis. The dialyzed solutions were assayed for enzyme activity.

Enzyme Characterization

The effect of different temperature, pH, and substrate concentration and incubation time on enzyme obtained after partial purification was studied. All experiments were done in triplicate and the mean values with standard error are reported.

Determination of Km and Vmax:

Km and Vmax were determined by plotting velocity against substrate concentration [S]. Following Michaelis-Menten equation was used to determine the value of Km:

V=Vmax[S]/Km+[S]

Reciprocal

1/V= [Km/Vmax] ×1/[S] +1/Vmax

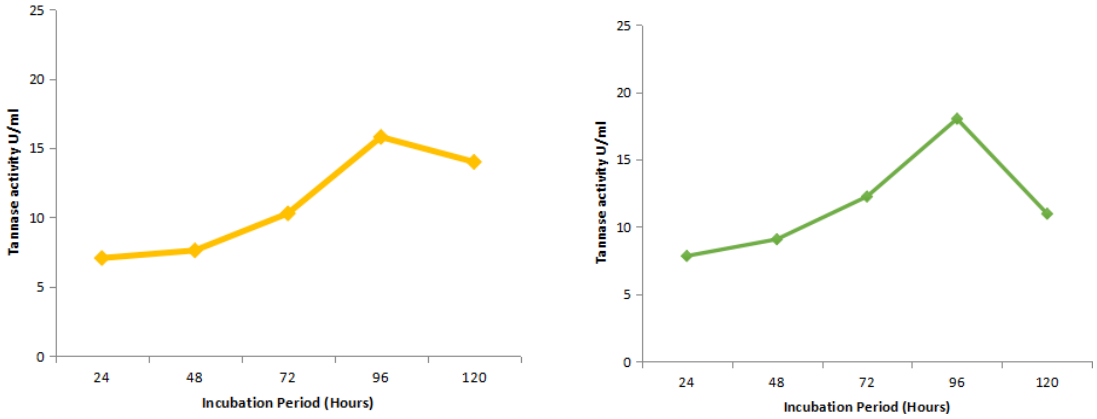
Results and Discussion:

In present work, study on the production of tannase from *A.niger* was carried out .Effect of different parameters was also observed on the production rate. Results are given in form of graphs.

Optimization of some parameters for maximum production of tannase

1-Effect of incubation period

The maximum tannase productivity was observed at the end of 96 h of incubation by using banana peels with an activity of 15.8U/ml/min and 18.02 U/ml/min enzyme activity was observed with using orange peels as substrate by *Aspergillus niger*. The decrease in enzyme activity after 96 h might be due to reduced nutrient level of medium as explained by Gautam *et al.* (2002).

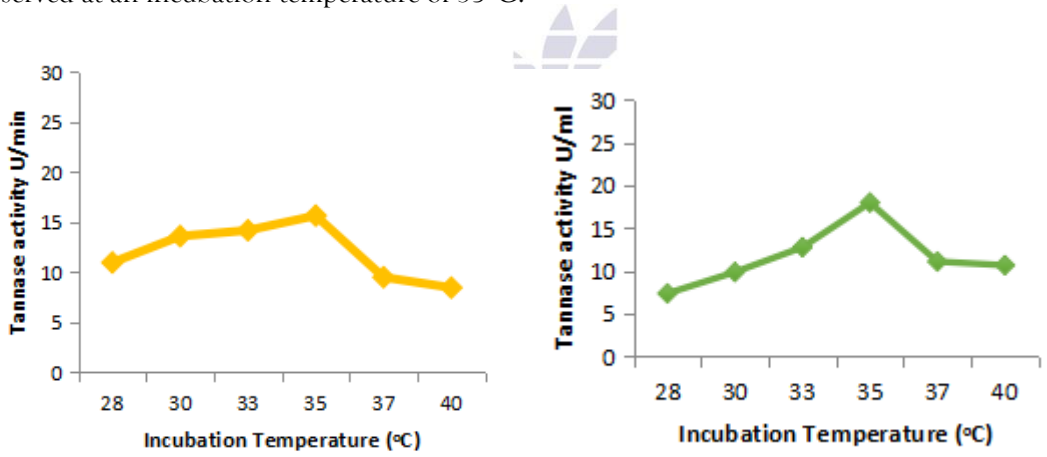


BANANA PEELS ORANGE PEELS

Figure 1: Effect of different incubation period on Tannase production by *Aspergillus niger* fermented with banana and orange peels substrate respectively

2-Effect of optimum temperature

The optimum temperature for tannase was found to be 35 °C, at which the enzyme productivity was highest i.e. 15.63 U/ml/min in banana peels and 17.96 U/ml/min in orange peel by using *Aspergillus niger*. Results are in accordance with Paranthaman et *al.* (2009) who reported that maximum tannase produced by *A. niger* was observed at an incubation temperature of 35°C.

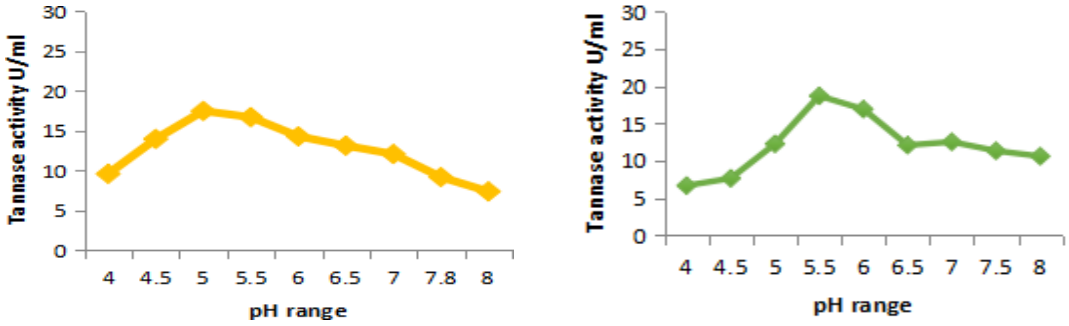


BANANA PEELS ORANGE PEELS

Figure 2: Effect of different incubation temperature on Tannase production by *Aspergillus niger* with fruit peels substrate respectively

3-Effect of optimum pH

The optimum pH values for the enzyme activity were found to be 5.0 with banana peels as solid substrate (17.47 U/ml/min) while with orange peels tannase production by *Aspergillus niger* was observed highest at 5.5 that was 18.68 U/ml/min. Similarly, Lekha and Lonsane (1997) indicated that optimal pH is around 5.5 and suggested that, any further increase in pH leads to decline in tannase production.



BANANA PEELS ORANGE PEELS

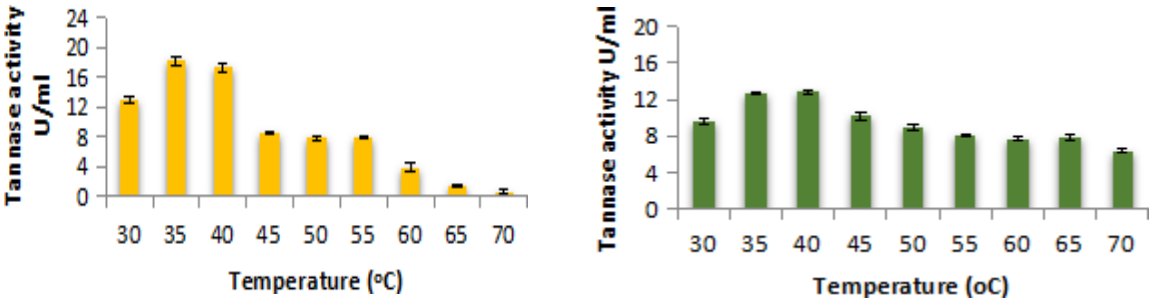
Figure 3: Effect of different pH range on Tannase production by *Aspergillus niger* fermented with fruit peels substrate respectively

Characterization of Partially purified enzyme

After partially purification of enzyme through ammonium sulphate precipitation method it is used for determining temperature stability, pH stability, and effect of substrate concentrations and incubation time on purified tannase .

1-Temperature optima of partially purified tannase

Partially purified tannase enzyme that was produced by *Aspergillus niger* in solid state fermentation media containing orange peels and banana peels showed highest activity at 40 °C that was 12.83 U/ml/min and 17.33 U/ml/min respectively.An increase in temperature beyond the optimum value caused a decrease in the catalytic rate of tannase as either the enzyme or substrate became denatured and inactive (Mukherjee and Banerjee, 2006).

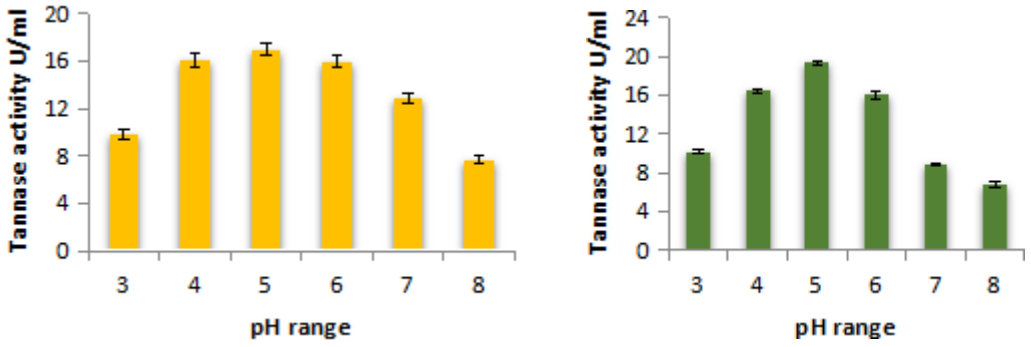


BANANA PEELS ORANGE PEELS

Figure 4: Temperature optima of partially purified tannase that produced by *Aspergillus niger* that allowed growing on fruit peels under solid state fermentation

2- pH optima of partially purified tannase

Enzymes are very sensitive to changes in pH and they function best over a very limited range, with a definite pH optimum. According to present study the pH stability for the partially purified enzyme was up to pH 5 i.e. 16.95 U/ml/min with banana peels and 19.35 U/ml/min with orange peels by *Aspergillus niger.*

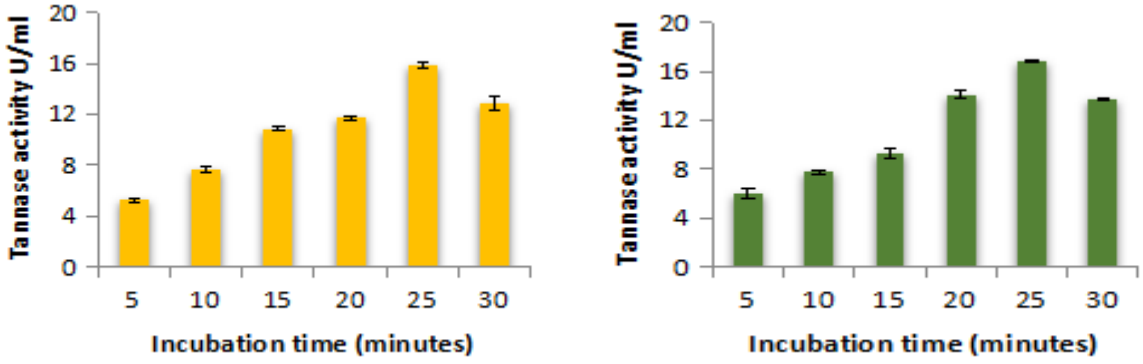


BANANA PEELS ORANGE PEELS

Figure 5: pH optima of partially purified tannase that produced by *Aspergillus niger* that allowed growing on fruit peels under solid state fermentation

3-Effect of incubation time on partially purified tannase

Enzyme activity of purified tannase that was produced by *Aspergillus niger using* solid media of orange peels and banana peels in fermentation were found to be optimum after 25 min of incubation time.

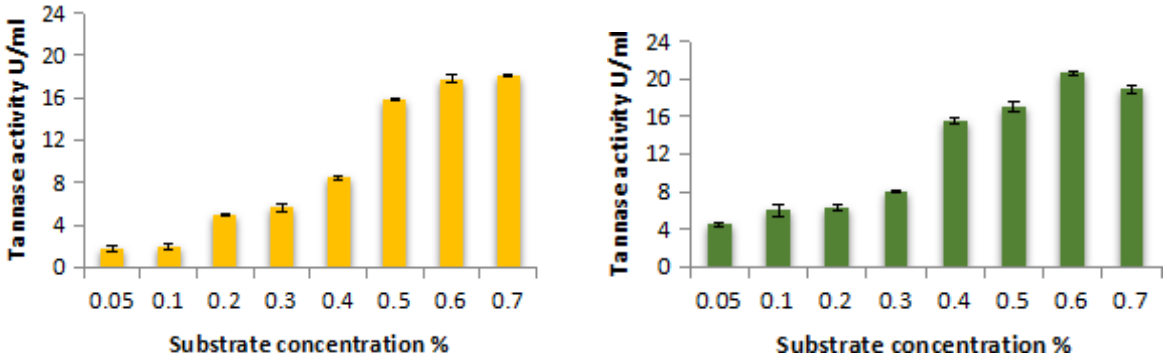


BANANA PEELS ORANGE PEELS

Figure 6: Effect of different Incubation time of partially purified tannase that produced by Aspergillus niger that allowed growing on fruit peels under solid state fermentation

4-Effect of different substrate concentration on partially purified tannase

The highest activity of tannase for stain *Aspergillus niger* that allowed to grow on banana peels and neem leaves respectively was achieved at 0.7% concentration of tannic acid that showed activity of 8.11 U/ml/min. At 0.6% concentration of tannic acid as substrate the activity of tannase enzyme was highest for stain *Aspergillus niger* that allowed growing on orange peels respectively with activity of 20.88 U/ml/min. Excessive tannic acid may act as repressor and prevents synthesis of mRNA. In addition, the increase of tannic acid causes an increase of heat building up and reduces aeration which in turn may decreases productivity of tannase (Banerjee *et al.*, 2005).



BANANA PEELS ORANGE PEELS

Figure 7: Effect of different substrate concentration of partially purified tannase that produced by

Aspergillus niger that allowed growing on fruit peels under solid state fermentation

5-K_m and V_{max} of enzyme

The V_{max} value was determined by Michaelis-Menten equation that was 0.58 U/ml and the Km value of tannase enzyme produced from *Aspergillus niger* by using banana peels as a substrate was found to be 0.831 g/ml. Tannase from *Aspergillus niger* had a calculated Vmax value of 0.83 and Km value of 0.155 for orange peel as substrate.

Conclusion:

The present work has been taken up with a view of exploring the possibilities of using peels of orange and banana as a substrate and *Aspergillus niger* as a microbial source for the production of tannase. The present investigation suggests that fruit peels such as orange and banana peels can be one of the best and cost effective alternatives to the costly pure tannic acid for industrial production of microbial tannase. From the results it was concluded that orange peels substrate was found to be more preferable for tannase production by *Aspergillus niger* as compare to banana peels substrate with at highest activity of 11.67 U/ml/min.

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