STUDIES ON THE PRODUCTION OF TANNASE BY *STAPHYLOCOCCUS AUREUS*.

Danjuma Isah, Aliyu Shugaba, Mohammed Adamu Milala

Department of Biochemistry,
University of Maiduguri, Maiduguri, Nigeria
Email: isahdanjuma2003@gmail.com

Abstract: Tannase is produced in the presence of tannic acid by different bacteria, examples *lactobacillus, staphylococcus aureus* and *yeasts,* is an inducible extracellular microbial enzyme: This study assessed the production of tannase by *staphylococcus aureus* in submerged fermentation (SMF) with tannic acid as substrate. The *staphylococcus aureus* specie was isolated and maintained on nutrient agar. Tannase production by *staphylococcus aureus* was screened for 120hours (5days) post-inoculation. The crude enzyme was used to determine the effect of pH, temperature and stability ($t_{1/2}$). The pH, temperature and incubation period optima of tannase production were found at 6.0, 50C and 24hrs. *Staphylococcus aureus* exhibited highest enzyme activity of 3.81U/min conversion respectively after 24h incubation period with 2.0g tannic acid as substrate. The enzyme was stable at temperature of 50°C for a period of 9hours. The half-life ($t_{1/2}$) value of enzyme was 3.17hours.From the result, tannase from *staphylococcus aureus* may be suitable and favorable for industrial application where operational temperature is 50°C and below.

Keywords: Tannase, Tannic acid, *Staphylococcus aureus*, SMF

INTRODUCTION

Tannin acyl hydrolase (EC 3.1.1.20), commonly referred to as tannase, is an important enzyme with various industrial applications. It is an extracellular hydrolase enzyme that is induced in the presence of tannic acid by various bacteria, examples by *lactobacillus* and *staphylococcus aureus* (Ayed and Hamdi; 2002) and Yeasts (Aoki e*t al;* 1976), (Belmares *et al.,* 2004; Ramirez-Coronel *et al.*, 2003). It catalyzes the hydrolysis of ester bonds in hydrolysable tannins such as tannic acid and gallic acid esters (Belmares *et al.,* 2004), releasing glucose and gallic acid (Banerjee, 2005). Tannase is extensively used in the preparation of instant tea, to improve the flavour of grape wine, for the clarification of beer and fruit juices, in coffee-flavoured soft drinks, and in the production of gallic acid (Aguilar *et al.,* 2001). Gallic acid can be used in the manufacture of ordinary writing inks and dyes, as a photographic developer, in the enzymatic synthesis of propyl gallate, in the tannery industry for homogenization of tannins, and for the production of pyrogallol and gallic acid esters (Banerjee *et al.*, 2005). However, currently, the most commercial application of tannase is in the manufacture of

instant tea, where it is used to eliminate water-insoluble precipitates (called "tea cream") (Aguilar *et al.*, 2001). Tannase can be obtained from plant, animal and microbial sources. The most important method to obtain the enzyme is via a microbial process, because the produced enzymes are more stable than similar ones obtained from other organisms (Murugan *et al.,* 2007). Of all microbes, filamentous fungi of the *Aspergillus* and *Penicillium* genus, however, constitute the major source.

These organisms are also used in the majority of research work (Belur *et al.*, 2011). The fungal species of *Aspergillus* and *Penicillum* are the most active microorganisms known, capable of producing tannase through submerged and solid state fermentation (Abdel-Nabey*et al.*, 2011). The use of submerged fermentation (SMF) is advantageous due to its better process control, and simple sterilization method (Mahapatra and Banerjee, 2009; Selwal *et al.,* 2012).
The present paper, reports the production of tannase from isolated fresh culture of *Staphylococcus aureus* in liquid culture medium by submerged fermentation technique.

Materials and Methods

Chemicals

All chemicals used were of analytical grade.

Isolation of Microorganisms

The microorganisms used for the study were of two species: *Lactobacillus* and *Staphylococcus aureus,* which were isolated and screened for their ability to utilize tannic acid as a sole carbon source with the subsequent production of tannase. The isolation and identification of the bacteria was carried out in the Veterinary Microbiology Laboratory, Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Maiduguri.

Screening of the Bacteria for Tannase Production

The ability of the bacteria to produce tannase was studied by observing the growth of the organism in the reaction flask, the change in pH and the change in dry weight. The reaction flask contained tannic acid in concentration of 0.5mg/ml, 1.0mg/ml, 1.5mg/ml and 2.0mg/ml dissolved in 1000ml of distilled water and the organisms was inoculated. The bioreactor was then allowed to ferment for 5 days, observing on daily with regards to the incubation time, the incubation temperature, the change in pH, concentrations of tannic acid. The growth of the organism was estimated on the basis of biomass dry weight (g/ml), based on this *staphylococcus aureus* was chosen for the study because it produced the desired pH and showed maximum growth i.e. it is capable of utilizing tannic acid as a sole carbon source.

Preparation of Culture Medium

The fermentation medium for tannase production contained tannic acid 2.0g dissolved in 1L distilled water. The culture medium (pH6.0) was sterilized at 121°C in a clinical autoclave for 20 minutes. After sterilization the flask was inoculated with the pre-inoculum *(staphylococcus aureus*) and then maintained on agar slants supplemented with 1 % (w/v) tannic acid as the sole carbon source.

Determination of Tannase Activity

Colorimetric assay was used to determine tannase activity, based on measuring the residual tannic acid content after enzymatic reaction as described by (Mondal *et al*;2001). 0.1 ml of enzyme solution was incubated with 0.3 ml of 1.0% (w/v) tannic acid and add 5 ml of 0.2 M citrate buffer (pH 6.0) at 50 °C for 10 min. The enzyme reaction was terminated by addition of 3ml of egg albumin solution, leading to precipitation of the remaining tannic acid. It is then centrifuged at 5000xg for 10 minutes at room temperature and precipitate was redissolved in 2ml of sodium dodecyl sulfate (SDS)-triethanolamine (1%w/v) SDS in 5%v/v triethanolamine) solution. The absorbance was measured at 530nm after addition of 1ml of $FeCl_3$ (0.13mol/l).One Unit of tannase enzyme was defined as the amount of enzyme which is required to hydrolyze 1mmol/L of ester linkage of substrate tannic acid in 1 minute at 50°C and pH 6.0.

Enzyme Characterization

Effect of Incubation Period

The tannase activity was assayed at different incubation period 24hrs, 48hrs, 72hrs, 96hrs and 120hrs with different substrate concentration.

Effect of pH

The tannase activity was assayed at different pH. The crude tannase enzyme was incubated at 50°C with buffers at various pH ranging from 4 to 9 at 30 minutes. Enzyme activity was determined as described by Mondal *et al*, (2001).

Effect of Temperature

The activity of tannase was assayed at different temperature range of 25-80°C. The crude tannase enzyme was incubated for 30 minutes. Assay of enzyme activity was determined as described above.

Stability of the tannase enzymes

Stability of the crude tannase enzyme was tested for stability studies by pre-incubating the enzyme solution at the optimum temperature (50°C) for 9hrs and assayed as described above at 30 minutes interval.

Results

The effect of incubation period (hr) (Fig.1) on tannase production by *staphylococcus aureus,* the incubation period was varied from 24 to 120hrs.The tannase activity began to increase from 0hr with increase in tannic acid concentration and optimum tannase activity of 3.81U/min was attained at 24hrs.As the incubation period increase enzyme activity started declining from 48hrs to 96hrs and relatively increase at 120hrs, though not up to that of 24hrs, as can be seen in Fig 1.

The effect of pH on tannase activity was varied from 4.0 to 9.0 (Fig.2), using 1N citric acid and 1N sodium citrate for pH 4 and 5; I N NaH_2PO_4 and 1N Na_2HPO4 for pH 6 to 9. The enzyme was active at slightly acidic pH of 6.0, with an optimum tannase activity of 2.96U/min (Fig.2).

The effect of temperature on tannase activity was varied from 25 to 80°C (Fig.3). With an increase in temperature, the tannase production increased and optimum activity of 2.96U/min at 50°C.

The stability of the tannase enzyme was determined at 30 minutes interval at 50°C for 9hrs.Activity started decreasing from 1hr up to the last hour. The stability of the tannase enzyme at 50 °C gave the half-life of 3.17hours as can be seen in Fig.4.

DISCUSSION

The effect of incubation period (hr) (Fig.1) on tannase production by *staphylococcus aureus,* the incubation period was varied from 24 to 120hrs.The tannase activity began to increase from 0hr with increase in tannic acid concentration and optimum tannase activity of 3.81U/min was attained at 24hrs.As the incubation period increased activity started declining from 48hrs to 96hrs and relatively increases at 120hrs,which may be due to accumulation of secondary metabolites. Optimum tannase activity of 3.81U/min in a medium containing 2.0g tannic acid was obtained at 24hrs .This finding corroborates with the works of Vaquero *et al*;(2004), who reported an optimum incubation period of 24hrs for tannase produced by *citrobacter sp.* Also the findings of Inoue and Hagermen, (1988) and Darah *et al*; (2011), who reported an optimum incubation period of 36hrs and 48hrs for tannase produced by A. aculeate and A. niger respectively, were in contrast to the present work. As incubation period increases enzyme activity started declining which may be due to effect of tannic acid on organisms, which when broken down may produces secondary metabolites, which accumulate and inhibit the secretion of tannase.

The effect of pH on tannase activity was varied from4 to 9(Fig.2). The enzyme was active at slightly acidic pH of 6.0, with an optimum tannase activity of 2.96U/min (Fig.2).This finding corroborates to the works of Pourrat *et al; (*1994), who reported an optimum pH of 6.0 in the case of tannase obtained from *P. Chrysogenum* and *A. Niger*. These findings were in contrast to the works of Lekha and Lonsane (1997) who reported that tannases are acidic proteins with an optimum pH of 5.5 and Rakesh *et al*(2007)who reported an optimum pH of 5.5 for tannase production by *A. Ruber.* There are reports of optimal pH of 5.0 for tannase from *A. Awamori* (Mahaptara *et al;* 2005) and pH of 5.5 for the tannase from *A Flavus and A.Oryzae* (Batra and Saxena;2005),which were in contrast to the current finding. The effect of temperature on tannase activity was varied from 25 to 80°C(Fig.3). With an increase in temperature, the tannase production increased and optimum activity of 2.96U/min at 50°C.This finding is in consonance to the works of (Aoki *et al;* 1976), who reported an optimum temperature of 50°C for tannase produced by *Candida sp* which is fungal specie. In the findings of (Mondal and Patil, 2000; Sabu *et al*; 2006), who also reported an optimum temperature of around 30°C and 36°Cfor the tannase by *Bacillus lichenformis and Lactobacilli* respectively which is in contrast with this work. With a further increase in temperature, there was a decrease in enzyme activity because each enzyme has its optimum temperature. Any temperature above the optimum it will eventually disrupt the weak ionic bonding and disulfide bonds between the amino acids in the active site of an enzymes.

The stability of the tannase enzyme was determined at 30 minutes interval at 50°C for 9hrs. Activity started decreasing from 1hr up to the last hour. The half-life ($t_{1/2}$) of the enzyme was determined. The stability of enzyme at 50°C gave a half-life of 3.17 hours as can be seen in the Fig.4 below .Similar finding by were related for tannase from several *A spergillus*sp (Mahapatra *et al.*, 2005) reported that the enzyme was stable at 40° C for more than 2 h, but it was not stable at temperature higher than 45°C, which is in contrast with this work. Thermostability is paramount in industrial application especially in drinks (acron wine), foods (instant tea) and pharmaceuticals (trimethoprim). The crude tannase was subjected to thermostability at 50°C for 9hrs. Half-life is defined as the time taken for half of the original crude enzyme to lose. The half-life of crude tannase obtained is 3.17hours which may suitable and favorable for industrial production. In conclusion, *Staphylococcus aureus* has a good potential of producing microbial tannase enzyme. This specie of bacteria is able to produce tannase in a medium containing tannic acid as the sole carbon source. Another advantage of this specie is that it can produce maximum enzyme within a short period of cultivation.

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ACKNOWLEDGEMENTS

The authors greatly acknowledge Mr. Isa Adamu Gulani, Veterinary Microbiology Laboratory, Veterinary Microbiology Department, Faculty of Veterinary Medicine, University of Maiduguri, Borno State, Nigeria. For the isolation and identification of microorganisms used in the research.

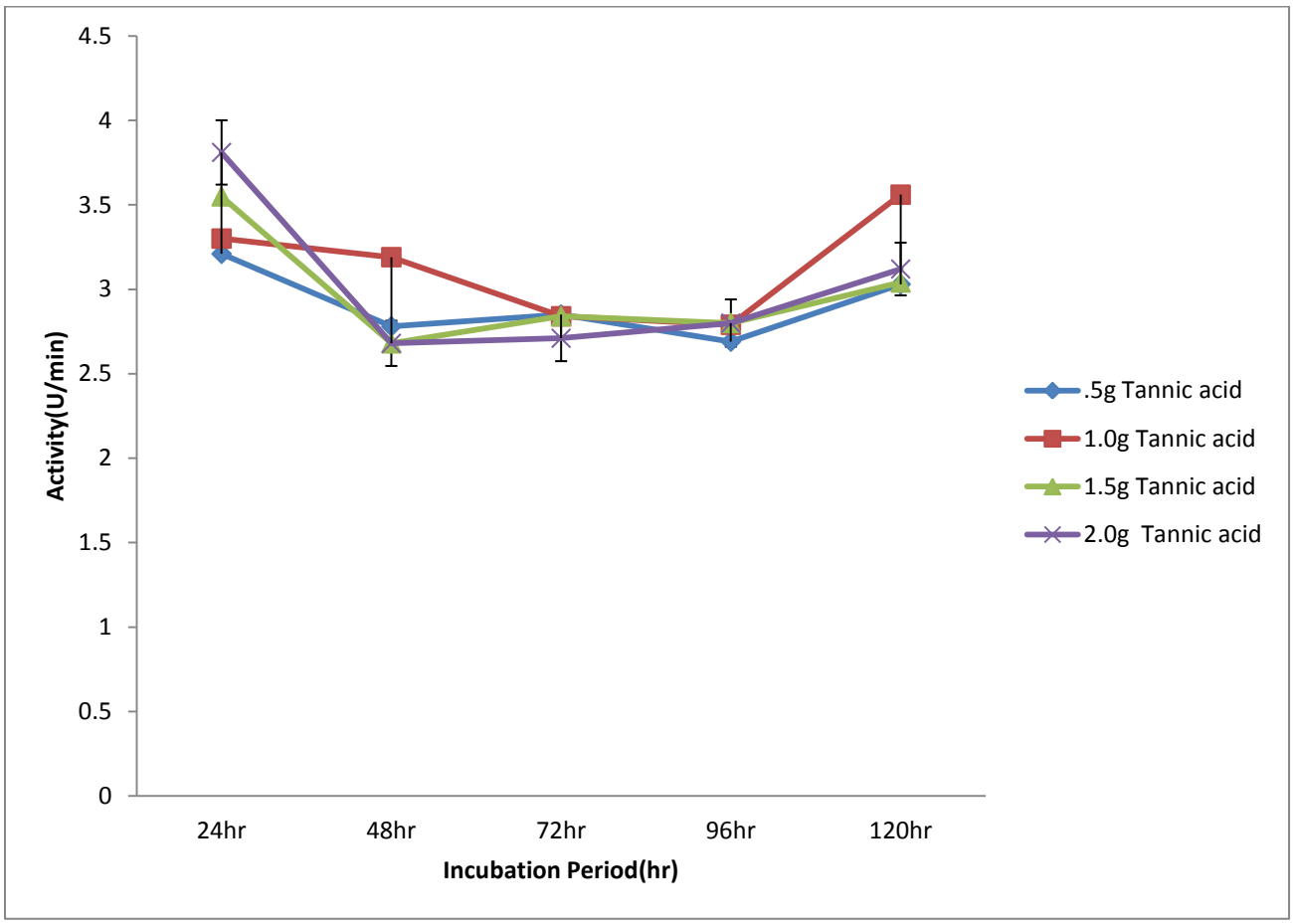

Figure .1 Production of Tannase by *Staphylococcus Aureus*

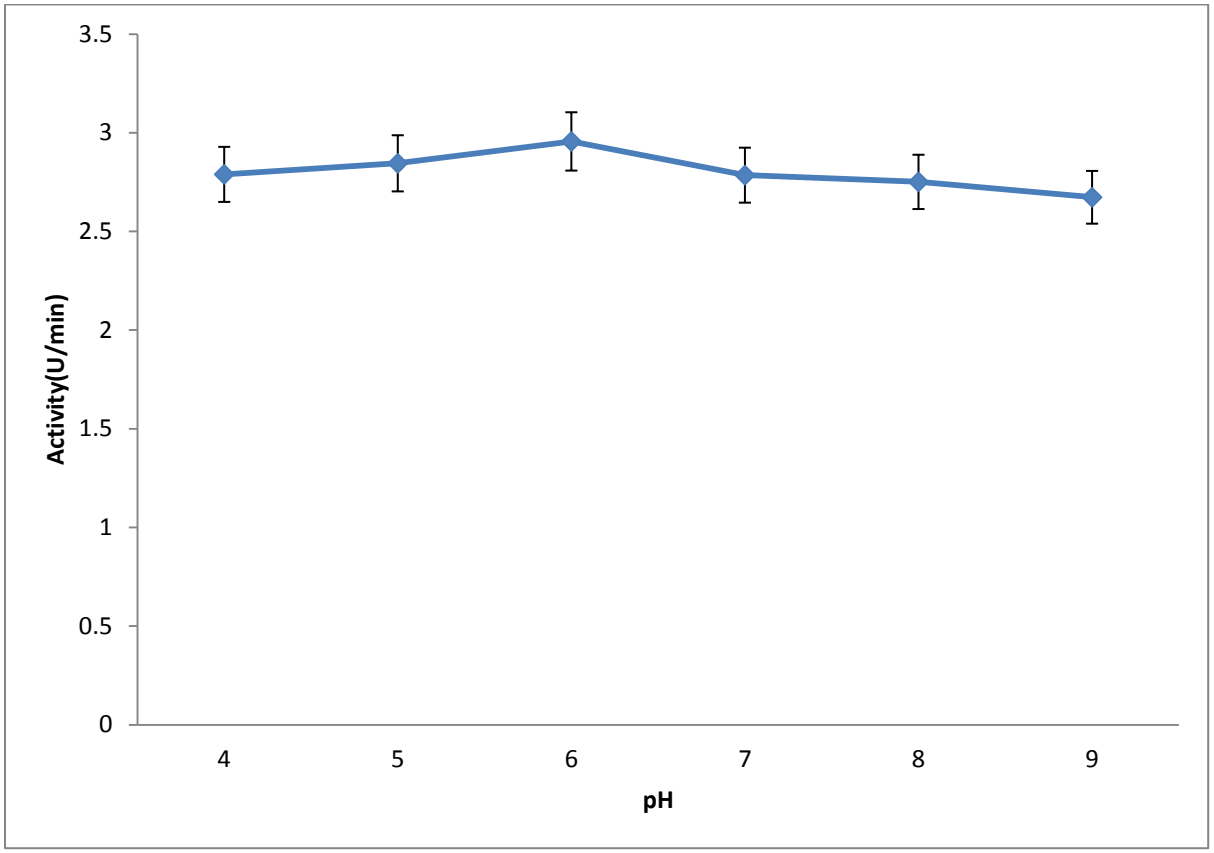

Figure.2. Effect of pH on Tannase Activity by *Staphylococcus Aureus*

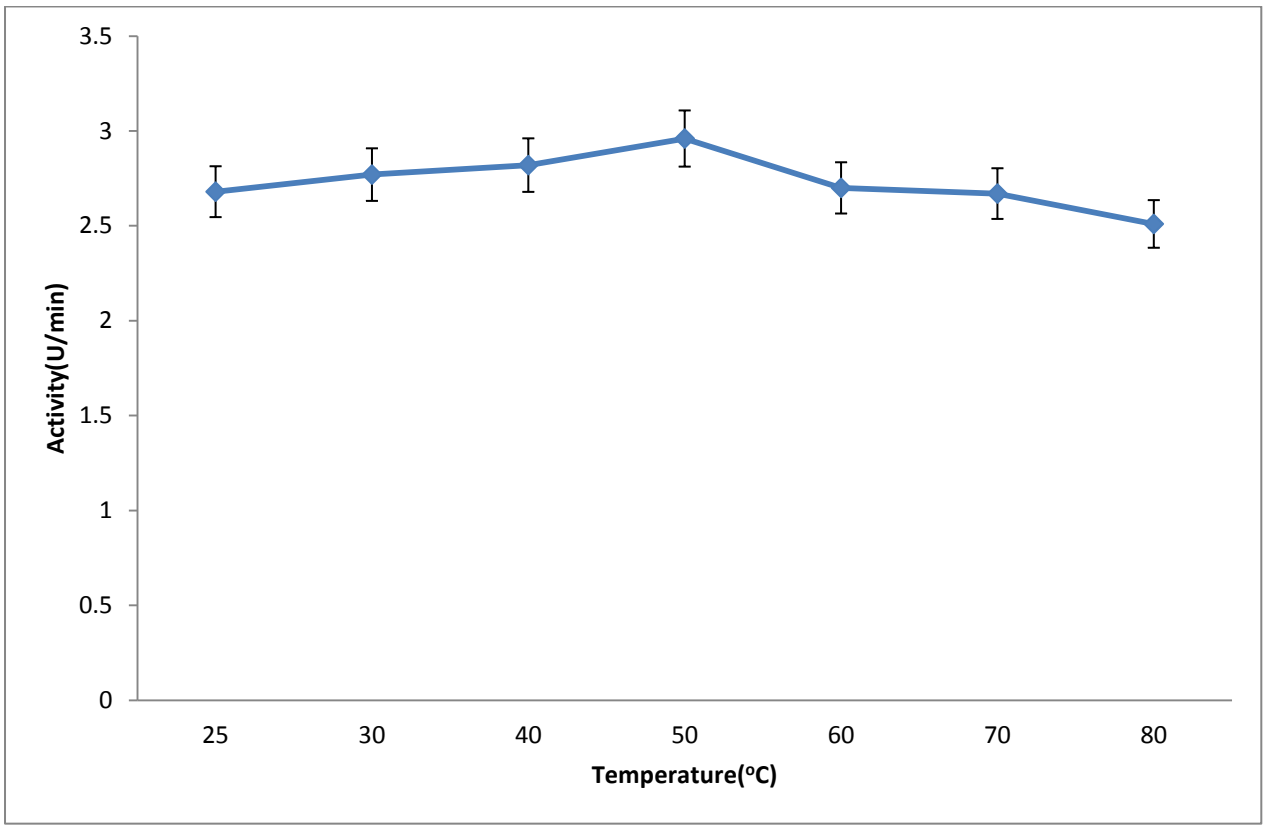

Figure.3. Effect of Temperature on Tannase Activity by *Staphylococcus Aureus*

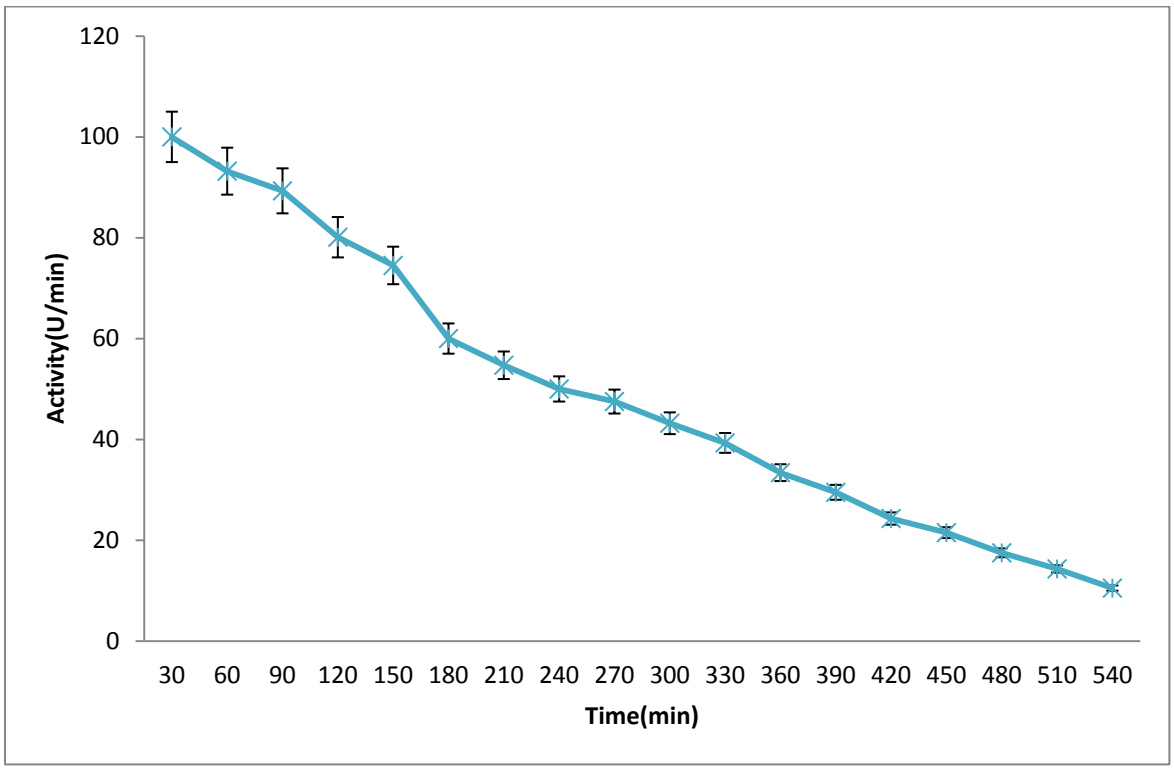

Figure. 4. Stability on Tannase Production by *Staphylococcus Aureus*

Reference to this paper should be made as follows: Danjuma Isah, Aliyu Shugaba, Mohammed Adamu Milala, (2017). Studies on the Production of Tannase by *Staphylococcus Aureus. J. of Sciences and Multidisciplinary Research,* Vol. 9, No. 2, Pp. 21-31