Production of tannase by utilizing Banana wastes as substrate

By

GAAYATHIRI DEVI E

(17PBO006)

Thesis submitted to the

Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-641043

In partial fulfilment of the requirements for the

Degree of Master of Science in Botany

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Signature of the Head of the Department

25/4/19

Signature of Supervisor

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CHAPTER-I

INTRODUCTION

Tannins are naturally occurring secondary metabolites, found in plants and are considered as the fourth most abundant plant constituent after cellulose, hemicellulose and lignin. They are water-soluble polyphenolic compounds, present in different plant parts such as leaves, fruits, bark, wood, seeds and roots. One of the major characteristic of tannins is its ability to form strong complexes with carbohydrates and proteins. Tannins are classified into two major groups, hydrolysable and condensed tannins (Belmares *et al*., 2004), which is based on two differences such as the sugar content and the availability as substrates to tannase. Hydrolysable tannins are compounds containing a central core of glucose called gallotannins surrounded by gallic acid units or with hexahydroxydiphenic acid, or its dilactone form, ellagic acid called ellagitannins. Condensed tannins are oligomers or polymers composed of flavan-3-ol nuclei. They are also called proanthocyanidins, because they are decomposed to anthocyanidins in heated ethanol solutions. The most frequent basic units of condensed tannins are derivatives of flavan-3-ols: (+)-catechin, (-)-epi catechin, (+)-gallo catechin and major polyphenols of green tea: (-)epigallo catechin (EGC) and (-)-epigallo catehin gallate (EGCG). Tannins protect the vulnerable parts of plants from microbial attack by inactivating the invasive microbial extracellular enzymes. Despite the antimicrobial properties of tannins, many fungi, bacteria, and yeast are quite resistant to tannins and can grow and develop on them. (Bhat *et al*., 1998).

Tannin acyl hydrolyses (E.C. 3.1.1.20) is an enzyme that cleaves ester linkages in hydrolysable tannins producing glucose and gallic acid. Tannase catalyses the breakdown of hydrolyzable tannins such as tannic acid, methyl gallate, ethyl gallate, *n*-propylgallate, and isoamylgallate. It is well known that tannase hydrolyses the ester bonds of tannic acid, although tannic acid is known to denature proteins. Tannases are an important group of enzymes involved in many industrial processes in the tannin reduction of food and beverage products. Tannase finds a wide range of applications in the production of gallic acid, an intermediate for trimethoprim, used in pharmaceutical industry, substrate for chemical synthesis of pyrogallol or ester galates which are used as food preservatives, manufacture of instant tea, clarification of

beer and beverages, reduction of anti-nutritional effects of tannins in animal feed and in decontamination of tannery effluents.

Tannase has immense potential for the practical utility in a wide range of industries, but the real use of this enzyme is at present limited owing to the elevated production cost. Hence, processes are essential for their economically feasible production (Beena *et al.*, 2010). The utilization of agro-industrial wastes, on one hand, provides alternative substrates and, on the other hand, helps to eliminate the pollution problems of waste disposal. Solid state fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of free flowing water and is an alternative cultivation system for the production of value added products from microorganism, especially enzymes as secondary metabolites. Agro industrial residues are generally considered the best substrates for the process of enzyme production. The major crop wastes like cotton stalks, jute sticks, coconut shell and coir pith are used in enzyme production. Several naturally occurring agricultural by-products such as wheat bran, coconut oilcake, groundnut oilcake, rice bran, wheat and paddy straw, sugar beet pulp, fruit pulps and peels, corn cobs, saw dust, maize bran, rice husk, soy hull, sago hampas, grape marc, coconut coir pith, banana waste, tea waste, cassava waste, apple pomace, peanut meal, cassava flour, wheat flour, corn flour, steamed rice etc., are also used as a cheap source for the production of the enzymes (Pandey *et al*., 2000). Availability of cheap substrates and scaling up of process parameters is the bottleneck in any aerobic cell growth and fermentation technology.

Banana is the second largest produced fruit after citrus, contributing about 16% of the world's total fruit production. Banana is a major crop of our country generating a various kind of agriculture waste after harvesting. Such kind of agro wastes are available in abundance but may possess some values. Banana peel is a rich source of starch (3%), crude protein (6-9%), crude fat (3.8-11%), total dietary fibre (43.2-49.7%), and polyunsaturated fatty acids, particularly linoleic acid and α- linolenic acid, pectin, essential amino acids (leucine, valine, phenylalanine and threonine) and micronutrients (K, P, Ca, Mg). Banana peels are also a good source of lignin (6-12%), pectin (10-21%), cellulose (7.6-9.6%), hemicelluloses (6.4-9.4%) and galactouroninc acid (Mohapatra *et al*., 2010).

PLATE-I

Banana waste as substrates used for the production of tannase

a. Banana spathe

b. Banana peel

Banana peel, an agro waste can be used as a substrate for ethanol production owing to its rich carbohydrate, crude proteins and reducing sugars. Moreover, banana peels are affordable and renewable low cost raw material. It acts as a storage house of potassium and pectin in abundance. In Banana inflorescence, the flowers are protected by large, brightly coloured, spirally arranged, boat shaped bracts called spathes. These are arranged in layers which enclose the male and female flowers . When the flowers get matured, the spathe begins to fall off. It forms the major agro waste in plantain field when compared to other parts and is rich in fibre and used as a cattle feed.

Tannase producing microorganisms

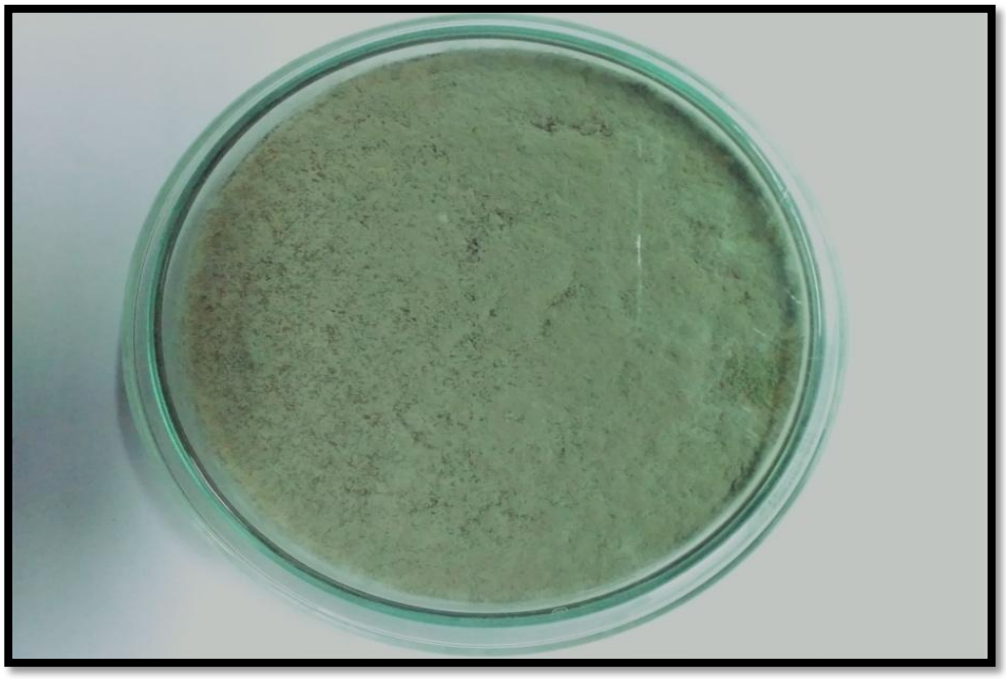
Tannase is an extracellular, inducible enzyme produced in the presence of tannic acid by fungi, bacteria and yeast. *Aspergilli* were reported as the best tannase producers in submerged and solid state fermentation. Few animals and plants also have been found to be the producers of tannase, but the most important sources to obtain the enzyme are microorganisms, because the enzymes produced by them are more stable than that obtained from other sources. Some of the organisms that produce tannase are *Aspergillus awamori, A. niger, A. oryzae, A. fumigatus, Penicillium notatium, P. digitatum, P. carylopilum, Trichoderma viride, T. haematum, Fusarium oxysporium, Rhizopus oryzae, Candida spp, Mycotorula japonica and Pichia spp.* Microorganisms degrade tannic acid, which makes a hydrolysis zone in screening medium that gives the indication of tannase presence (Kmar *et al*., 2010).The first step in the development of microbial enzyme production is the lineage selection. Extracellular enzymes were preferred because they are easily extracted and do not require expensive extraction methods (Couri *et al*., 1998).

Fungal inoculant (*Aspergillus nidulans)*

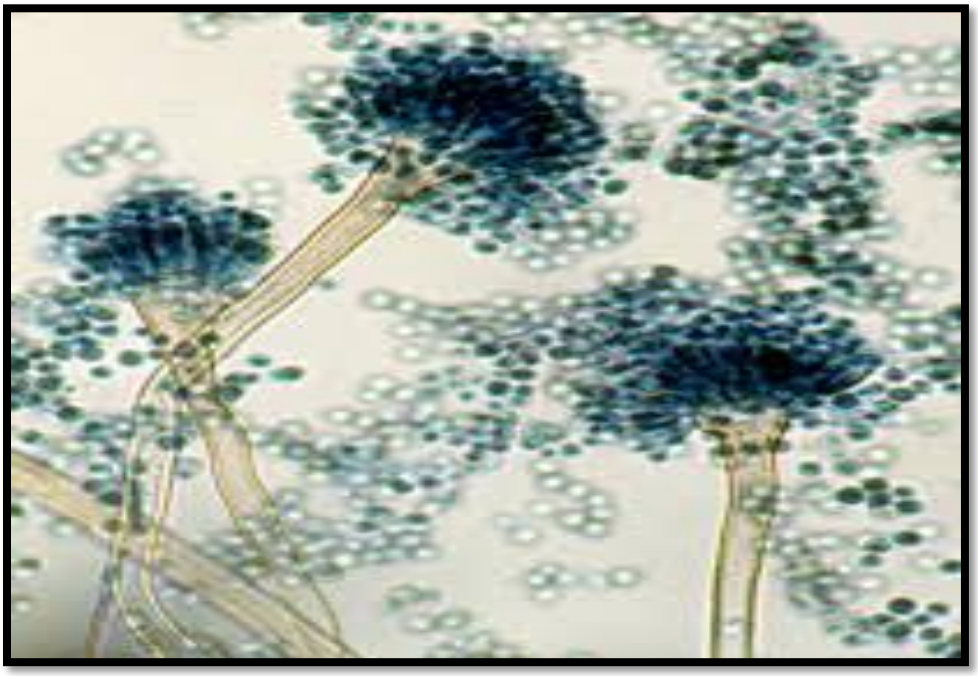
The fungi *Aspergillus nidulans* belongs to the class Eurotiomycetes, Order Eurotiales and comes under the Family Trichocomaceae. *Aspergillus nidulans*, a sexually reproducing member of the Aspergillaceae, is a haploid eukaryote which rapidly forms colonies on simple media and can therefore be treated as a microorganism. Hyphae are long tubular structures that extend solely at their tips and are typically partitioned into individual cellular compartments by the formation of septa.

PLATE-II

a. Plate culture of the fungi *Aspergillus nidulans*



b. Mycelium with conidia of *Aspergillus nidulans*



The asexual spores are uninucleate, green color. Filamentous fungi grow in nature on solid substrates like wood, stem, root and leaf of plants in the absence of free water and also have the intrusion power for penetrating deep into the intercellular space for better utilization of the substrate. Due to the increasing industrial application of tannase, new researches are necessary to find new microbial sources with high production of tannase. According to this, the aim of this study entitled **"Production of tannase by utilizing Banana wastes as substrate"** was undertaken with the following objectives.

- To determine the tannase activity on banana waste as substrate.
- To study the production of tannase using *Aspergillus nidulans*
- To optimize the fermentation conditions namely substrate concentration, incubation period, temperature and pH.

CHAPTER-II

REVIEW OF LITERATURE

The available literature pertaining to the present investigation has been reviewed and presented under the following headings.

- Tannin
- Tannase activity
- Substrate
- Factors affecting tannase activity
    - Incubation time
    - Temperature and pH
- Microorganisms

Tannin

Spencer *et al.* (1988) during their observations on Polyphenol complex at ion defined tannins as naturally occurring water soluble polyphenols of varying molecular weight, which differ from most other natural phenolic compounds in their ability to precipitate proteins from solutions. Harvey and Allan (1992) during their study on biochemistry and nutritional properties of tannins reported that, tannins are considered plant secondary substances as they are not involved in metabolic pathways. After lignins they are the second most abundant group of plant phenolics. The presence of a large number of phenolic hydroxyl groups enables them to form large complexes, mainly with proteins, and to a lesser extent with other macromolecules like cellulose and pectin. Tannins are hydrosoluble and high molecular weight polyphenolic compounds.

Bhat, (1998) reported that tannins are water-soluble polyphenolic compounds having wide prevalence in plants. Some microbes are, however, resistant to tannins, and have developed various mechanisms and pathways for tannin degradation in their natural milieu. The microbial

degradation of condensed tannins is, however, less than hydrolysable tannins in both aerobic and anaerobic environments. A number of microbes have also been isolated from the gastrointestinal tract of animals, which have the ability to break tannin-protein complexes and degrade tannins, especially hydrolysable tannins.

Mohapatra *et al.* (2007) stated that tannase is an industrially important inducible enzyme that hydrolyzes ester and depside linkage in hydrolysable tannin to gallicacid and glucose. Aguilar *et al.* (2007) stated that high concentration of phenolic compound such as tannin present in microorganism, contributed to the decomposition of organic materials and plant tissues. They also reported that condensed tannins are oligomeric and polymeric proantho cianidins that are conformed by unit esofflavan-3-ol(catechin) or flavan-3-4-ol linked to each other by C-C bonds. Catechin and epicatechin are the main monomeric units of condensed tannins which are condensed to form polymers. Polymeric chains could be formed by two or more than 50 units. Condensed tannins cannot be used as substrate for tannase. Major constituents of these compounds are cyaniding and delphinidin that are the responsible for the astringent flavoin fruits and wine. Aguilar *et al*. (2007) stated The tannins have the ability to precipitate macromolecules (such as proteins, cellulose, starch, etc.,) and minerals by forming strong complexes. Tannins are the second most important group of natural phenolic compounds after lignin.

Robledo *et al*. (2008) revealed that ellagi tannins are obtained mainly from trees such as oak (*Quercus sp*.) and chesnut (*Casta neadentata*), shrubs and fruits such as the pomegranate (*Punica granatum)* and red raspberry. Ellagi tannins are capable of reducing congenital defect, reduce the possibilities of heart attacks, stop proliferation of viruses and prevent the appearance of cancer cells. Costa *et al*., (2012) inferred that phenolic compounds such as gallic acid pyrogallol, methyl gallate and tannic acid are considered as an important tannase inducer.

Tannase Activity

Lekha and Lonsane (1997) stated that several fungal species such as *Aspergillus spp* and *Penicillium spp*. are capable of producing a large amount of tannase enzyme. They also observed maximum tannase yield with wheat bran and tannic acid as substrate after 72h of

incubation under SSF conditions using *Aspergillus niger* PKL104. Bajpai and Patil (1997) during their study on enzyme microbiology showed that methylgallate, gallic acid, and pyrogallol can induce the production of tannases and their investigation on the influence of other carbon sources resulted in lower levels of total activity.

Seth and Chand (2000) examined tannic acid concentration, agitation speed and pH during fermentation and they identified these parameters as an important one for inducing cell growth and enzyme synthesis by *Aspergillus awamori.* Maximum gallic acid accumulation of $40.3g^{-1}$ was obtained in 24 h incubation period with an initial substrate concentration of $45g^{-1}$. Gustavo et al. (2001) have reported higher tannase activity of 67.5 U/g dry substrate using wheat bran enriched with 0.8% tannic acid as substrate.

According to Saxena (2004) *Aspergillus fumigatus* (8.3 IU/ml), *Aspergillus versicolor* (7.0 IU/ml), *Aspergillus flavus* (4.95 IU/ml) and *Aspergillus caespitosum*(4.47 IU/ml) were potent producers of tannase among *Aspergillu*s strains. Among *Penicillii, Penicillium charlesi* (4.82 IU/ml), *Penicillium variable* (4.70 IU/ml) and *Penicillium crustosum* (4.7 IU/ml) were found to be the potent tannase producers. Aguilar *et al.* (2004) examined the Modeling and verification process parameters for the production of tannase by *Aspergillus oryzae* under submerged fermentation using agro-wastes. They reported that Tannase (EC 3.1.1.20), also known as tannin acyl hydrolase, is a water-soluble enzyme that has catalytic activity on hydrolyzable tannins and gallic acid esters. The enzyme catalyzes the hydrolytic cleavage of ester linkages present in tannic acid and yields gallic acid and glucose as end products.

Rana and Bhat (2005) reported maximum tannase production after 96 h of incubation by *Aspergillus niger* under solid state fermentation. Malgireddy *et al.* (2005) isolated *Aspergillus terrus* and studied the effect of different carbon sources (Glucose, Fructose, Galactose, Sucrose, Maltose, Mannose, Xylose, Lactose and Glycerol) and nitrogen sources (yeast extract, beef extract, peptone, casein, $NaNO_3$, $AgNO_3$) on the growth and tannase production under solidstate fermentation. Maximum enzyme production of 46.7 U/mg.m was observed in sucrose as carbon source and in yeast extract (46.1 U/mg) as nitrogen sources.

Mohapatra *et al*. (2006) used crude extracts of *Anacardium occidental* for the production tannase and they observed that the strain *Bacillus subtilis* KBR6 produced 0.6

Uml^{-1} tannase. Vijayalakshmi *et al.* (2009) examined the production of tannase by *Aspergillus flavus* in submerged fermentation. They observed higher tannase production of 70.0U/g min in different medium in the presence of 2% tannic acid at 35°C and 96h of incubation.

Paranthamam *et al.* (2008) investigated the production of tannase by *Aspergillus oryzae* using sugarcane baggasse and rice straw as substrates. The results of the study revealed that the maximum crude enzyme activity of 60.5U/gm/min was observed in sugarcane baggasse and rice straw used in 1:1 ratio at an optimum pH of 5.5, temperature of 35°C and at 72h incubation period respectively. Rodriguez *et al*., (2008) characterized tannase activity in cell-free extracts of *Lactobacillus plantarum* $CECT748^{T}$. The result of the investigation revealed maximum tannase activity of $11.4Uml^{-1}$ in methyl gallate as substrate at pH 5.0 and at 30°C.

Awan *et al*. (2010) reported that the enzymes are the biological catalysts, which are responsible for carrying out various biochemical reactions and, therefore, play an important role in the various aspects of life and its processes. Gomez *et al*. (2011) observed that tannase catalyzes the hydrolysis of ester bonds from tannic acid. Tannase production by seven xerophilic strains of *Aspergillus* was evaluated under solid state and submerged culture. Among xerophilic strains, *A.niger* GHI strain produced highest enzyme activity of 313.11U/L followed by *A. rugulosus* NS4 with 228.13U/L.

Kuppusamy *et al.* (2012) examined the optimum condition for maximum tannase enzyme production in submerged fermentation using *Aspergillus foetidus* with an initial tannic acid concentration of 3% (W/V) at 35°C and at a fermentation period of 96h. Tamarind seed powder and red gram husk gave a maximum tannase production of 71.48U/ml and 67.68U/ml respectively. Hamdy and Fawzy (2012) demonstrated the ability of *Aspergillus niger* to utilize *Ficus nitida* leaves and crude tannic acid extract to produce tannase. The result of the study revealed that the maximum activity of 81.5 U in *Ficus nitida* leaves as substrate.

According to Kumar *et al.* (2013) microbial tannase is more stable than tannase produced from other sources like plants or animals. Tannase from fungal sources are reported to be active in a wide range of Ph and temperature. Tannase production reached its maximum within 24 to 36h against crude tannin extract obtained from *Anacardium occidentale* by using

Aspergillus niger. Activation energy of the immobilized enzyme was lower than that of the free enzyme. Anitha and Arunkumar (2013) carried out purification and characterization of tannase enzyme (E.C.3.1.1.20) from *Mucor spp*. The specific activity of the purified enzyme was found to be 950.6U/mg and the molecular weight as 68kDa.

Shanmugapriya *et al*. (2014) investigated the production of tannase enzyme by *Aspergillus niger* and *Aspergillus flavus.* The fungal culture produced high yield of extracellular tannase (30.05±1.32U/ml and 23.43±1.43U/ml) under solid state fermentation using *Syzygium cumini* seed powder as substrate. Shajitha and Nisha (2018) studied the production of tannase from *Trichoderma viride* by using fruit peels as substrate and reported that the maximum production of tannase was found in 4% Pomegranate peel (6.07±0.004Umg^{-1}protein and 1.79±0.009Umg^{-1}protein) at an extra and intracellular level.

Substrates

Kumaran *et al*. (1997) used crop residues like paddy, wheat, millet, sorghum pulps, oil seed crops, mustard stalks, cobs, cotton stalks and jute sticks as substrates for the production of tannase enzyme. Silva *et al*., (2002) reported that agroindustrial wastes and byproducts such as orange bagasse, sugarcane bagasse, wheat bran and other food processing waste are effective substrate for de-polymerizing tannase production by solid state fermentation. Production of tannase by *Rhizopus oryzae* and *Aspergillus foetidus* from the powdered fruit of *Terminalia chebula* and *Cassalpinia digyna* has been reported by Mukherjee and Banerjee (2004).

Pandey *et al*. (2005) utilized palm kernel cake and tamarind seed powder for the production of tannase under solid state fermentation by *Aspergillus niger* and the maximum yield obtained was 53.5% in a 5% tannic acid concentration at 30°C and at 96h incubation period. Malgireddy *et al.* (2005) during their study on optimal conditions for production of Tannase from newly isolated *Aspergillus terrus* under solidstate fermentation revealed that the maximum tannase production was observed with wheat bran (41.6 U/mg) and minimum in cotton seed powder (37.6 U/mg) as substrate. Krishna (2005) during his study on solid-state fermentation systems reported that, fungi are well known to be favoured by a moist environment, for their growth while filamentous fungi are known to grow at water deficient substrates.

Vegetable residues such as coffee wastes, grape wastes, cashew wastes, wheat bran, rice bran, etc., supplemented with tannic acid were used as substrates for tannase production employing SSF. The utilization of agro-industrial wastes, on one hand, provides alternative substrates and, on the other hand, helps to solve pollution problems by eliminating the need for disposal of the wastes. The nature of the substrate employed is the most important factor affecting fermentation processes, and its selection depends upon several factors mainly related to cost and availability and, thus, may necessitate the screening of several agro industrial residues (Couto and Sanroman 2006).

Kumar *et al.* (2007) isolated tannase producing fungal strains from different locations including garbages, forests, orchards, etc. The strain giving maximum enzyme yield was identified to be as *Aspergillus ruber*. The tannase production was studied under solid state fermentation using different tannin rich substrates like ber leaves *(Zyzyphus mauritiana*), jamun leaves (*Syzygium cumini*), amla leaves (*Phyllanthus emblica)* and jawar leaves *(Sorghum vulgaris*). In solid state fermentation, the maximum production of tannase was found to be at 30°C after 96 h of incubation in (jamun leaves) as substrate. Muslim (2008) have reported that pure tannic acid is a very costly substrate and is not suitable for large-scale production of the enzyme. In this respect crude tannin could be cost effective and suitable for the commercial production of the enzyme. Agro-residues and forest products are generally considered the best source of tannin-rich substrate. The use of inexpensive substrates can economize the process of production. Therefore the agricultural wastes containing tannin could be considered as an alternative source of tannic acid for producing this enzyme.

Sun (2011) investigated for the production of cellulase by Trichoderma viride GIM 3.0010 in solid-state fermentation using banana peel reported the maximum activities of filter paper activity (FPA), carboxy methyl cellulase sodium activity (CMCase) and β-glucosidase (BG) reached 5.56, 10.31 and 3.01 U/gds, respectively. These results indicated that banana peel provided necessary nutrients for cell growth and cellulase synthesis and proved that natural banana peel provided all the nutrients needed by the organism for cell growth and enzyme production. Kapoori and Iqbal (2012) studied the production of tannase using *Trichoderma harzianum* MTCC 10841 under submerged fermentation with rich tannin materials like Amla (*Phyllanthus emblica* bark, leaves and fruit), Amalt ash (*Cassia fistula* leaves), Ber (*Zyzipus maurtiana* leaves), Eucalyptus (*Eucalyptus glogus* bark and leaves), Jamun(*Syzgium cumini* bark

and leaves), Guava (*Psidium guazava* bark and leaves), Keekar (*Acacia nilotica* leaves), Mango (*Magnifera indica* leaves), Mulberry (*Morus macroura* leaves), Tamarind (*Tamarindus indica* seed) and Pomegranate (*Punica granatum* rind) as carbon sources. Amla fruit, Tamarind seed, Jamun leaves, Mulberry leaves and Keekar leaves proved to be best substrates than tannic acid and the optimum pH and temperature for the tannase enzyme production were found to be 5.5 and 40ºC respectively.

Lal *et al*. (2012) investigated the production of tannin acyl hydrolase (E.C.3.1.1.20) from *Aspergillus niger* isolated from bark of *Acacia nilotica* and they reported highest tannase activity of 196.913Uml^{-1} at pH 5.0.Natarajan and Rajendran (2012) studied tannin acyl hydrolase (tannase) production by *Lactobacillus plantarum* MTCC1407 in submerged and solid state fermentation process. They observed maximum tannase activity of 5.319Ugds^{-1} in coffee husk as substrate. Bhoite and Murthy (2013) in their study carried out SSF with coffee pulp as substrate to screen positive fungal organism for production of tannase and reported *Penicillium spp.*CFR303 to be potential tannase producer with 66.5 ± 0.9% tannin degradation.

Inamdar (2014) in their study reported that tamarind seed powder contained considerable amount of tannin, It was found that the *Rhizopus* spp. isolated from humus under the *Tamarindus indica* tree possess capability to produce tannase because most of the fungal strains growing under the tree uses the tamarind tree litters as nutrient. Mahdi *et al*. (2014) in their study examined different agricultural residues for tannase production by *Aspergillus niger* and reported that the maximum enzyme activity of 4012.5 U/mg was observed in wheat bran mixed with tannic acid. Among wheat bran, corn bran, wheat straw, soy bean bran, tea leaves, *Punica granatum* peels and crushed barks of *Acacia nilotica* with tannic acid, a significant enzyme activity was recorded in tea leaves (3382.5 U/mg) followed by *Punica granatum* peel (3358 U/mg).

Malgireddy and Nimma (2015) on their investigation studied the effect of different agro wastes such as red gram husk, green gram husk, groundnut waste, cotton seed waste, wheat bran, rice bran, coconut powder, corn starch powder, *Cicer aritinum* on the production medium by *Aspergillus terrus*. They observed the tannase production was found to be maximum in wheat bran (41.6 U/mg), and minimum in cotton seed powder (37.6 U/mg) as substrate. Souza *et al.*

(2015) analyzed the production of tannase by *Aspergillus spp.* gm4 under solid-state fermentation using different leaves (mango, jamun and coffee) and agricultural residues (coffee husks, rice husks and wheat bran). They reported that among the different substrates used, the high yield of tannase was obtained in jamun leaves (1.44 U mg^{-1}) as substrate, followed by mango leaves (0.99 U mg^{-1}), after 4 days of incubation.

Bharathiraja *et al*. (2016) have revealed that utilization of agricultural wastes as substrates for producing industrially pivotal products such as enzymes, polysaccharides, organic acids, aroma, and flavor compounds by growing microorganisms on these wastes is a valuable technique, with a great economical advantage. Viswanath *et al*. (2016) during their investigation on the optimization of factors for the production of tannase from *A. niger* MTCC 5898 using cashew testa as a substrate reported the maximum of 25 U/g DS tannase activity in solid state fermentation. Shajitha and Nisha (2018) during their study on tannase production from agro-wastes by *Trichoderma viride* reported the maximum tannase activity of 4.34±0.004Umg^{-1} protein in 3% Guava leaves and the least activity of 0.06±0.005 Umg^{-1} protein at 1% Eucalyptus leaves as substrates.

Factors affecting tannase activity

Incubation time

Sharma *et al.* (2007) studied the optimization of tannase production by statistical techniques using *Aspergillus niger* in submerged fermentation. The effect of concentrations of tannic acid, sodium nitrate, agitation rate and incubation period on tannase production was studied and found that 5% tannic acid, 0.8% sodium nitrate, 150 rpm agitation and 48 hours were optimum conditions and gave a maximum tannase activity of 19.7 U/ml. Beniwal *et al.* (2010) reported the optimization of culture conditions for tannase production by *Aspergillus awamori* MTCC 9299 and reported that the maximum yield of tannase production was obtained at pH of 5.0, incubation temperature of 35°C, agitation speed of 125 rpm and 48 hours of incubation period.

Malgireddy *et al.(*2005) during their study on Optimal conditions for production of Tannase from newly isolated *Aspergillus terrus* under solid state fermentation reported that the

incubation period of the medium varied from 24 h to 168 h. Maximum yield of tannase (42.4 U/mg) was obtained after 3 days (72 hours) of incubation. Kumar *et al*. (2006) during their study on the production of tannase from *Aspergillus ruber* under solid-state fermentation using Jamun (*Syzygium cumini*) leaves reported that the enzyme production started after 48 h of incubation and increased with time peaking at 96 h (64 U/g) and thereafter, the enzyme production started decreasing.

Banerje*et al.* (2007) reported maximum tannase production at 36 h in 5 L ferment or using *A. aculeatus* DBF 9. Similarly, Lokeshwari and Raju (2007) reported maximum production (20.26 U/Ml) at 36 h from *A. niger*. Paranthaman *et al.* (2009) reported maximum tannase production in 96 h in submerged fermentation while working with *Aspergillus flavus.*

Tan Wee Yee *et al.* (2011) during their study on process parameters influencing tannase production by *Aspergillus niger* using mangrove (*Rhizophora apiculata*) bark in solid substrate fermentation observed the maximum yield of 4.97U/g on the 6^{th} day of cultivation with fungal growth achieved at 4.28 mg/g of fermented substrate. Lal and Gardner (2012) carried out their investigation to determine the best harvesting time for tannase from production culture by *A. niger* and observed maximum tannase activity (162.3 U/ml) on the 7^{th} day (168 hrs) of incubation. Reddy and Reddy (2015) in their study evaluated the effect of incubation period on tannase production, by the *Aspergillus terreus* isolate for different time durations ranging from 24 –168 hr and observed maximum tannase production of 32.5 U/ml when incubated for 72 h.

Shete and Chitanand (2015) isolated and screened for 10 fungi (F1- F10) and found that F5 showed highest capacity of tannin hydrolysis with hydrolysis capacity (HC) 2.09 at 72hr of incubation. Shajitha and Nisha (2018) during their study on tannase production from different agro-wastes as substrate by *Trichoderma viride* reported that the extracellular and intracellular enzyme activity showed an increasing trend in Guava and Eucalyptus leaves as substrates in different concentration (1%, 2%, 3% and 4%) up to 9 days of incubation and after that it declined gradually.

Temperature and pH

Batra and Saxena (2005) studied on different temperature range of the tannase production (30°C–70°C) and reported that the optimum temperature as 60°C for *A. flavus, A.*

fumigatus, *A. versicolor* and *P. variable*, whereas *A. caespitosum*, *P. charlesii*, *P. crustosum* and as 40°C for *P. restrictum*. Sabu *et al.* (2005) reported tannase activity was found to increase with the increase in pH and the optimum activity was at pH 6 (3.2 U/mL). Mahapatra *et al.* (2005) reported the production of tannase using *A. awamorinakazawa* and exhibited optimum activity at 35°C and at a pH of 5.0. Tannase obtained from *A. versicolor* was more stable in a broad temperature range of 30°C –0°C.

Cruz-hernández *et al.* (2006) studied the evaluation of culture conditions for tannase production by *Aspergillus niger* GH1. They observed *A. niger* GH1 produced the highest tannase level (2291 U/L) in SSF at 30°C during the first 20 h of culture at tannic acid concentration of 50 g/L. Mahendran *et al.* (2006) studied the purification and characterization of tannase using *Paecilomyces variotii* and reported that the enzyme was stable at a pH from 5 to 7. Banerjee *et al* (2007) investigated the possible use of wheat bran as substrate for production of tannase and reported a maximum tannase activity of 8.16 U/g at 30°C by *Aspergillus aculeatus* DBF9.

An increase in the temperature increases the rate of reaction, since the atoms in the enzyme molecule have greater energies and a greater tendency to move. However, the temperature is limited to the usual biological range. As the temperature rises, denaturation processes progressively destroy the activity of enzyme molecules. This is due to the unfolding of the protein chain after the breakage of weak (hydrogen) bonds, so that the overall reaction velocity drops. For many proteins, denaturation begins to occur at 45°C to 50°C. Some enzymes are very resistant to denaturation by high temperature, especially the enzymes isolated from thermophilic organisms found in certain hot environments. (Dutta , 2008)

Chhokar *et al.* (2010) during their study on the effect of additives on the activity of tannase from *Aspergillus awamori* MTCC9299 reported that the temperature of 30°C and pH of 5.5 were optimum for tannase activity (2.88 U/ml). Iqbal and Kapoor (2012) studied the effect of temperature on tannase enzyme production using *T. harzianum* in the temperature range from 25°C to 60°C and the optimum temperature was found to be 40°C and was stable at 40ºC retaining about 71% of original activity for 2 hours. Costa *et al.* (2012) obtained the results for tannase enzyme production using *A. tamarii* in submerged culture fermentation and the optimum temperature was found to be 35ºC and was stable at 40ºC for 2 hours.

Malgireddy and Nimma (2015) studied on the effect of various temperatures (26°C, 28°C, 30°C, 32°C, 34°C and 36°C) on the growth and tannase production and observed maximum tannase production of 46.2 U/mg at 30°C at 72h and there was an decreased yield of 31.6 U/ml when there was an increase in incubation temperature to 36°C. Lata and Rani (2016) studied the effect of incubation temperature on tannase production from *Penicillium duclauxii* in the temperature range 30°C - 50°C and the optimum incubation temperature for tannase enzyme production of 29.87 U/ml was observed at 30°C. Shajitha and Nisha (2018) studied the production of tannase from *Trichoderma viride* by using fruit peels as substrate and reported that the optimized conditions for the production of tannase from *Trichoderma viride* was found to be 30ºC, 5.0 pH and 9 days of incubation in the 4% substrate concentration.

Microorganisms producing tannase

Bajpai and Patil (1997) in their work reported that microorganisms can continuously produce tannase in large quantities, resulting in an increased yield with the establishment of optimized fermentation methods. Tannase is produced in the presence of tannic acid by several filamentous fungi, mainly *Aspergillus* and *Penicillium* species. Holker *et al.* (2004) during their study on biotechnological advantages of laboratory-scale solid-state fermentation with fungi reported that several enzymes of industrial importance have been extracted from the fungi belonging to the genus *Aspergillus*. The importance of this genus is so much, that it has been studied as a model organism for fungal enzyme production. Cruz-Hernandez *et al.* (2005) isolated and evaluated tannin-degrading strains from the Mexican desert and reported that the xerophilic fungus *Aspergillus niger* GH1 was found to be an efficient producer of tannase.

Mata-Gómez1 *et al*. (2009) during their study on a Novel Tannase from the Xerophilic fungus *Aspergillus niger* GH1 reported the use of A. niger GH1 has been shown to be very promising in tannin-rich wastes treatment, to enhance the biological activity of tea, to produce important potent phenolic antioxidants, etc. According to Beena *et al*. (2010) the tannase has immense potential for the practical utility in a wide range of industries, but the real use of this enzyme is at present limited owing to the elevated production cost. Hence, processes are essential for their economically feasible production by utilizing aerobic *Aspergillus* species

under solid state growth at the industrial level of tannase production. Melo *et al.* (2013) in their study reported that the species belonging to the genera *Aspergillus* and *Pencillium* existed in greater number and among *Aspergillus* species, the best tannase producers were *A. japonicus* 246A (16.45 U/mg), *A. tamarii* 3 (12.95 U/mg), and *Aspergillus sp*. 47 (5.98 U/mg). Among *Penicillium species* only *Penicillium sp.* 121 produced moderate amounts of the enzyme (1.41 U/mg). Kapoor and Iqbal (2013) isolated 84 fungal strains from soil around Cassia tree. Among the 84 isolates, *Trichoderma harzianum* showed maximum tannase activity of 31.36 Uml^{-1} with 1:0 tannic acids as substrate. Panesar *et al*. (2016) have reported that among the various natural sources, microorganisms are the preferred sources for enzyme production due to a number of advantages. Owing to the high cost of production arising from the fermentation medium and process conditions, the trend has been shifted towards the use of agro industrial wastes for production of cost-effective enzymes.

Applications

Lekha and Lonsane (1997) stated that tannase enzymes are used in the preparation of gallic acid, instant tea, acron wine, coffee flavoured soft drinks, high-grade leather tannin, clarification of beer and fruit juice, detanification of food and to clean-up highly polluting tannin from the effluent of leather industry. According to Conesa *et al.* (2001) the tannase enzymes are capable of degrading the plant cell wall by breakage of poly-phenolics such as dehydrodimer cross links present in the cellwall. Kauppinen *et al*. (2001) reported that the tannase produced by the species of *Aspergillus* and *Pencillium* are used for the cleavage of polyphenolics such as dehydrodimer present in the cell wall of plants and helps in the wall digestibility.

According to Boadi and Neufeld (2001) tannase enzymes are used as inhibitor of foam in tea and as a clarifying agent in the production of beer and fruit juices. Aguilar *et al*. (2001) stated that tannase is used in the manufacturing of instantaneous tea and to eliminate insoluble precipitates formed when the beverage is cooled at $4^{\circ}C$. Precipitates are formed by interaction of phenolic compounds and caffeine. Tannase treatment breaks the ester bonds of polyphenols avoiding its polymerization and complex with caffeine. Chemical processes for diminution of precipitates in tea can delete some aromatic compounds. However, the enzyme treatment

produces a cold water soluble tea with high content of aromatic compounds and appropriate color.

Ayed *et al*. (2002) examined the enzyme activity found in *Lactobacillus plantarum* strain. They inferred that the enzyme plays an important role in production of fermented foods, including dairy products, silage, pickled vegetables, meat and fish products and it was proposed as a potential probiotic. Microbial production of tannase is most preferable in pharmaceutical and food industries due to maximum production in the shortest period of cultivation. Belmarse *et al.* (2004) reported that the tannase enzyme is used in beer and wine industries to remove chill haze formation. It is also used to reduce the anti-nutritional effects of poultry and animal feed along with food detanification and industrial effluent treatment.

According to Hernandez *et al*. (2005) tannase enzyme is used in the food industry for the production of gallic acid, a substrate for the chemical synthesis of pyrogallol, which in turn is used in a variety of industrial sector, such as a developer in photography, to make colloidal solutions of metals, as a mordant for wool, for staining leathers, in process engraving, in the manufacture of various dyes and in the dying of fur, hair etc., Aissam *et al*. (2005) carried out their study on some varieties of sorghum and they found that it possess high content of tannins and are useful as animal feed and also for bioethanol production due to its antimicrobial effect. The treatment of sorghum with tannase decreases the tannin content and can thus be used as a complement for animal feed and for bioethanol production. Tannase has been used in environmental biotechnology for the treatment of tannery effluent.

Rout and Banerjee (2006) reported a reduction of 25% of tannin content in pomegranate juice by treatment with tannase. Reduction of bitter in fruit juices by enzymatic treatment possess the advantage of increasing the quality of the beverages. High concentration of tannins in fruits, such as blueberry, pomegranate and raspberry, leads to the formation of sediment, color and bitter taste during the storage of its juices. Enzymatic treatment with tannase is commended one. Aguilar *et al*. (2007) observed the Tannase gene and the enzyme activity can be employed for the identification of *Staphilococcus lugdunensis* in humans and to prevent the colon cancer. Noguchi *et al*. (2007) employed tannase for the production of molecules with therapeutic applications, such as prunioside with anti-inflammatory activity.

According to Yu and Li (2008) gallic acid is used in pharmaceutical industry as an intermediary compound for the synthesis of trimethoprim. In chemical industry it is used as substrate for the chemical or enzymatic synthesis of propyl gallate and other antioxidant compounds with varying applications. Gallic acid is used in manufacture of semiconductor, inks and as photographic developer. Thus,the studies have demonstrated that gallic acid possess important therapeutic properties. Lu *et al.* (2009) reported that tannase treated green tea showed better colour stability and organoleptic properties. Murugan and Sohaibani (2010) reported that tannase has potential use in the treatment of tannery effluents and pre-treatment of tannin containing animal feed.

Srivasta and Kar. (2010) found that the tannase is used to remove haze, improves color, bitterness and astringency of the juice upon storage. Tannase can be used for degradation of the phenolic compounds and thus increase the catalytic activity of cellulose as reported by Tejirian and Xu (2011). Dykstra *et al*. (2011) stated that potential application of tannase is used in the manufacture of laundry detergent as an additive and in the leather industry to homogenize the tannins.

CHAPTER- III

MATERIALS AND METHODS

Collection of agrowaste

Banana agro wastes like banana peel and banana spathe were collected from the plantain field of Paramathi velur Taluk , Namakkal District, Tamilnadu. Both the peel and spathe were dried in shade and ground into fine powder and sieved and kept in sterile containers until used.

Microorganism

The fungus *Aspergillus nidulans* was taken from the laboratory of Botany Department, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, identified based on their morphology, mycelia structure and spore formation (Barron, 1968; Ellis, 1976, and Domsche and Gams,1972)

Growth medium for fungal culture

Potato dextrose agar medium (PDA) - (Riker And Riker, 1936).

- Peeled potato - 250 g
- Dextrose - 20 g
- Agar - 15g
- Distilled water - 1000 ml
- pH - 6 to 6.5

Peeled Potato was made into thin chips, boiled in 500ml water and after extraction, dextrose was added into the extract. The agar was melted separately in other half of water and mixed with the above solution. The volume was made into 1000ml. The medium was poured into sterile petriplates (15 ml/ plate) and fungal cultures were inoculated in the centre of the petriplates. Agar slants were prepared to maintain the fungal (*Aspergillus nidulans*) culture.

Enzymology

Preparation of culture medium

The basal medium used throughout the present investigation was composed of (g/l):

- NH_4NO_3 - 0.5%
- NaCl - 0.1%
- $MgSO_4.7H_2O$ - 0.1%
- Tannic acid - 4%
- Distilled water -1000ml
- pH - 5.0

100 ml of tannin containing liquid medium was dispensed in 250 ml Erlenmeyer flasks and sterilized at 1 atm for 15 minutes. After cooling, 1 ml of chloramphenol (10,000 ppm) was added. The pH of the medium was maintained at 5.0, after sterilization. The medium was inoculated with 5g (disc) of the fungus, *Aspergillus nidulans* with banana spathe and peel powder as substrate at different concentrations (1%, 2%, 3% and 4%). The enzyme activity was analysed on the 6^{th}, 8^{th}, 10^{th}, and 12^{th} days of incubation.

Preparations of culture filtrate as enzyme source (Extracellular)

The mycelium was filtered through Whatman No.1 filter paper using a Buchner funnel under suction and the clear filtrate was used as a source of extracellular enzyme.

Preparation of cell free enzyme source from fungal mycelium (Intracellular)

The fungal mycelium was washed with distilled water twice. A quantity of 0.5g of the washed mycelia mat was macerated in 5 ml of citrate buffer, pH 5.0 in a pre chilled mortar and pestle with a pinch of acid washed sand. The homogenate was centrifuged in a refrigerated centrifuge at 5,000g for 10 minutes. The supernatant served as an intracellular enzyme source.

Estimation of enzyme activity (Mondal and Pati, 2000)

Enzyme solution (0.1 ml) was incubated with 0.3 ml of 1.0% tannic acid in 0.2M citrate buffer (pH 5.0) at 40°C for thirty minutes and then the reaction was terminated at 0°C by the addition of 2ml BSA (1mg/ml), which precipitates the remaining tannic acid. A control reaction was also done side by side with heat denatured enzyme. The tubes were then centrifuged at 5,000x g, for 10min.The precipitate was dissolved in 2ml of SDS-triethanolamine 1% w/v, (SDS in 5% v/v of triethanolamine) solution and the absorbency was measured at 550nm after the addition of 1ml of $FeCl_3$ (0.13 M).A standard graph was prepared with gallic acid in the concentration range of 50µg/ml.

Estimation of protein (Lowry *et al*., 1951)

Principle

The blue colour developed by the reduction of the phosphomolybdic phosphotungstic components in the folin-ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry's method.

Reagents

- 2 per cent sodium carbonate in 0.1 N sodium hydroxide (Reagent A)
- 0.5 per cent copper sulphate ($CuSO_4 2H_2O$) in one per cent potassium sodium tartarate (Reagent B).
- Alkaline copper solution: 50 ml of A and 1 ml of B were mixed prior to use (Reagent C).
- Folin-ciocalteau reagent (Reagent D).

Protein Solution (Stock Standard)

50 mg of Bovine Serum Albumin (Fraction V) was weighed accurately and dissolved in distilled water and the volume was made upto 50 ml in a standard flask.

Working Standard

10 ml of the stock solution was diluted to 50 ml with distilled water in a standard flask (one ml of this solution contains 200 mg protein).

Procedure

- Extraction is usually carried out with buffers used for the enzyme assay.
- A quantity 500 mg of the sample was ground well with pestle and mortar in 5 ml of the buffer.
- The supernatant was centrifuged and used for protein estimation.

Estimation of Protein

- 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard was pipetted out in a series of the test tubes and 0.1 ml of the sample extract was pipetted out in the test tube.
- The volume was made up to 1 ml in all the test tubes. A tube with one ml of water served as the blank.
- 5 ml of reagent C was added to each test tube including the blank, mixed well and allowed to stand for 10 minutes.
- Then, 0.5 ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30 minutes. The blue colour developed was read at 660 nm.
- The amount of protein in the sample was calculated by drawing a standard graph.

Calculation

The amount of protein in the enzyme assay was expressed in mg^{-1} g^{-1}.

Optimization of culture conditions for enzyme production

The test fungal strains were inoculated in the nutrient medium (production medium) and was subjected to various pH, temperature, incubation period, and agro wastes like Banana spathe and peel as substrate to assess the optimal production of the enzyme. All sets have been performed in triplicates and the statistical errors have been determined.

Effect of Incubation period on Enzyme Production

Fermentation period was an important parameter for enzyme production. So, the enzyme analysis was carried out at an incubation period of 6, 8, 10 and 12 days.

Effect of pH on enzyme production

The test strain was inoculated in 100ml of production medium which was prominently adjusted by using 1N HCl or 1N NaOH to various pH ranges of 4.0, 4.5, 5.0, 5.5, 6.0 and kept in static condition at 30°C. The experimental set up was done in triplicates and the enzyme activity was estimated at 6^{th}, 8^{th}, 10^{th} and 12^{th} day of incubation.

Effect of temperature on enzyme production

The test strain was inoculated in 100 ml of production medium and incubated at varying temperature ranges of 30°C, 35°C, 40°C, 45°C and 50°C under static condition. All sets have been performed in triplicates and the enzyme activity was estimated at 6^{th}, 8^{th}, 10^{th} and 12^{th} day of incubation.

Effect of agro wastes as substrate on enzyme production

The test strain was incubated in production medium containing various concentration (1%, 2%, 3% and 4%) of agro wastes [banana peel and spathe] as substrates. All sets have been performed in triplicates and the enzyme activity was estimated at 6^{th}, 8^{th}, 10^{th} and 12^{th} day of incubation.

Statistical analysis

Standard errors of means of all the replicates of each variable were computed using computer software, Microsoft excel. Data's regarding the tannase activity on different substrates were statistically analysed using 2 way analysis of variance (ANOVA), followed by LSD method to delineate mean differences. Treatments were compared using Duncan's Multiple Range Test (DMRT). All the experiments were repeated three times. Based on the results, inferences were drawn. (Panse and Sukhatme, 1978).

CHAPTER-IV

RESULT AND DISCUSSION

The result pertaining to the present investigation are discussed under the following heads:

1. Effect of banana waste as substrate on tannase production
2. Effect of temperature on enzyme production in Banana waste as substrate
3. Effect of pH on enzyme production in Banana waste as substrate

1. Effect of banana waste as substrate on tannase production

Among the banana wastes, banana spathe and peel were used as substrates in different concentration (1%, 2%, 3%, and 4%) at an intracellular and extracellular level. The enzyme showed an increase in the activity upto 10 days of incubation and gradually decreased after that.

Effect of banana spathe as substrate on tannase production

Tannase activity showed an enhancement up to 10^{th} day of incubation by *A. nidulans*, after that its activity declined. Among the different concentrations (1%, 2%, 3% and 4%) of the substrate, highest tannase activity was recorded in 3 percent (1.052 ± 0.003 Umg^{-1} protein) followed by 4 percent (0.831 ± 0.003 Umg^{-1} protein) on the 10^{th} day of incubation by *A.nidulans*, when compared to the control (0.472 ± 0.024 Umg^{-1} protein) at an intracellular level and the least activity of 0.135 ± 0.008 Umg^{-1} protein was exhibited in 1 percent Banana spathe on the 6^{th} day of incubation. A significant level of tannase activity was observed in 1 percent (0.548 ± 0.023 Umg^{-1} protein) and 2 percent (0.529 ± 0.007 Umg^{-1} protein) which were on par with each other on the 10^{th} day of incubation and the least value was recorded as 0.158 ± 0.010 Umg^{-1} protein in 1 percent Banana spathe on the 6^{th} day of incubation compared to the control at an extracellular level (Table – I & Figure- I a & b)It may be inferred from the statistical investigation (mean± standard deviation) of the data that the production of tannase was found to be significantly enhanced in 10^{th} day at a intracellular and extracellular level ($p<0.05$)

Jana *et al*. (2012) used tannin rich plant residues (Haritaki, Pomegranate, Tea leaf waste, Tamarind and Arjun fruit) as substrate for tannase production by *Penicillium purpuogenum* PAF6. Among them, tamarind seed powder showed the maximum tannase activity. The present result is in accordance with the view of **Kapoor and Iqbal (2012)** who

reported that the enzyme showed appreciable activity with pomegranate rind (97%) as substrate. Similar result was observed by Jana *et al.* (2012) the enzyme productively was maximum in tamarind seed followed by Haritaki, Pomegranate, Tea leaf waste and Arjun fruit after 48 h of cultivation at 30°C.

TABLE-I

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on Banana spathe waste as Substrate

Banana spathe as Substrate	Concentrations	Days			
		6	8	10	12
Intracellular	Control	0.103 ± 0.011	0.193 ± 0.004	0.472 ± 0.024	0.169 ± 0.010
	1%	0.135 ± 0.008	0.212 ± 0.012	0.593 ± 0.006	0.446 ± 0.002
	2%	0.299 ± 0.005	0.324 ± 0.117	0.639 ± 0.041	0.283 ± 0.008
	3%	0.137 ± 0.003	0.431 ± 0.003	1.052 ± 0.003	0.247 ± 0.005
	4%	0.168 ± 0.001	0.223 ± 0.166	0.831 ± 0.003	0.245 ± 0.003
Extracellular	Control	0.074 ± 0.008	0.176 ± 0.002	0.471 ± 0.006	0.131 ± 0.001
	1%	0.158± 0.010	0.396 ± 0.002	0.520 ± 0.001	0.284 ± 0.006
	2%	0.247± 0.004	0.399 ± 0.013	0.529 ± 0.007	0.381 ± 0.028
	3%	0.402 ±0.002	0.478 ± 0.001	0.548 ± 0.023	0.375 ± 0.005
	4%	0.180± 0.003	0.271 ± 0.001	0.409± 0.001	0.371 ± 0.008
SEd		**0.003**			
CD ($p<0.05$)		**0.007**			

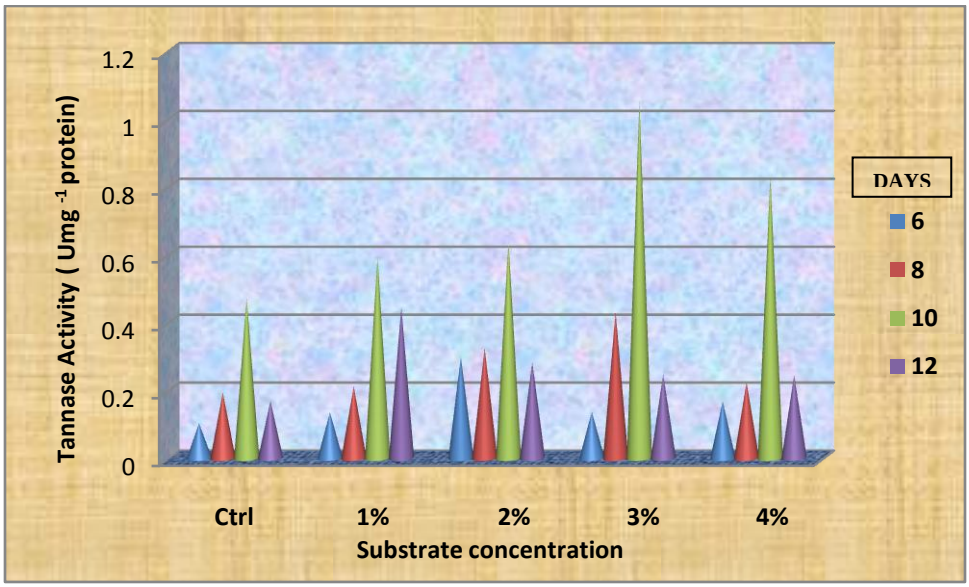
Results are the mean ± standard deviation of triplicates

Umg^{-1} = μ molgallic acid released min^{-1} mg^{-1} protein

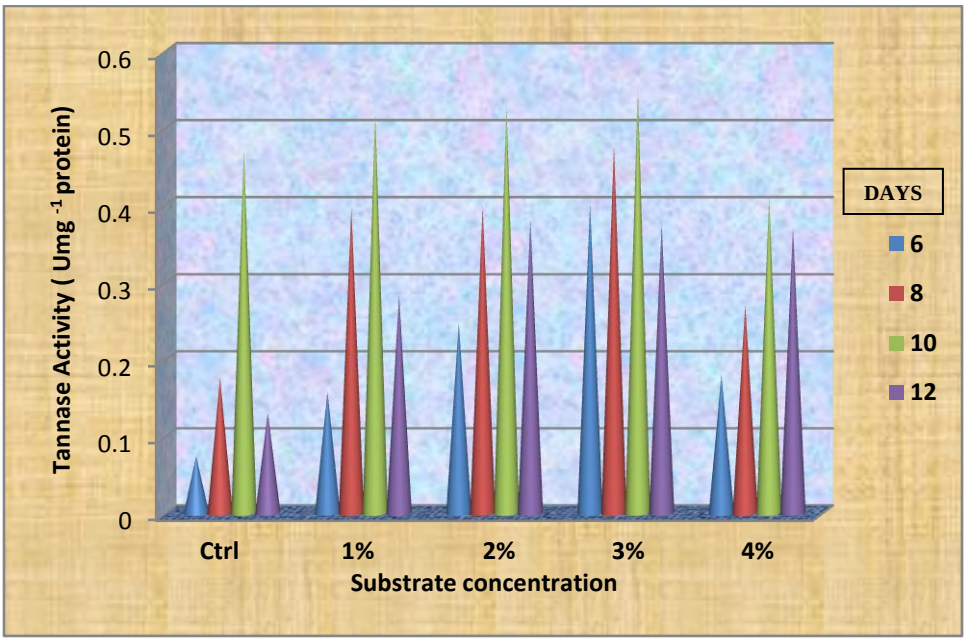
FIGURE -I

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on Banana spathe as substrate

a. At Intracellular level



b. At Extracellular level



Effect of Banana peel as substrate on enzyme production (Table – II & Figure -II a & b)

Tannase production by *A.nidulans* showed a pronounced activity of 0.817± 0.034 Umg^{-1} protein at 3 percent followed by 0.647 ± 0.002 Umg^{-1} protein at 4 percent in banana peel as substrate on the 10^{th} day of incubation when compared to the control (0.502± 0.009 Umg^{-1} protein) at an intracellular level. The least activity of 0.143 ± 0.008 Umg^{-1} protein was exhibited in 1 percent Banana peel on the 6^{th} day of incubation, compared to control (0.083 ± 0.002 Umg^{-1} protein). At an extracellular level tannase activity was much pronounced in 3 percent (0.612 ± 0.028 Umg^{-1} protein) followed by 2 percent (0.456 ± 0.025 Umg^{-1} protein) compared to the control (0.302 ± 0.036 Umg^{-1} protein) on the 10^{th} day of incubation and the least value was recorded as 0.146 ± 0.003 Umg^{-1} protein in 4 percent on the 6^{th} day of incubation compared to control (0.131 ± 0.003 Umg^{-1} protein).

It may be inferred from the statistical investigation (mean± standard deviation) of the data that the production of tannase was found to be significantly enhanced in 10^{th} day of incubation.

Pandey *et al*. (2005) reported highest yield of tannase in Palm kernel cake by *Aspergillus niger* and in Jamun leaves as substrate by *A.ruber* (Kumar *et al*., 2007). Banerjee *et al*. (2007) found maximum tannase production at an extracellular level by *Aspergillus aculaetus* after 72h incubation. The present result is related to the findings of Shanmugapriya *et al.* (2014). They reported that the fungal cultures *Aspergillus flavus* and *Aspergillus niger* produced high yield of extracellular tannase (1.32U/ml and1.43 U/ml) under solid state fermentation using *Syzygium cumini* seed powder as substrate. Hamdy and Fawzy (2012) reported highest tannase activity of 9.14 Uml^{-1} by *Aspergillus niger* in *Ficus nitida* leaves as substrate at 30°C and at pH 5.0.

Kuppuswamy *et al.* (2012) reported that among the powdered agro wastes like green-gram husk, black-gram husk, red gram husk, tea dust, tamarind seed powder, groundnut shell and rice bran, the tamarind seed powder and red-gram husk exhibited a maximum tannase production of 67.59 U/mL and 76.49 U/mL after an incubation period of 96 hr. Similar result was reported by Kaur and Katyal (2017) who observed the maximum tannase production of 35.62 U/mL on spent tea as substrate.

TABLE-II

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on Banana peel waste as Substrate

Banana peel as Substrate	Concentrations	Days			
		6	**8**	**10**	**12**
Intracellular	Control	0.083 ± 0.002	0.141 ± 0.002	0.502± 0.009	0.321 ± 0.042
	1%	0.143 ± 0.008	0.185 ± 0.003	0.584± 0.001	0.510± 0.010
	2%	0.143 ± 0.009	0.212 ± 0.015	0.612 ± 0.017	0.520± 0.008
	3%	0.129 ± 0.003	0.336 ± 0.003	0.817± 0.034	0.664 ± 0.002
	4%	0.123 ± 0.006	0.148 ± 0.003	0.647 ± 0.002	0.426 ± 0.082
Extracellular	Control	0.131 ± 0.003	0.146± 0.002	0.302 ± 0.036	0.273± 0.076
	1%	0.169± 0.004	0.207± 0.012	0.416 ± 0.025	0.363 ± 0.041
	2%	0.197 ± 0.007	0.241± 0.003	0.456 ± 0.025	0.372 ± 0.001
	3%	0.198 ± 0.010	0.252 ± 0.003	0.612 ± 0.028	0.381 ± 0.001
	4%	0.146 ± 0.003	0.185 ± 0.001	0.315 ± 0.002	0.296 ± 0.014
SEd CD ($p<0.05$)		**0.004** **0.008**			

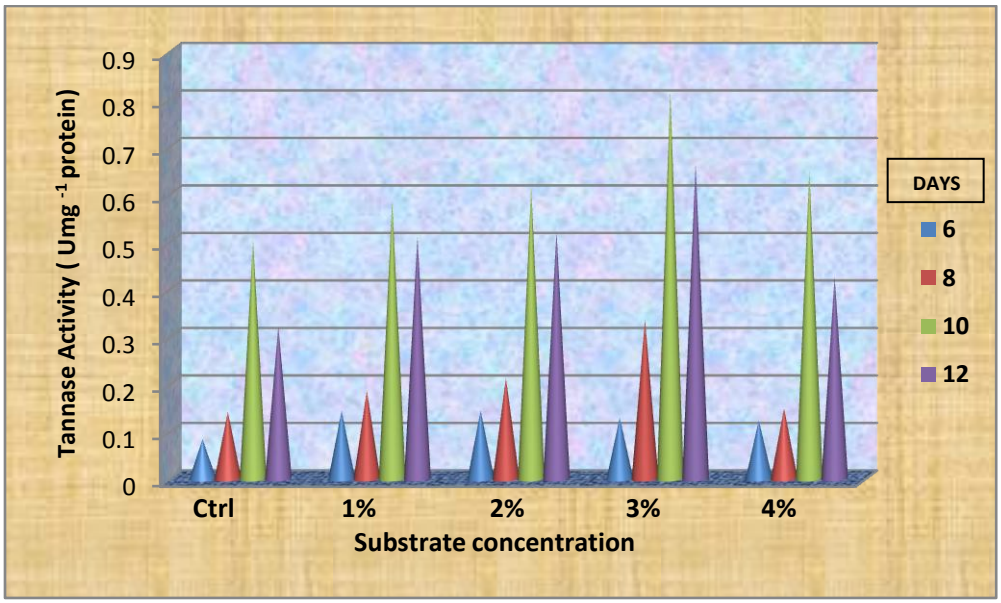
Results are the mean ± standard deviation of triplicates

Umg^{-1} = μ molgallic acid released min^{-1} mg^{-1} protein

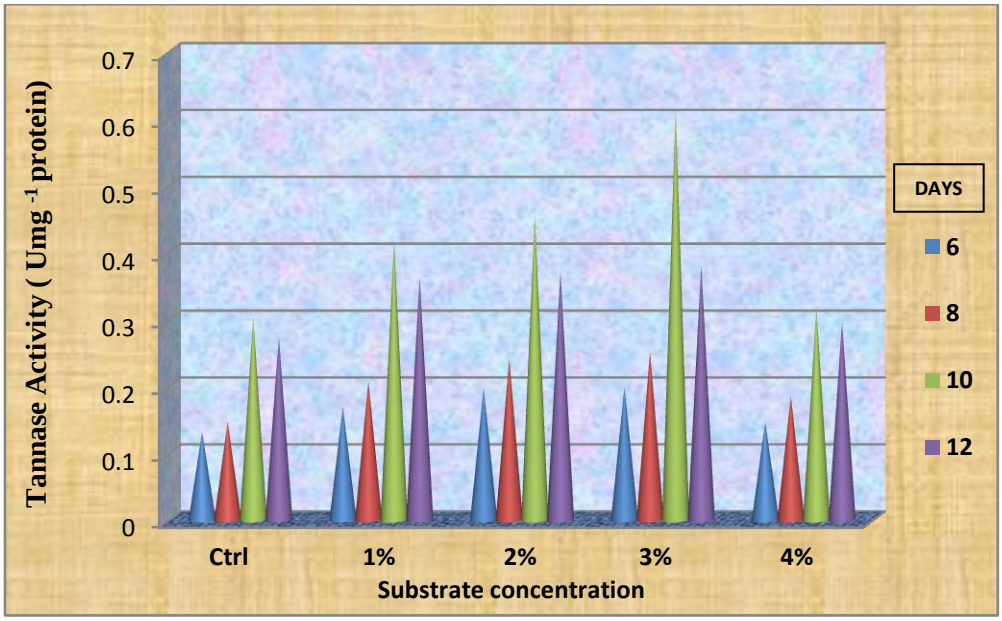
FIGURE -II

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on Banana peel as substrate

a. At Intracellular level



b. At Extracellular level



2. Effect of temperature on enzyme production in Banana waste as substrate

Temperature play an important role in the enzyme production as it is extremely significant in microorganism's growth regulation and physiological activity and microbial product formation and it varies from each microorganism. Even the slight changes in the growth temperature, may affect enzyme production (Syarifah *et al.,* 2012). Temperature is directly related to the metabolic activities of the microorganism and it affects the proper growth and product formation of the organism (Sandhya and Kurup, 2013). Optimal temperature is defined as that temperature which results in maximum velocity of the enzymatic reaction above which, the rate of reaction decreases due to thermal inactivation (Battestin and Macedo, 2007).

TABLE-III

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on different temperature at 3% concentration on Banana spathe and peel as substrate

Substrare		30ºC	35ºC	40ºC	45ºC	50ºC	SEd CD ($p<0.05$)
Banana Spathe	**Intra cellular**	0.093±0.033	0.132±0.024	0.225±0.055	0.220±0.005	0.135±0.020	**0.020**
	Extra cellular	0.092±0.022	0.126±0.012	0.248±0.015	0.185±0.004	0.101±0.015	**0.042**
Banana Peel	**Intra cellular**	0.064±0.011	0.165±0.051	0.219±0.022	0.188±0.018	0.123±0.012	**0.022**
	Extra cellular	0.140±0.351	0.164±0.021	0.423±0.032	0.252±0.036	0.127±0.010	**0.047**

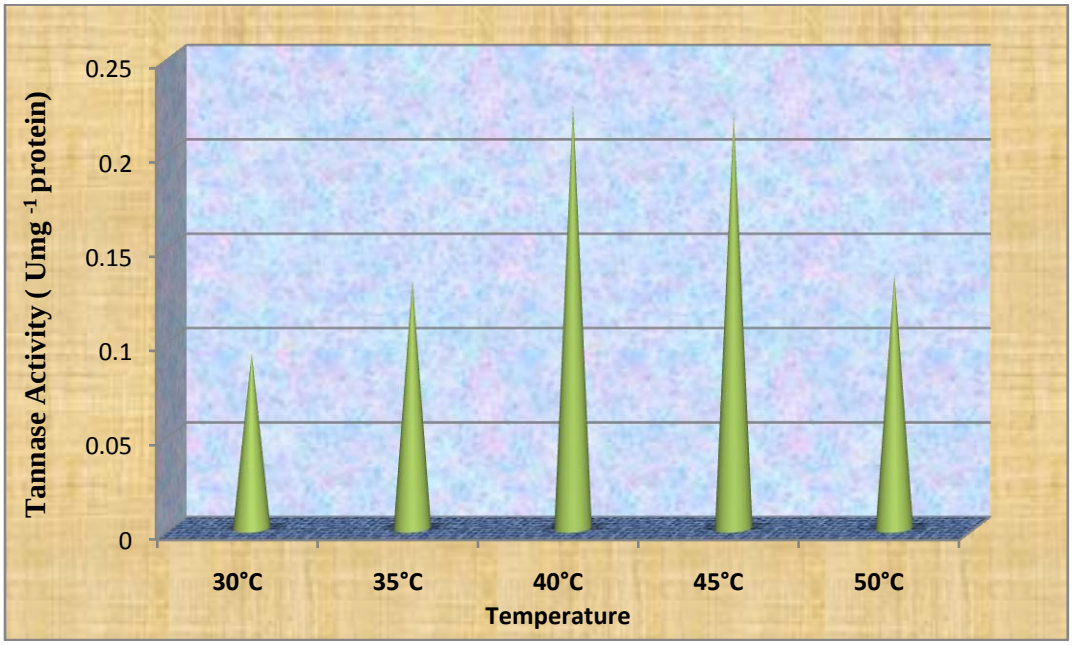
Results are the mean ± standard deviation of triplicates

Umg^{-1} = μ molgallic acid released min^{-1} mg^{-1} protein

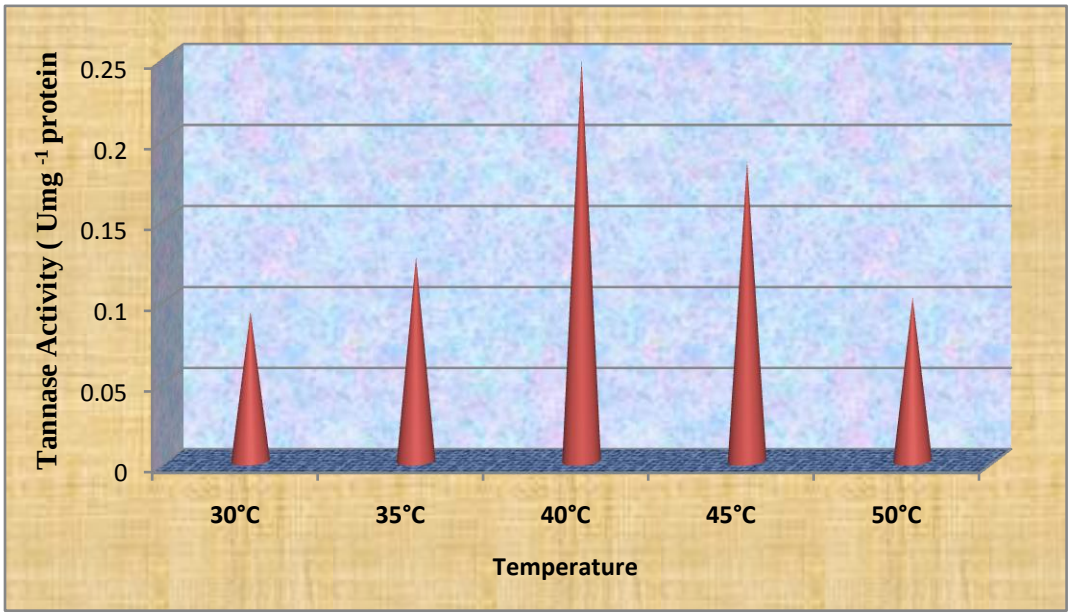
FIGURE-III

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on Banana spathe as substrate on different temperature

a. At Intracellular level.



b. At Extracellular level



The enzyme activity at both extracellular and intracellular level by *A.nidulans* at different temperature ranges was found to increase for 30°C to 40°C and peaked at 40°C and further increase in the temperature showed a decreasing trend. (Table–III & Figure–III a & b).

An enzyme production by *A.nidulans* in Banana spathe was found to be increased from 0.093±0.033 to 0.225±0.055 Umg^{-1}protein and 0.092±0.022 to 0.248±0.015 Umg^{-1} protein production from 30°C to 40°C at both intracellular and extracellular level and further increase in temperature after 40°C declined its activity to 0.135±0.020 and 0.101±0.015 Umg^{-1} protein production at 50°C on the 10^{th} day of incubation.

In Banana peel as substrate, the highest tannase activity of 0.064±0.01 to 0.219±0.022 Umg^{-1} protein production from 30°C to 40°C and further increase in temperature after 40°C declined its activity to 0.123±0.012 Umg^{-1} protein production at 50°C on the 10^{th} day of incubation at an intracellular level and found to be increased from 0.140±0.351 to 0.423±0.032 Umg^{-1} protein production from 30°C to 40°C and further increase in temperature after 40°C declined its activity to 0.127±0.010 Umg^{-1} protein production at 50°C on the 10^{th} day of incubation at an extracellular level. (Table–III & Figure–IV a & b).

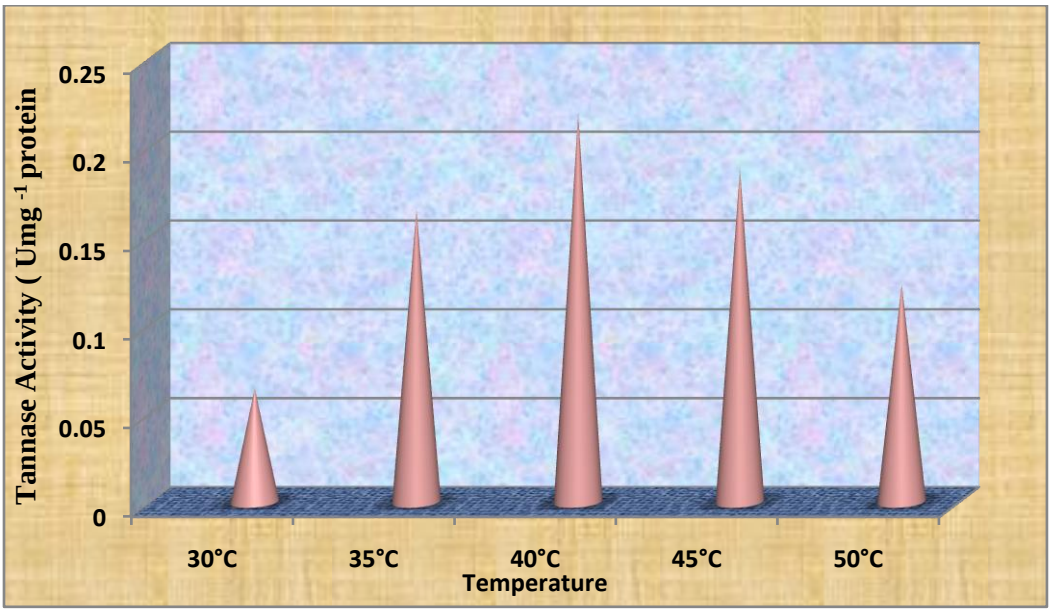
Similar view was expressed by Sabu *et al*. (2005) who reported that *Aspergillus niger* ATCC 16620 exhibited the maximum tannase production at the incubation temperature of 35°C (48 U/g/ min) and further decreased considerably at higher temperatures. The present work is on par with the result of Kumar *et al.* (2007) who observed maximum tannase production (64/U/g) at 30°C.

The present result coincides with the results of Malgireddy and Nimma (2015) who observed maximum tannase production (46.2 U/mg) at 30°C by *Aspergillus terreus*. Lal and Gardner *et al.* (2012) reported the maximum tannase activity of 195.0 U/ml at an incubation temperature of 30°C by *Aspergillus niger*.

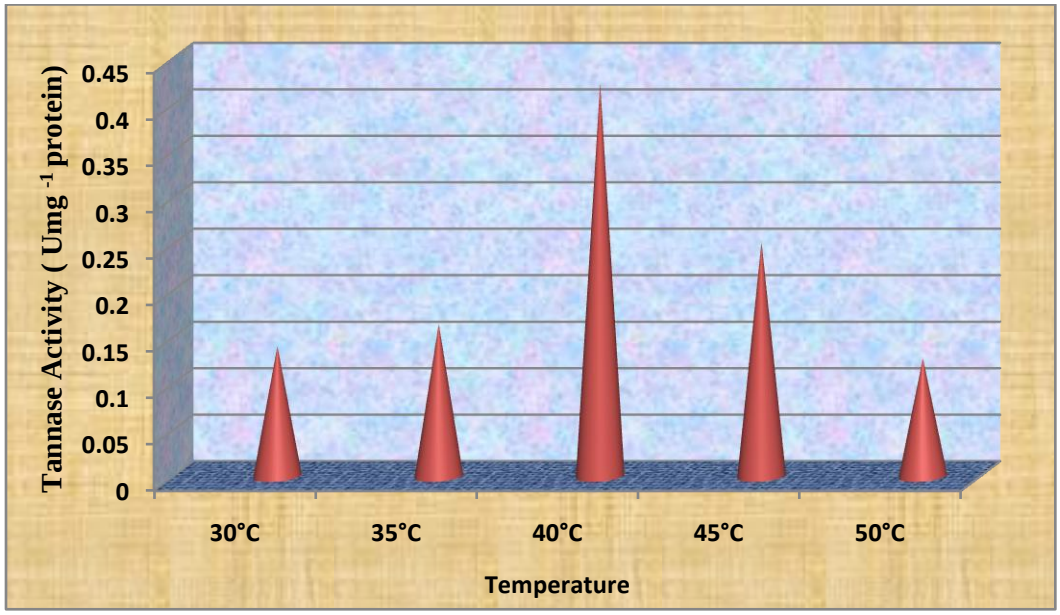
FIGURE-IV

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on Banana peel as substrate on different temperature

a. At Intracellular level



b. At Extracellular level



3. Effect of pH on enzyme production in Banana waste as substrate

TABLE-IV

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on different pH at 3%Banana spathe and peel as a substrate at 30°C

Substrate		4.0	4.5	5.0	5.5	6.0	SEd CD ($p<0.05$)
Banana Spathe	Intra cellular	0.018±0.016	0.087±0.033	0.208±0.010	0.142±0.008	0.085±0.018	0.023
	Extra cellular	0.074±0.007	0.083±0.007	0.353±0.010	0.247±0.070	0.051±0.029	0.048
Banana Peel	Intra cellular	0.017±0.019	0.086±0.007	0.345±0.019	0.257±0.039	0.260±0.024	0.025
	Extra cellular	0.085±0.011	0.194±0.040	0.399±0.051	0.199±0.050	0.111±0.006	0.052

Results are the mean ± standard deviation of triplicates

Umg^{-1} = μ molgallic acid released min^{-1} mg^{-1} protein

There was a continuous increase in production of tannase between range of pH from 4.5 to 5.0 and optimal enzyme units were observed at pH 5. 0. Beyond this range there was a considerable deterioration in the enzyme production by *Aspergillus nidulans*. This might be due to the fact that pH affects enzyme activity by determining the nature of amino acid at active site, which undergo protonation and deprotonation and by the confirmational changes induced by ionization of amino acid.

The enzyme activity at both extracellular and intracellular level by *A.nidulans* at different temperature ranges was found to increase for 30°C to 40°C and peaked at 40°C and further increase in the temperature showed a decreasing trend. (Table–III & Figure–III a & b).

An increase in the production of tannase was observed between the pH ranges 4.5 and 5.0 respectively in both spathe and peel. The tannase activity in Banana spathe at an intra and extracelluar level showed an enhanced activity from 0.018±0.016 to 0.208±0.010 Umg^{-1} protein production and from 0.074±0.007 Umg^{-1} protein to 0.353±0.010 Umg^{-1} protein production up to a pH of 5.0 and after that its activity decreased (0.085±0.018 Umg^{-1} protein and 0.051±0.029 Umg^{-1} protein) at a pH of 6.0 by *A.nidulans* on the 10^{th} day of incubation. (Table-IV & Figure – V a & b)

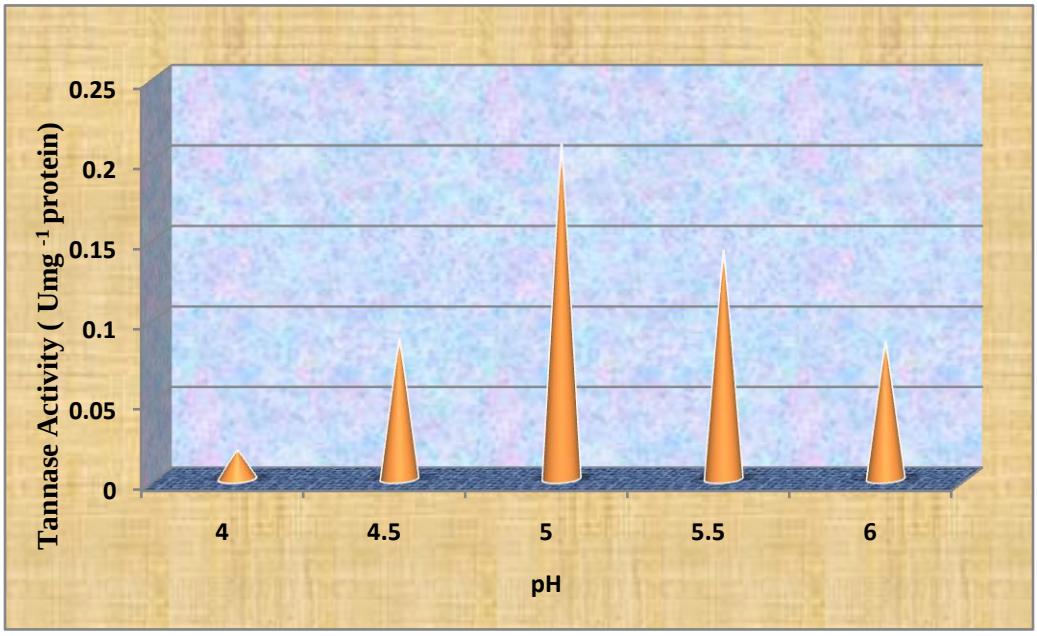
The tannase activity at an intra and extracellular level showed an enhanced activity from 0.017±0.019 Umg^{-1} protein to 0.345±0.019 Umg^{-1}protein and from 0.085±0.011 Umg^{-1} protein to 0.399±0.051 Umg^{-1}protein in Banana peel as substrate up to a pH 5.0 and after that its activity decreased to 0.260±0.024 Umg^{-1} protein and 0.111±0.006 Umg^{-1} protein at a pH of 6.0 by *A.nidulans* on the 10^{th} day of incubation. (Table-IV & Figure –VI a & b)

The present result is related to the findings of Kumar *et al. (*2007) who obtained a maximum tannase activity of 68U/g at pH 5.5. Similary, Sabu *et al.* (2005), Lokeswari *et al.* (2007) and Mangrola *et al.* (2014) reported the optimum pH of 5.5 for tannase production by *Aspergillus niger* ATCC 16620 and *Aspergillus niger*. The present result is related to the findings of Lokeshwari and Kumar (2013) who obtained a maximum tannase activity of 32.62 U/ml by *Aspergillus oryzae* at pH 5.0.

FIGURE-V

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on Banana spathe as substrate on different pH

a. At Intracellular level



b. At Extracellular level

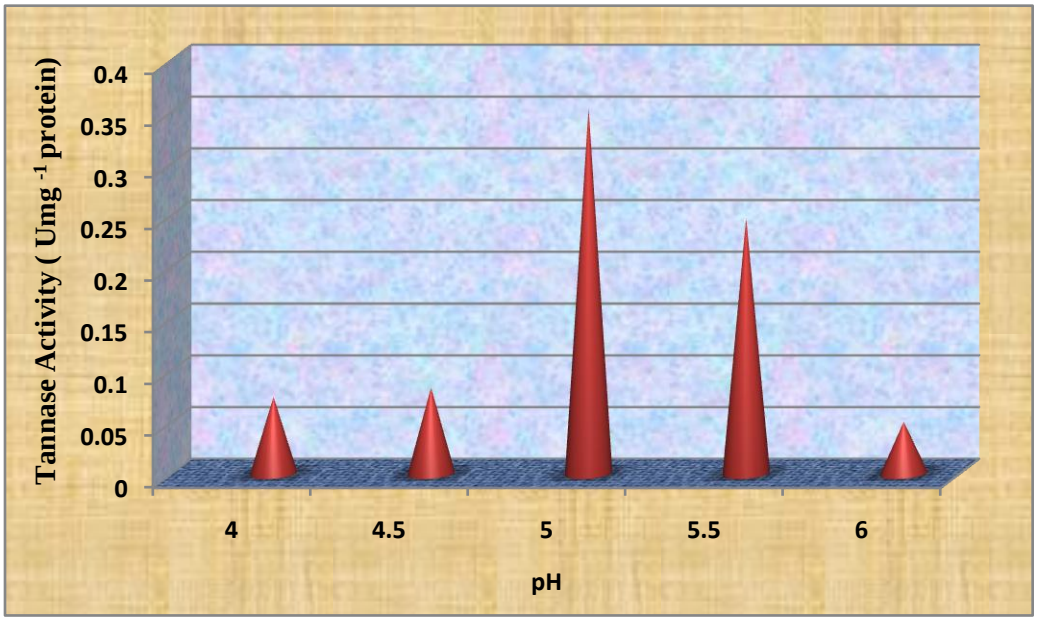
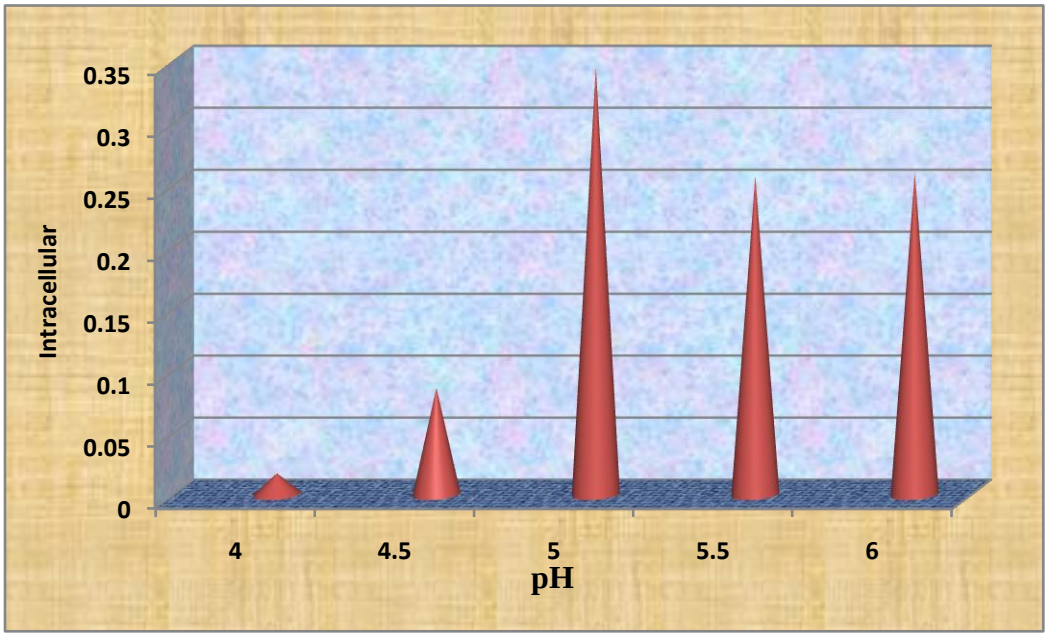


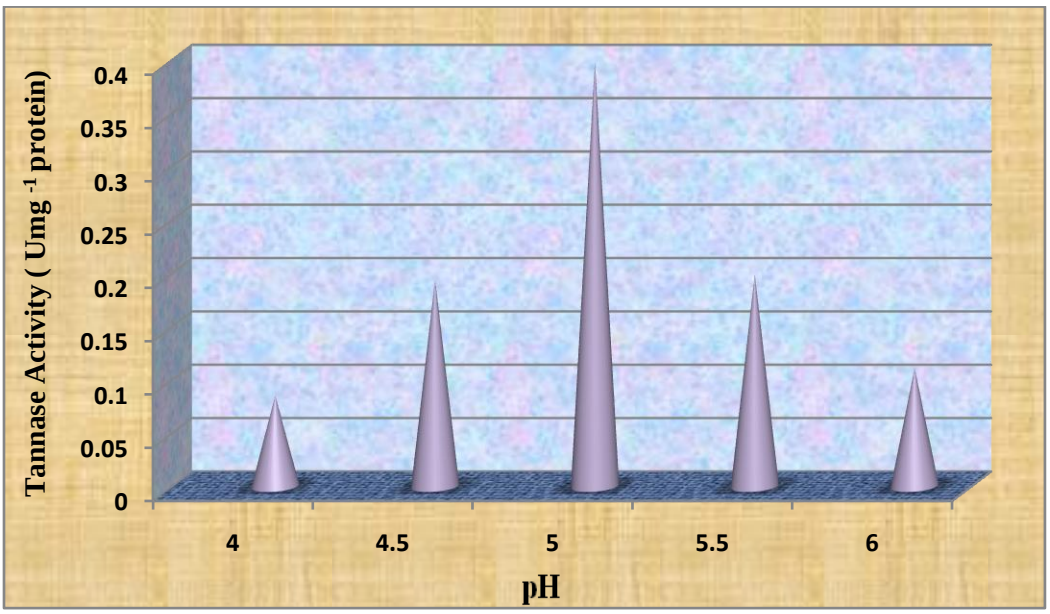
FIGURE- VI

Tannase activity (Umg^{-1} protein) of *Aspergillus nidulans* on Banana peel as substrate on different pH

a. At Intracellular level



b. At Extracellular level



Optimized conditions of tannase production (Table V)

Aspergillus nidulans analyzed for the production of tannase with banana waste as substrate (peel and spathe) of 1, 2, 3and 4 percent concentrations, different pH ranges (4.0, 4.5, 5.0, 5.5, 6.0) and different temperature ranges (30°C, 35°C, 40°C, 45°C and 50°C) for a period of 6, 8, 10 and 12 days of incubation. An enhanced activity was observed in 3% percent banana peel waste as substrate at a pH of 5.5 and in 40°C temperature.

Table-V

Overall Summary of Optimization of Tannase from *Aspergillus nidulans*

Parameters	Tannase (Umg^{-1} Protein)
Banana waste as substrate (3% banana spathe)	1.052±0.003
pH (5.5)	0.257±0.039
Temperature (40°C)	0.423±0.032

CHAPTER - V

SUMMARY AND CONCLUSION

Tannins are water-soluble polyphenolic substances and have ability to bound proteins that form insoluble or soluble tannin-protein complexes. As a consequence, tannins are able to make complex with polysaccharides (cellulose, hemicelluloses and pectin) nucleic acids, steroids, alkaloids, and saponins (Maheri-sis *et al.*, 2011).

Tannin acyl hydrolase (EC 3.1.1.20), commonly referred to as tannase, is an important enzyme with various industrial applications. It possess both esterase and depsidase activities and catalyzes the hydrolysis reactions of the ester bonds, in particular hydrolyzes the ester and depside bonds present in galloyl groups of gallotannins and hexa hydroxyl diphenoyl groups of ellagitannins (Daniel *et al*., 1991). Tannase is widely utilized in industrial sector, food and in pharmacy.

Banana is the largest produced and maximum consumed amongst the fruits cultivated in India. It is known as the 'common man's fruit'. It is highly nutritive and very delicious. India ranks first amongst the banana cultivating countries of the world with an annual production share of 25 % of the total harvest. The land of our country is suitable for banana production. Its fruit is a healthy diet and demandable in local markets as well as the free waste could be utilized to produce bio-products which will contribute directly in our national economy. When the flowers get matured, the spathe begins to fall off. It forms the major agrowaste in plantain field when compare to other parts and is rich in fibre and used as a cattle feed. Women can also be employed in production of different bio-products from banana wastes and thus, they can contribute to their livelihood improvement

The present research was undertaken to investigate the influence of different concentrations of Banana spathe and peel waste on tannase activity by *Aspergillus nidulans* on 5, 7, 9, and 11 days of incubation. To achieve this goal, an *in vitro* investigation was carried out to assess the enzyme production by the efficient tannolytic fungus *Aspergillus nidulans* obtained

from the Department of Botany, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-43.

The results of the investigation are summarized below.

1. Among the different concentration (1, 2, 3 and 4%) of the substrate highest tannase activity was recorded in 3% banana spathe followed by 4% on the 10^{th} day of incubation of *A.nidulans*, compared to control at an intracellular level.
2. The highest tannase activity was recorded in 3% followed by 4% compared to control at an intracellular level using banana peel as substrate on the 10^{th} day of incubation by *A.nidulans*.
3. An enzyme production at an intracellular level b*y A.nidulans* was found to be increased from 30°C to 40°C and further increase in temperature after 40°C declined its activity on the 10^{th} day of incubation.
4. There was a continuous increase in the production of tannase between ranges of pH from 5.0 to 5.5 and the optimized enzyme units were observed at pH 5.5.

Conclusion

In most of the countries where the economy is largely based on agriculture and farming practice is very intensive, accumulation of agricultural residues is a serious problem. Submerged fermentation technology using non-pathogenic microorganisms which can produce hydrolytic enzymes such as tannase will be advantageous for the proper utilization of these residues. Utilization of agricultural residues through biotechnology is becoming more and more significant with the dual goal of waste disposal and value addition. Since microbial activity especially fungal activity is the key aspect in this area, there is enormous opportunity for the cost effective production of tannase, which is an important enzyme in the food and pharmaceutical industry.

In conclusion, tannase has now been extensively used in different biochemical industries. The selected fungi *Aspergillus nidulans* used in this study is able to synthesize high amounts of tannase through submerged fermentation. Exploitation of banana wastes (peel and spathe) could be a source of cheaper substrate for industrial production of microbial tannase. The present investigation suggests that agro residues such as spathe and peel of banana can be one of the best and cost effective alternatives to the costly pure tannic acid production of microbial tannase *Aspergillus.*

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