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TANNASE ENZYME EXTRACTION FROM *Aspergillus niger* UNDER SOLID STATE FERMENTATION USING LEAVES AS THE SUBSTRATE AND THEIR APPLICATION ON ANTIBACTERIAL ACTIVITIES.

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***Abstract*:** Tannase enzyme producing fungal spores were isolated from spoiled bread. The extracellular fungal spores that producing maximum yield of enzyme was identified as *Aspergillus niger*. Enzyme production was studied under solid state fermentation using different rich tannin substrates like indian almond leaves (*Terminalia catappa*), amla leaves (*Phyllanthus emblica*), jamun leaves *(Syzygium cumini)* and tamarind leaves (*Tamarindus indica*). Compared to all of these substrates indian almond leaves were found to be the best substrate for enzyme production under solid state fermentation (SSF). The maximum production of tannase was found to be at 30°C after 72hrs of incubation. The crude enzyme was separated by using the column chromatography from that fractions were analyzed that serine ($0.2R_f$) amino acid from indian almond leaves substrate using thin layer chromatography (TLC), and jamun leaves substrate has tannase enzyme functional groups using Fourier-transform infrared analysis (FTIR). Then the tannase enzyme was shows the higher inhibitory activity against the *Pseudomonas sp.*, while applied on antibacterial activities.

***Keywords*:** Tannase, *Aspergillus niger*, indian almond leaves, solid state fermentation, antimicrobial activities.

INTRODUCTION

Tannase is a key enzyme in the degradation of gallotannins, a type of hydrolysable tannins. The systemic name of this enzyme is Tannin acylhydrolase. Other name in common use includes Tannase S and Tannin acetylhydrolase. Tannase (tannin acyl hydrolase) specifically breaks the galloyl ester bond of tannins and produce gallic acid and glucose. Because of this property, the enzyme has been extensively used in the fields like foods, beverages, pharmaceutical and

chemical industries, wine making, beer chill proofing etc. It is present in a diverse group of microorganisms, plants, animals. However microorganism and plants are mainly used for commercial production of tannase enzyme. Microorganisms have been reported to produce tannase capable of hydrolyzing tannins to gallic acid during fermentation. Tannins are present in the many leaves, shrubs, roots, seeds, fruits etc... These were produced the tannins in higher number and these can be used as the solid substrates for tannase enzyme production.

Solid state fermentation is defined as the fermentation process occurring in the absence or near absence of free water that employs the natural substrate as the inert support for production of enzymes like tannase, amylase, pectinase etc… Solid State Fermentation is a batch process using natural heterogeneous materials containing complex polymers like lignin, pectin, lignocelluloses, tannin. Tannase production by solid state fermentation (SSF) is more advantages over submerged (or) liquid surface fermentation. Tannase in SSF was due to better growth of *Aspergillus niger* in solid state fermentation. Resulting in higher biomass production and more efficient biosynthesis of enzymes under conditions without catabolic repression moreover, the breakdown of enzymes by contaminating proteases was eight times (SMF) than in solid state fermentation.

The most important means for obtaining tannase is in the use of microorganisms because they can produce enzymes continuously and in large qualities in addition, the enzymes thus obtained are more stable compound to those obtained by other means. The main genera of fungi known as producers of tannase are *Aspergillus*, *Penicillum*, *Fusarium* and *Trichoderma*. Tannase from fungal sources to be highly active over a wide range of PH and temperature.

Although tannase production by *Aspergillus niger* can occur in the absence of tannic acid concentration as high as (20% w/v). Without having a deleterious effect on both the growth and enzyme production. The crude tannin obtained from various natural resources is used for tannase production to minimize the making cost.

The crude enzyme were to be extracted using different types of leaves *Syzygium cumini* (Jamun leaves), *Terminalia catappa* (Indian almond leaves), *Phyllanthus emblica* (Amla leaves), *Tamarindus indica* (Tamarind leaves**).** The present study aims to extraction of tannase enzyme from the *Aspergillus niger* under solid state fermentation by using different types of dried leaves as the substrate and their antimicrobial application on antimicrobial activities.

MATERIALS AND METHODS

Culture collection:

A.niger used in the present study was isolated from the spoiled bread and characterization was carried out by 1gm of black spores from spoiled bread and mix with sterile distilled. Aqueous and spread plate over Rose Bengal agar and incubate in room temperature for 3-4 days. After the growth culture was characterized and maintained at 4℃.

Tannin degradation by *A.niger*:

- *A.niger* has higher tannin degrading capacity and produce tannase
- Mix 0.2% of tannic acid to SDA (Sabouraud Dextrose Agar) and platted with *A.niger* for the tannin degradation

Raw material:

Leaves of *Terminalia catappa* (Indian almond), *Syzygium cumini* (Jamun), *Tamarindus indica (*Tamarind) and *Phyllanthus emblica* (Amla) was taken from the local farmer field. Leaves were properly dried at 60℃ and powdered in mixer/grinder.

Tannin estimation of substrate:

For tannin estimation, prepare the leaves extract using solvent (Methanol) 50ml and mix 5gm of dried leaves powder and kept at 4℃ for overnight incubation. Then filtrate the extract using

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Whattman no.1 filter paper and further used for the tannin analysis in substrate.

1) Ferric chloride test**:** Suitable quantities of various leaves substrates (2ml) were taken and tested for the presence of tannins with $fecl_3$ formation of greenish-black colour indicates the presence of tannins.

2) Lead acetate test**:** 1ml of filtrate was added to 3drops of the lead sub acetate solution. A cream gelatinous precipitate indicates the presence of tannins.

Inoculum preparation:

Sporulated culture lawn of the *A.niger* was picked up and mixed well in sterilized distlled.H_2O with 0.1% (Tween 80).

Tannase production by solid state fermentation:

Five grams of each substrate was taken in 250ml Erlenmeyer flask. These act as an inert material and also provide nutrients to the organism. The moisture content of the culture medium was kept as 60% (v/w) using Mineral salt solution (KNO_3 0.2%), (KH_2PO_4 0.1%), ($MgSO_4.7H_2O$ 0.05%), (Kcl 0.05%) with PH 6.0. the flask were sterilized at 121℃ for 30mins and cooled. After cooling 1ml of spore cell suspension (5×10^8 spores/ml). And incubated at 30℃ for 96 hours.

Enzyme extraction:

After the incubation period, 25ml of 1% (w/v) Nacl was added to the flasks and the enzyme was extracted from solid substrate by shaking at 200rpm for 1hour in rotatory shaker. Then the extract

was passed through the whattman no.1 filter paper and filtrate the substrate. The filtrate was collect in vials and preserved at 4℃ for further enzyme analysis.

Enzyme assay:

1) Column chromatography:

Sample: The samples to be taken are the tannase crude enzyme of jamun, Indian almond, and amla leaves extract.

Stationary phase: silica gel.

Mobile phase: Methanol.

Stationary phase was prepared in 3 different column tubes and left over night for settlement. Then the stagnant sol vent was removed and add tannase enzyme sample of 3 different substrates.

Then the mobile phase (methanol) added gradually until the final fractions separated. From the eluted fractions dark colored fractions were taken, and proceed for TLC, FTIR analysis.

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2) **Thin layer chromatography (TLC):**

Thin Layer Chromatography is a technique used to isolate non-volatile mixtures. The experiment is conducted on a sheet of aluminium foil, plastic, or glass which is coated with a thin layer of adsorbent material. The material usually used is aluminium oxide, cellulose, or silica gel. On completion of the separation, each component appears as spots separated vertically. Each spot has a retention factor (R_F) expressed as: R_F = dist. travelled by sample / dist. travelled by solvent.

3) **FTIR Analysis:**

- Fourier Transform Infrared (FTIR) Spectroscopy is commonly referred to as FTIR
- This infrared spectroscopy method is used to identify organic, polymeric, and in some cases, inorganic materials. The FTIR test relies on infrared light to scan samples and observe bond properties
- Powdered sample mixed with potassium bromide and placed in a pellet (KBr pellet)

Enzyme application on antimicrobial activities

- **Preparation of broth cultures:**

Tannase enzyme was active against several organisms such as *E.coli, Staphylococcus aureus, streptococcus sp., Klebsiella sp., Pseudomonas sp., Bacillus sp.,* etc… in these organisms (*E.coli,*

Pseudomonas sp., S.aureus) were taken and prepare the nutrient broth culture and incubate over night at 37℃ and used for the antibacterial analysis.

- **Antimicrobial activity:**

Antimicrobial activity was performed by agar well diffusion method. The 20µl of overnight broth culture was swabbed on MHA plates with sterile cotton swabs and allow the plates for 2-3 minutes, the well was punctured with well puncture, and then the 20µl of tannase crude enzyme was mix with the 1ml of DMSO (dimethyl sulfoxide) loaded on the well.

RESULT AND DISCUSSION

Isolation of *A.niger*:

A.niger was isolated from the spore from spoiled bread were platting on SDA that produces the black spores and the growth of *A.niger* was confirmed by the LPCB (Lacto phenol cotton blue) method (that has carbon black/dark brown colour of spores with large hyphae that distinguish the *niger* from other sp).

Degradation of tannin by *A.niger*:

A.niger has highest tannin degradation capacity Break tannin protein complex and degrades tannins especially hydrolysable tannins.

Determination of tannin from leaves substrate:

Ferric chloride: By addition of $FeCl_3$ the substrate extract was turned into greenish black colour. That indicates the presence of tannins in the substrates leaves.

Lead acetate: By addition of lead sub acetate the substrate extract produces the creamy gelatinous precipitate. That indicates the presence of tannin in the substrate leaves.

Enzyme production:

Crude enzyme was prepared from the solid state fermentation after the 96hours of incubation at 30℃.

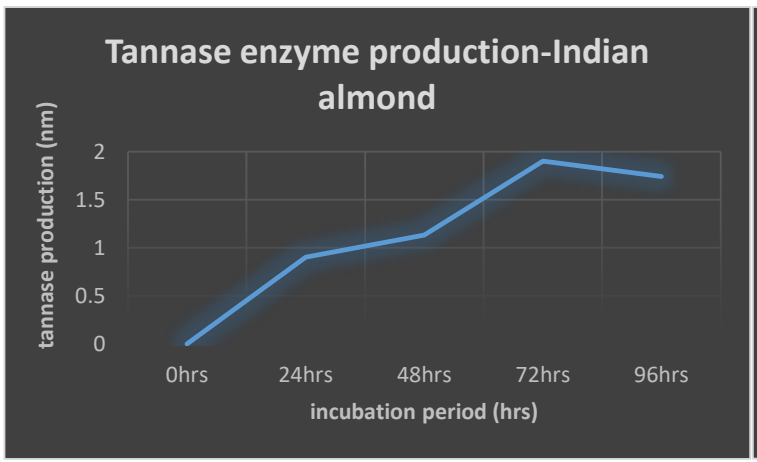


Figure 1: Indian almond leaves as substrate

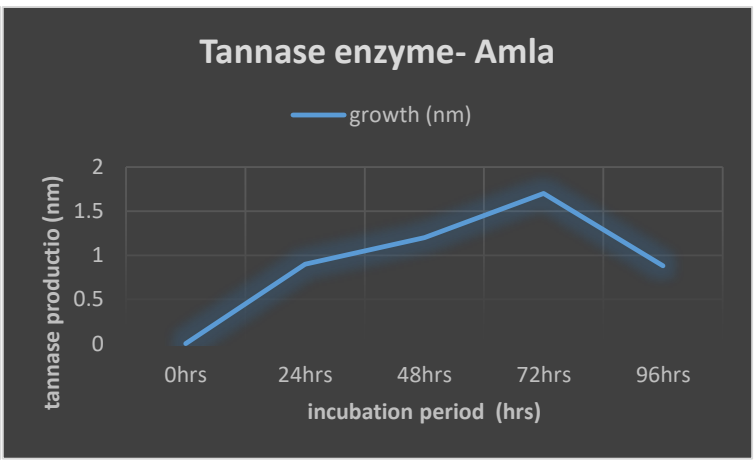


Figure 2: Amla leaves as substrate

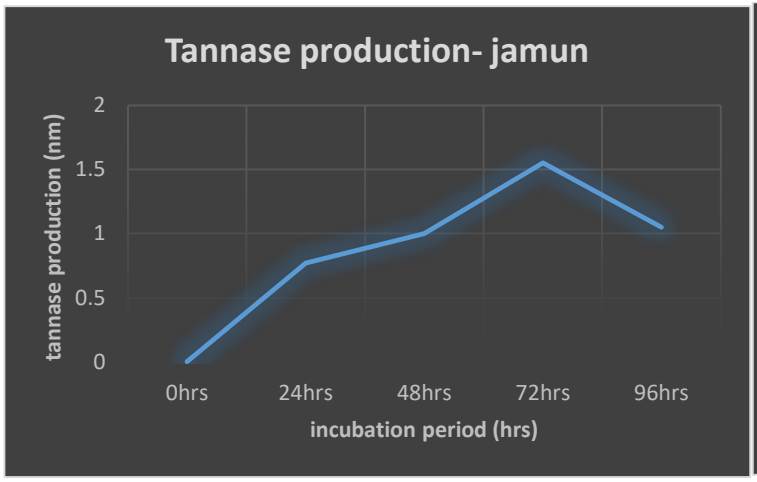


Figure 3: Jamun leaves as substrate

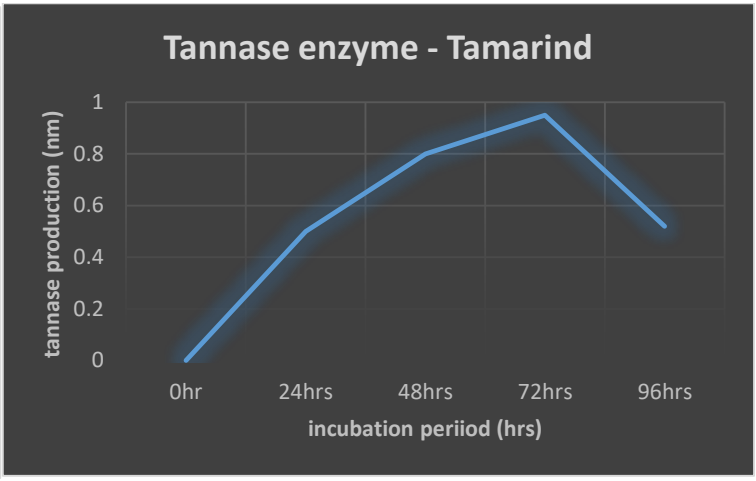


Figure 4: Tamarind leaves as substrate

Thus enzyme production was starts after the 48 hours of incubation period in all the substrate that introduced. The highest amount of enzyme production was at (fig-1) Indian almond leaves substrate (1.90nm), (fig-2) Amla leaves substrate (1.7nm), (fig-3) Jamun leave substrate (1.55nm) and (fig-4) tamarind leaves substrate (0.95nm) at the 72hrs of incubation.

Enzyme assay

1) **Column chromatography:** The tannase crude enzyme produced using the different leaves substrate. In that effective production of tannase by the three substrates were taken for the identification of the individual compound from the mixture of compounds.

From that dark colored fractions were to be taken for the further analysis, they are, Thin layer chromatography, FTIR (Fourier-transform infrared spectroscopy).

2) **Thin layer chromatography (TLC):** The amino acids present in the tannase enzyme was serine and glycine.

R_F = dist. travelled by sample / dist. travelled by solvent. Was shown in (table 1)

Table 1: determination of amino acids present in tannase.

S.no	TYPES OF LEAVES SUBSTRATES	Rf VALUE	Amino acids	Presence of Amino acids in tannase
1.	Indian almond leaves	0.2	Serine	+
2.	Amla leaves	0.14	Lysine	_
3.	Jamun leaves	0.36	Threonine	_

- Indian almond leaves as substrate = 1.7/6.9

  R_F = 0.2

The amino acids present in Indian almond leaves substrate was the serine, thus the serine was found in the tannase enzyme.

- Amla leaves as substrate = 1/6.

  RF = 0.14

The amino acids present in the amla leaves substrate was the lysine, thus the lysine was not found in the tannase enzyme.

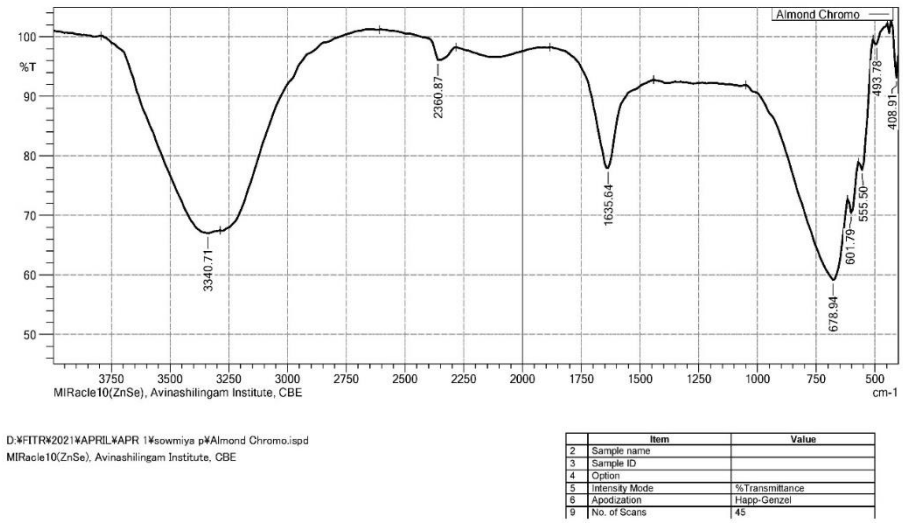
- Jamun leaves as substrate = 2.5/6.9

  RF = 0.36

The amino acid present in the Jamun leaves as substrate was the threonine, thus the threonine was not found in tannase enzyme.

Among these three substrates, tannase enzyme produced by Indian almond leaves as a substrate was only has the amino acid (serine).

FTIR Analysis: The Graph (fig-5, 6, 7) represents Fourier Transform of Infra-Red analysis for different samples of Tannase Enzyme. The substrates contains Indian almond leaves, amla leaves, and Jamun leaves. The main functional groups present in these materials are secondary amines, carbon dioxide, alkene, amine, halo compound etc…From these, three main groups (secondary amines, alkene, and halo compounds) are present in the sample of jamun leaves. So this sample shows the higher stability than the others.



	Item	Value
2	Sample name	
3	Sample ID	
4	Option	
5	Intensity Mode	%Transmittance
6	Apodization	Happ-Genzel
9	No. of Scans	45

Figure 5: Determination of FTIR analysis on tannase enzyme produced by Indian almond leaves substrate

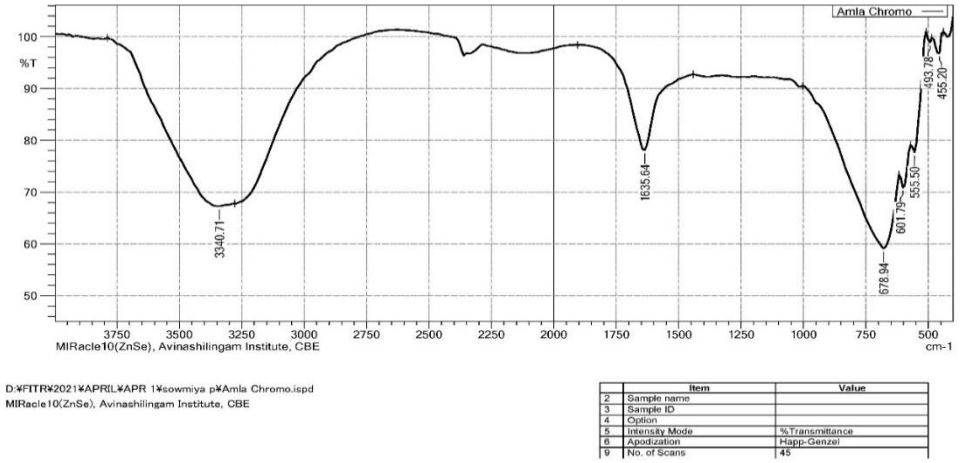


Figure 6: Determination of FTIR analysis on tannase enzyme produced by Amla leaves

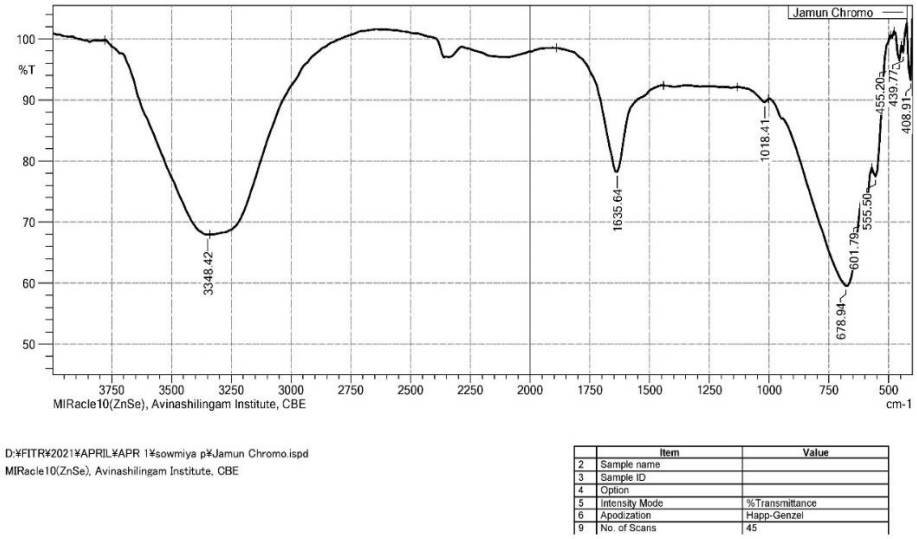


Figure 7: Determination of FTIR analysis on tannase enzyme produced by jamun leaves as substrate

These were the figures of FTIR analysis of tannase enzyme produced by different solid substrates.

Antibacterial activities of tannase enzyme:

Table 2: Determination of antibacterial activity of tannase crud enzyme

S.No	SUBSTRATE	ORGANISMS (mm)					
		E.coli	Standard	*S.aureus*	Standard	*Pseudomonas*	Standard
1.	AMLA	-	9	4	7	9	11
2.	INDIAN ALMOND	-	9	4	10	9	11
3.	JAMUN	5	9	7	10	-	10
4.	TAMARIND	-	15	-	8	4	13

From the results obtained from table 2: the sample *Syzygium cumini* (jamun) were effective against *S.aureus*, *Phyllanthus emblica* (amla) were effective against *Pseudomonas sp.*, *Terminalia catappa* (indian almond) were effective against *Pseudomonas sp.*, *Tamarindus indica* (tamarind) were effective against *Pseudomonas sp.*, Most of the samples were effective inhibitory against the *Pseudomonas sp.*, and some of them were show intermediate inhibitory activity against *S.aureus* and the streptomycin was taken as positive control and it was sensitive to all the three organisms that have taken.

DISCUSSION

The present is aimed at the production of tannase enzyme by *A.niger* Solid state fermentation offers a number of conventional medium for the enzyme production. The production medium is often simple using natural substrate like amla leaves, Indian almond leaves, jamun leaves, and tamarind leaves. Jamun leaves and amla leaves were serves good substrate, Here in this study we are reporting Indian almond leaves and tamarind leaves as the solid substrates for the production of tannase. The taken leaves were served as the good substrates for enzyme production. The organism (*A.niger)* taken for the enzyme production was tannin degradable and that produced the tannase

SUMMARY AND CONCLUSION

Tannase enzyme having the great industrial applications. It can be produced by fungal spore *A.niger* and by several tannin contained leaves such as *Terminalia catappa* (Indian almond), *Phyllanthus emblica* (amla), *Syzygium cumini* (jamun), *Tamarindus indica* (tamarind). The highest enzyme production was detected at 30℃ and 72hrs.

A.niger was spore producing fungi that produces the tannase enzyme by breaking down the tannin content in the substrate.

The tannase crude enzyme was produced by adding 25ml of 1% of Nacl and filtrate the crude enzyme by whattman no.1 filter paper and stored at 4℃ for further enzyme analysis. The growth curve of tannase enzyme production was determined and the compounds present in tannase enzyme was detected by thin layer chromatography (TLC) method and Fourier-transform infrared (FTIR) method from the eluents of column chromatography method.

Antibacterial activity of tannase enzyme was detected against the clinical pathogens by the agar well diffusion method by the diffusion of organisms and enzyme.

Tannase enzyme has major diversity owing to the diverse area of applications on food, feed industry, beverage and brewing industries , chemical and pharmaceutical firms extending from the making of acorn wine, instant tea, gallic acid, and flavored cool drinks.

The enhanced activity of enzyme production was by *A.niger* and varieties of leaves as the solid substrates, the maximum amount of enzyme was produced at 72hrs of incubation after that the production was reduced. The amino acids in the produced enzyme was serine by *Terminalia catappa* (Indian almond leaves), and functional group was secondary amines, alkenes, amines and hallo

compounds by *Syzygium cumini* (jamun leaves). The applications over the antimicrobial activities shows sensitive to *Pseudomonas sp.*, Hence the present study concluded that *Terminalia catappa* (Indian almond) leaves substrates produces the higher amount of tannase enzyme.

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