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Optimal conditions for production of Tannase from newly isolated Aspergillus terrus under solidstate fermentation

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Abstract
Tannase production under solid-state fermentation was investigated using isolated *Aspergillus terreus.* Among all agro –industrial waste material evaluated, wheat bran supported maximum tannase production. Solid material regulated the enzyme production and yield was improved with the supplementation of carbon and nitrogen sources to the solid medium. Maximum enzyme production was achieved with 1.5% Sucrose and 1.75% yeast extract. Glucose did not repress enzyme production but inorganic nitrogen sources showed little negative impact. The physiological fermentation factors such as pH of the medium (pH 3.5) moisture content (60%), incubation time (72 h) and inoculum level (3ml) played a vital role in tannase production. The enzyme production was found to be associated with the growth of the fungal culture.

Keywords: solid State fermentation, tannase, Aspergillus terreus, enzyme extraction

1. 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 with molecular masses ranging from 0.3 to 5 kDa (Haslam, 1989). In nature, tannins are widely distributed worldwide among different families of higher plants including tara, gall, oak, sumach, trillo, myrobalan and so on. They are also found in common foods such as tea, cashew nut, hazelnut, walnut, grape, mango, strawberry, raspberry, blackberry, etc. and high concentrations of tannins are present in different plant parts such as leaves, fruits, bark, wood, seeds and roots (Li *et al*., 2006). 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 (Lekha and Lonsane, 1997; Auguilar and Gutierrez-Sanchez, 2001; Belmares *et al*., 2004), which is based on two differences such as the sugar content and the availability as substrates to tannase. Condensed tannins do not have sugar residues comprising of only flavan-3-ol or flavan-3-4-diol polymers, while hydrolysable tannins are polyesters of a sugar moiety (or other non-aromatic polyhydroxy compounds) and organic acids. These compounds undergo hydrolytic cleavage on treatment with dilute acids to release the respective sugar and acid moiety. If the acid component is gallic acid or ellagic acid, the compounds are known as gallotannins or ellagitannins (Serrano *et al*., 2009). Most ellagitannins are mixed esters comprising of both hexahydroxydiphenic acid (forms ellagic acid when hydrolyzed with elimination of water) and gallic acid.

It was earlier established that high molecular mass tannins have a stronger anti-nutritional effects and lower biological activities. The plausible reason is that high molecular mass tannins form complexes with proteins, carbohydrates and digestive enzymes such as amylase, lipase, protease, pectinase and cellulase, and thus reduce the nutritional values of feeds (Chung *et al*., 1998). Thus, tannins have toxic or anti-nutritional implications on ruminants, which reduce their feed intake with lower nutrient digestibility and protein bioavailability (Barry *et al*., 1986; Lowry *et al*., 1996). In contrast, small molecular mass tannins including monomeric, dimeric and trimeric tannins are considered less anti-nutritional and are readily absorbed by the animal (Butler and Rogler, 1992). It was

demonstrated that medicinal herbs exhibited noticeable biological and pharmacological activities such as anti-carcinogenic, antitumor, antiviral and inhibition of lipid peroxidation due to the presence of small molecule tannins (Okuda *et al*., 1992). Further, *in vitro* and *in vivo* investigations indicated that ellagic acid significantly inhibits cancer formation in the colon, oesophagus, liver, lung, and skin of rats and mice, and is thus identified as a possible chemotherapeutic agent against human carcinogenesis (Lee, 1992). Tannic acid and ellagic acid sulfate were earlier demonstrated to exhibit anti-HIV activity (Mizumo *et al*., 1992). Considering the benefits of small molecule tannins, the biodegradation of gallotannins and ellagitannins can be explored using microbial tannases.

Tannase (tannin acyl hydrolase, EC 3.1.1.20), is an industrially important inducible enzyme produced by different filamentous fungi like *Aspergillus, Penicillium, Fusarium* and *Trichoderma* species (Iibuchi *et al.*, 1967; Rajakumar and Nandy, 1983; Lekha and Lonsane, 1994; Bajpai and Patil, 1996) along with bacteria (Deschamps *et al*., 1983; Skene and Brooker, 1995) and yeasts (Aoki *et al*., 1976). 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 hexahydroxydiphenoyl groups of ellagitannins (Daniel *et al*., 1991). 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.

The present investigation was aimed at isolation of fungal cultures from soil samples collected from different biosphere zones of India and screening of different isolates available in the lab culture collection for identifying a promising tannase producing culture. Further, the optimization of production conditions for a promising culture was standardized under solid state fermentation.

Materials and Methods

Chemicals

All chemicals were of analytical grade purchased from Himedia Biosciences Mumbai and all the substrates for SSF were procured from the local dhal mills.

Microorganism

Aspergillus terreus was isolated from soil collected from different biospheare zones of India. The culture was maintained on a Potato Dextrose Agar plates containing tannic acid and maintained at 4^0 C and sub-cultured at monthly intervals.

Preparation of Spore Suspension:

Since tannase is an induciable enzyme, pre-induced inoculum was used which was prepared using potato dextrose agar medium where 2% tannic acid was used as a sole carbon source. Induced inoculum was prepared with 2ml spore suspension and was incubated at 30 ^{0}C under shaking condition for 72h.

Substrates

Different agro-industrial waste materials were collected from the local dhal mills and processed. Red gram husk, Green gram husk, Ground nut waste, Cotton seed waste, Wheat bran, Rice bran, Coffee husk, Tamarindus seed powder, Cashew apple bagasse, Coconut powder, Corn powder and Cycer aritinum were used.

Enzyme extraction

The enzyme was extracted according to the method described by Bradoo *et al.*, 1996. Fermented medium was mixed thoroughly with 50 ml phosphate buffer in orbital shaker for 10 min and squeezing through a cloth, separated the extract. This process was repeated two times and extracts were centrifuged at 5000 rpm for 20 min. The supernatant was used as enzyme source for tannase assay.

Measurement of protein activity

Protein activity was determined by using lowery *et al* (1959) method.

Measurement of tannase activity

Tannase activity produced by *Aspergillus terreus* isolate was assessed using the rhodanine method (Sharma *et al.*, 2000).

The method is based on the formation of a pink colored chromogen with gallic acid released by the action of tannase on methyl gallate, which was read at 520 nm using a spectrophotometer (Shimadzu UV 160A, Japan).

One unit of tannase activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under the defined reaction conditions.

Optimization of fermentation process

Factors like selection of solid substrate, initial moisture content, inoculum level, incubation temperature, initial pH, incubation time, amount of substrate, various carbon and nitrogen additives etc, are known to influence the production of metabolites during SSF. Experiments were conducted to improve the production of tannase by *Aspergillus terreus*, by optimizing the following parameters. These were optimized by changing one independent variable while fixing the others at a certain constant level (Tunga *et al*., 1998; Gresham and Inamine, 1986). All the experiments were conducted in triplicate and the average values are represented.

The optimum conditions obtained in each parameter were applied to the subsequent experiments. The following parameters were investigated on the production of tannase. Effect of different substrates, Effect of incubation time, Effect of inoculum level, Effect of pH, Effect of Temperature, Effect of moisture content , Effect of different carbon sources, Effect of different nitrogen sources, Effect of salt solution level, Tannase production with all the optimized parameters

Effect of different substrates

The results in the present study may varied with the type of agro waste. This could be attributed to solid materials dual roles supply of nutrients to the microbial culture, different agro industrial waste materials Red gram husk, Green gram husk, Ground nut waste, Cotton seed waste. Wheat bran, Rice bran, Coffee husk, Tamarindus seed powder, Cashew apple bagasse, Coconut powder, Corn starch, Cicer aritinum

were used for the production of tannase. 10g of substrate was taken into 250ml Erlenmeyer flasks and the flasks with the production medium (pH 5.0) were inoculated as above and incubated at 30^0 C for 72 h. The general procedure mentioned earlier was followed for tannase production and assay.

Results and Discussion

Screening for Tannase Producers

About 100 fungal strains isolated from soil samples collected from different biosphere zones of India were screened for tannase activity on petri plates supplemented with tannic acid as substrate. About eight fungal isolates were short-listed which exhibited positive tannase activity (**Figure 1**). The zone diameter of the tannase activity exhibited by the fungal isolates on tannic acid supplemented plates is shown in **Figure 2**.

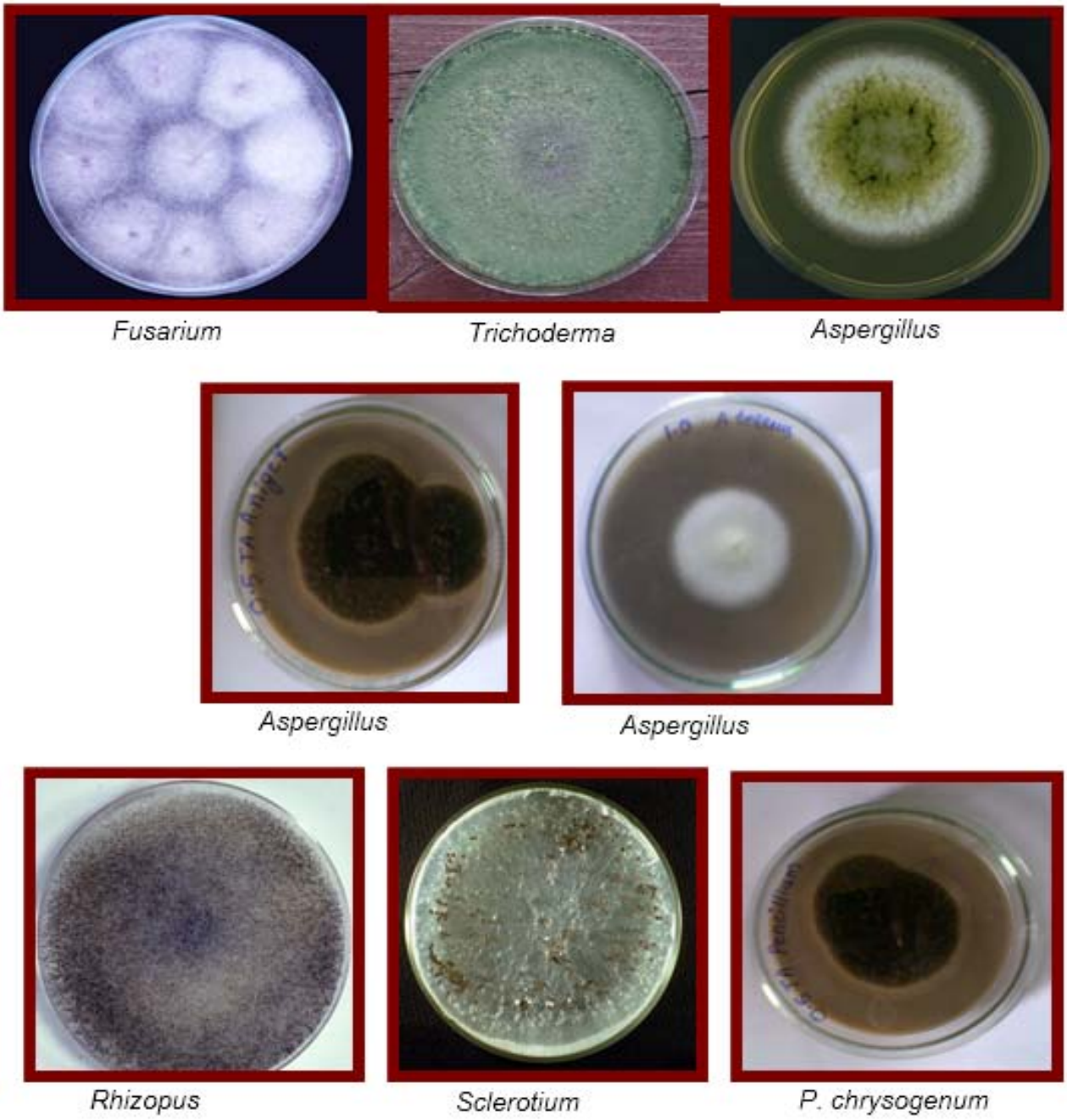

Fig 1: Positive cultures exhibiting tannase activity

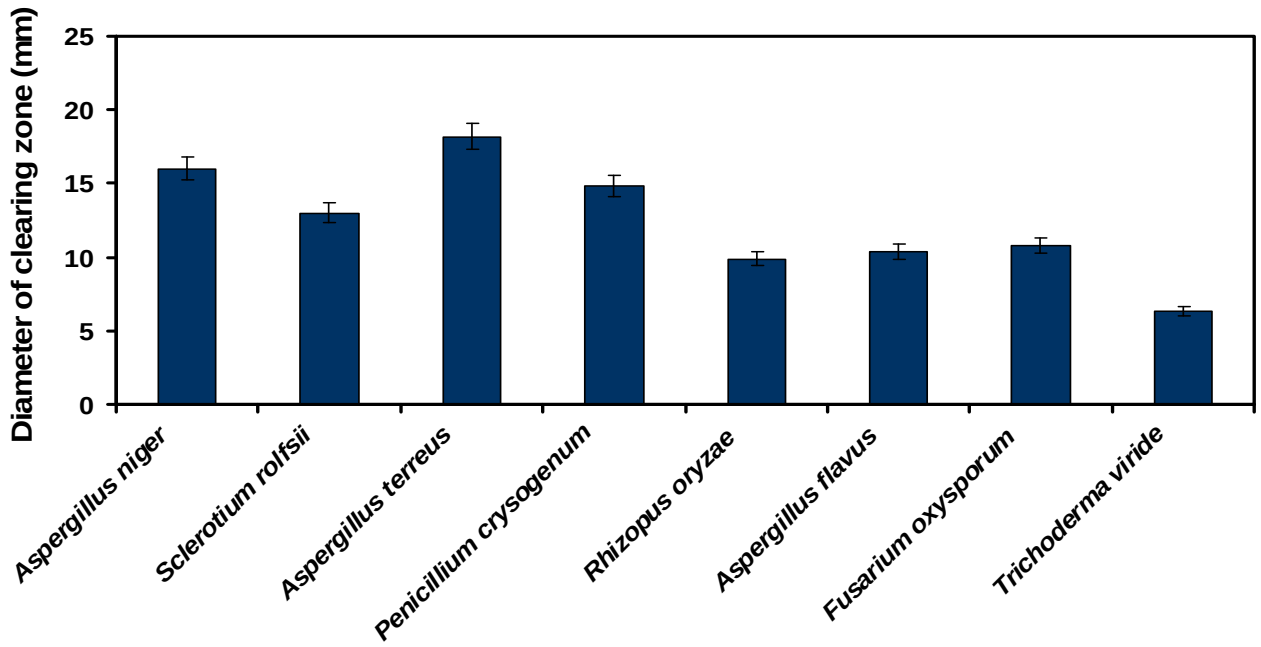

Fig 2: Zone diameter of tannase producing fungal strains

These eight short-listed fungal strains were further subjected to screening for checking tannase activity by submerged fermentation in mineral salts medium. The enzyme activity shown by the individual fungal isolates is shown in **Figure 3**.

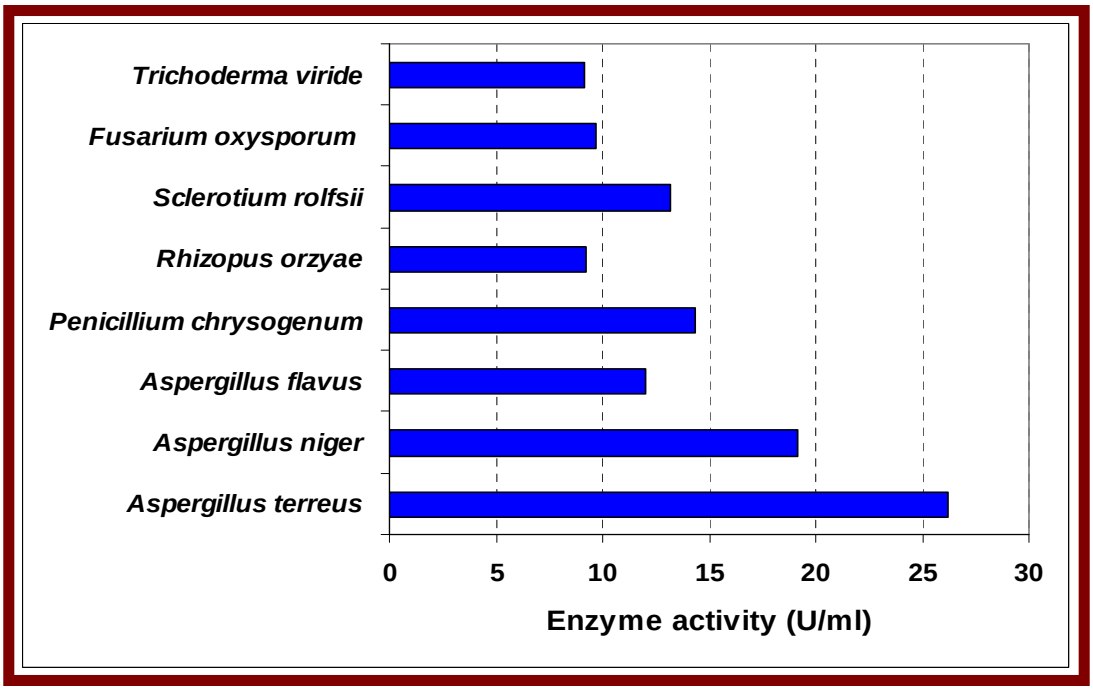

Fig 3: Tannase activity measurement for fungal strains

Optimization of Solid State Fermentation Conditions For Tannase Production

1. Effect of different substrates

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* were supplemented to the production medium at 0.1-1 % level. It was observed that the tannase production varied with the type of agro waste. Maximum enzyme production was observed with wheat bran (41.6 U/mg), while minimum tannase production (37.6 U/mg) was noticed with cottonseed powder as substrate (**Figure 4**).

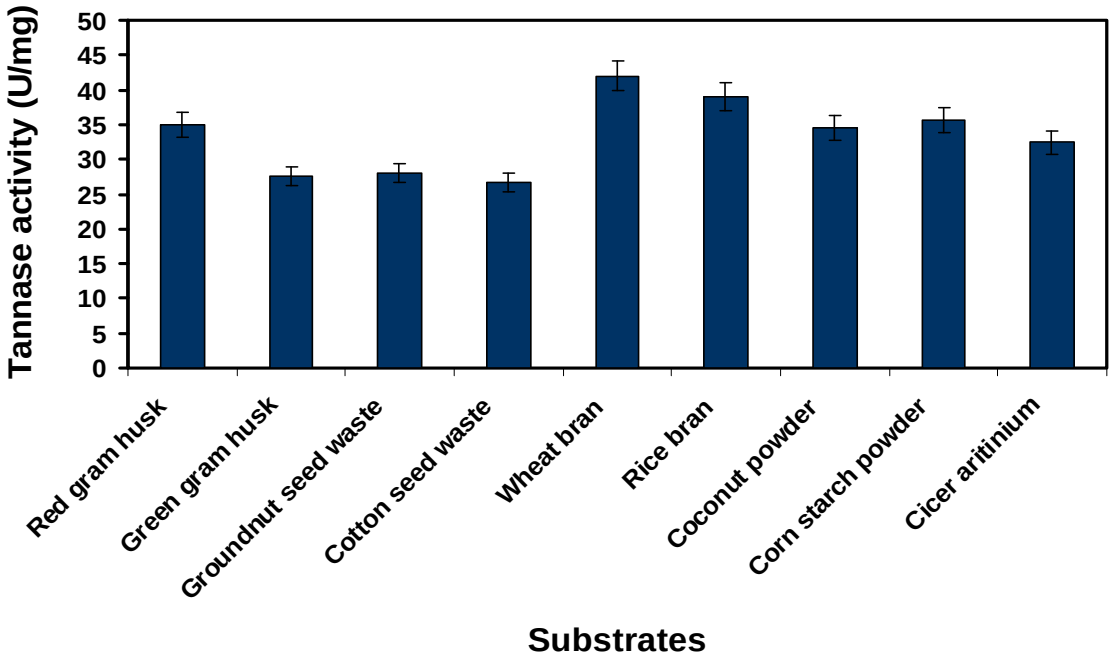

Fig 4: Effect of different agro wastes as solid substrate on tannase production

2. Effect of incubation time

To evaluate the effect of different incubation period on tannase production, the incubation period of the medium was varied from 24 h to 168 h. Maximum yield of tannase was obtained after 3 days (72 hours) of incubation i.e. 42.4 U/mg (**Figure 5**).

3. Effect of inoculum level

The initial microbial load to a medium does affect the growth and in turn favours metabolite production. To study the effect of inoculum level, experiments were conducted by adding inoculum in volume ranging from 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 ml to the production medium. The results indicate that tannase production was increased with increase in level of inoculum up to 3 ml level (44.8 U/mg) and further increase in inoculum level did not increase the tannase production (**Figure 6**).

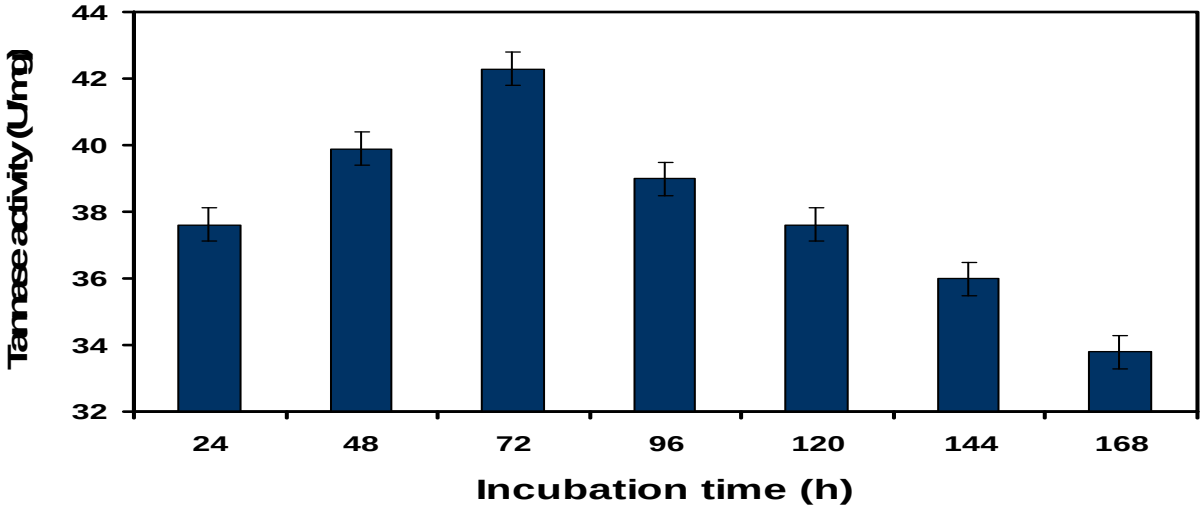

Fig 5: Effect of incubation time on tannase production

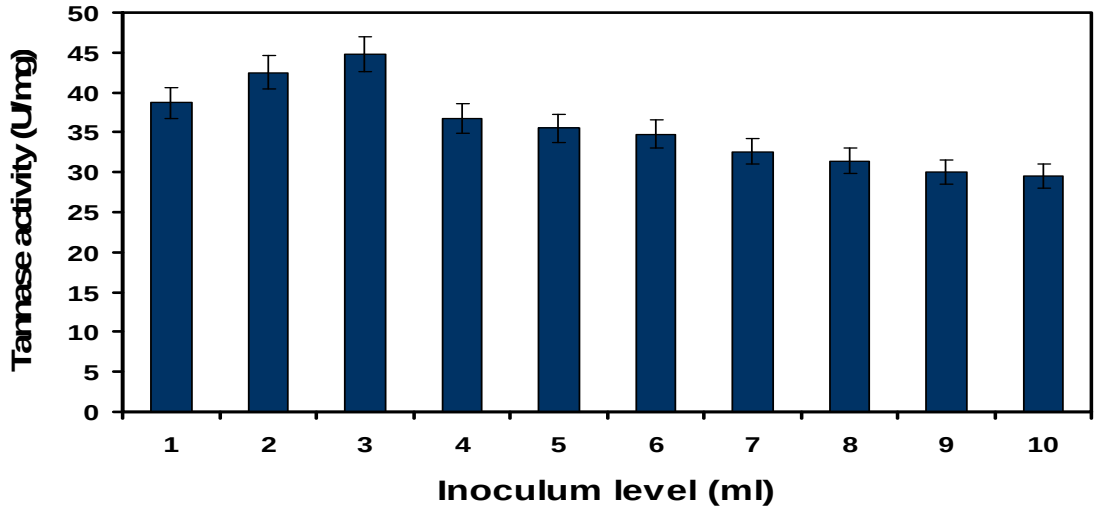

Fig 6: Effect of inoculum level on tannase production

4. Effect of pH

The effect of initial medium pH on tannase yield was studied by varying the initial pH values (1.0-12.0) of the production medium. The organism produced reasonable amounts of tannase in basic and in highly acidic conditions, and a highest yield of 44.5 U/mg was recorded at pH 4.0 (**Figure 7**).

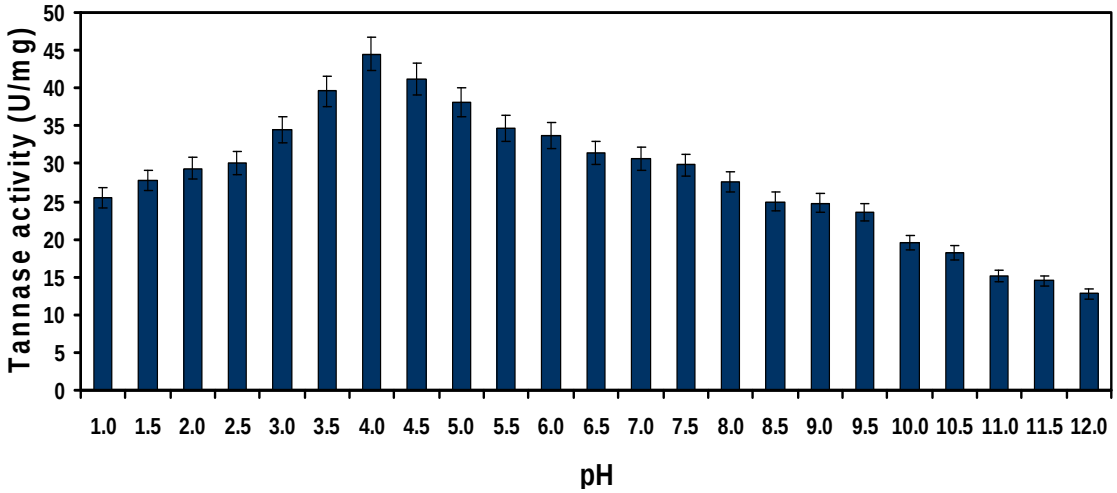

Fig 7: Effect of pH on tannase production

5. Effect of Temperature

The effect of various temperatures (26°C, 28°C, 30°C, 32°C, 34°C and 36°C) on the growth and tannase production were studied. The results indicated that the organisms grew over a wide range of temperatures (26°C to 36°C). Maximum tannase production (46.2 U/mg) was observed at 30°C at 72 h. An increase in incubation temperature to 36°C decreased the yield to 31.6 U/ml. Hence the optimum incubation temperature for tannase production is 30°C (**Figure 8**).

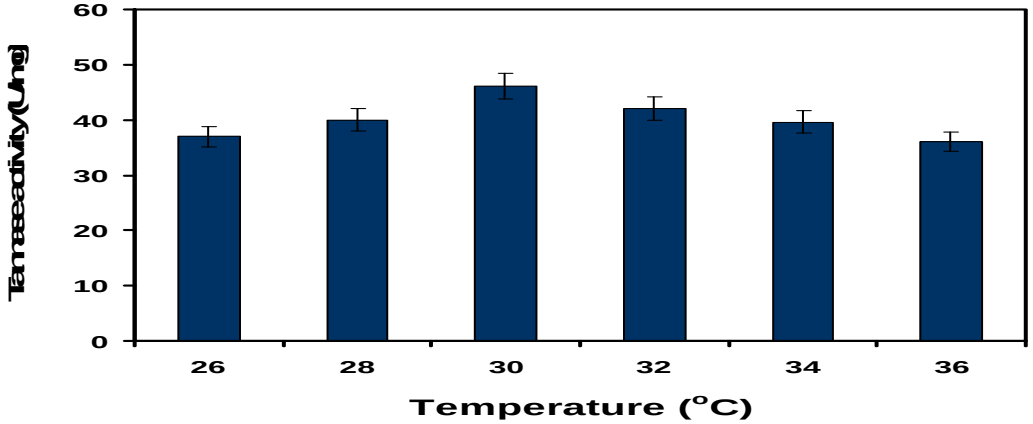

Fig 8: Effect of temperature on tannase production

6. Effect of moisture level

The effect of moisture content on the growth and tannase production was studied at different moisture levels (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%). Maximum enzyme production was observed with 6 % moisture content (46.2 U/mg). Further increase in moisture level in the fermentation medium resulted in reduction of tannase production (see **Figure 9**).

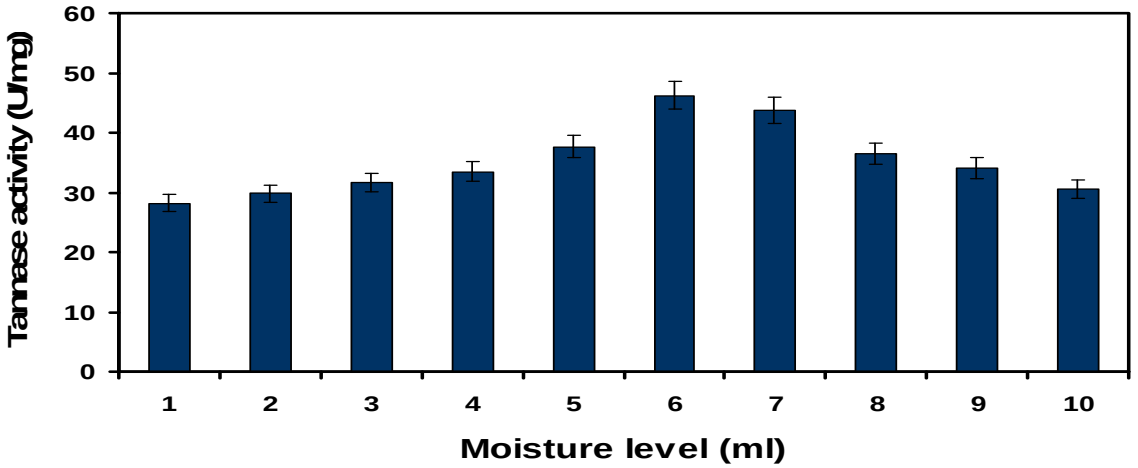

Fig 9: Effect of moisture level on tannase production

7. Effect of different carbon sources

The effect of different carbon sources (Glucose, Fructose, Galactose, Sucrose, Maltose, Mannose, Xylose, Lactose and Glycerol) were studied on the growth and tannase production. Maximum enzyme production was observed with sucrose (46.7 U/mg) (**Figure 10)**.

8. Effect of different nitrogen sources

The effect of different nitrogen sources (yeast extract, beef extract, peptone, casein, $NaNO_3$, $AgNO_3$) on the growth and tannase production were studied. Maximum enzyme production was observed with yeast extract (46.1 U/mg) (**Figure 11)**.

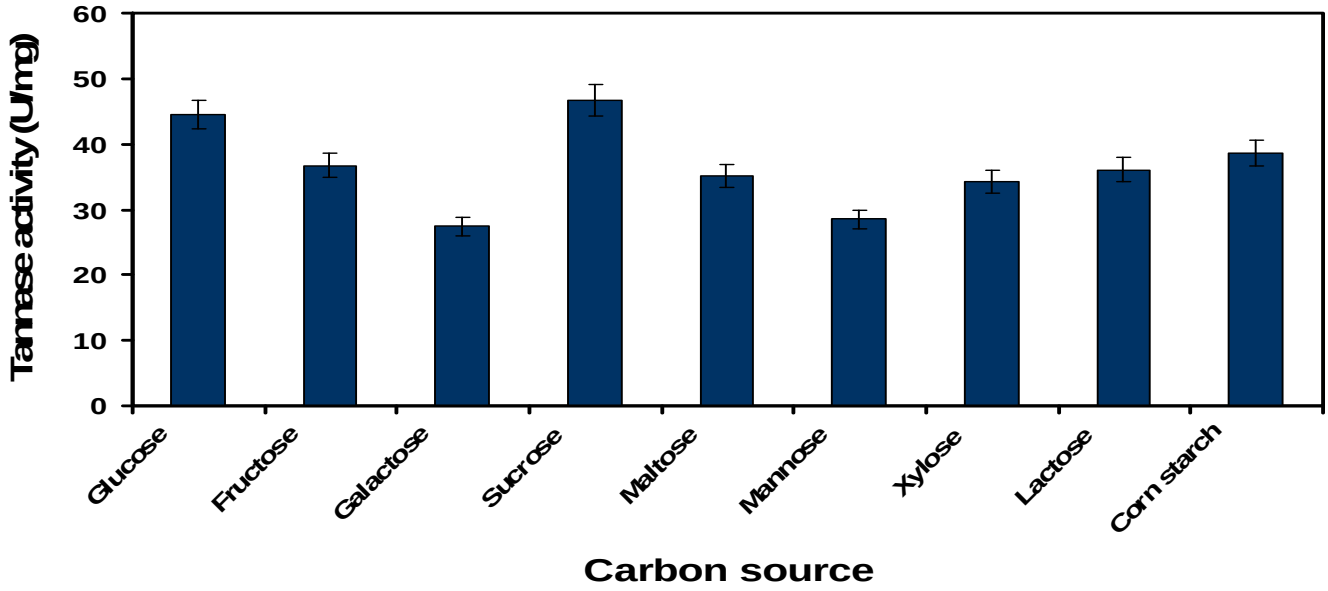

Fig 10: Effect of different carbon sources on tannase production

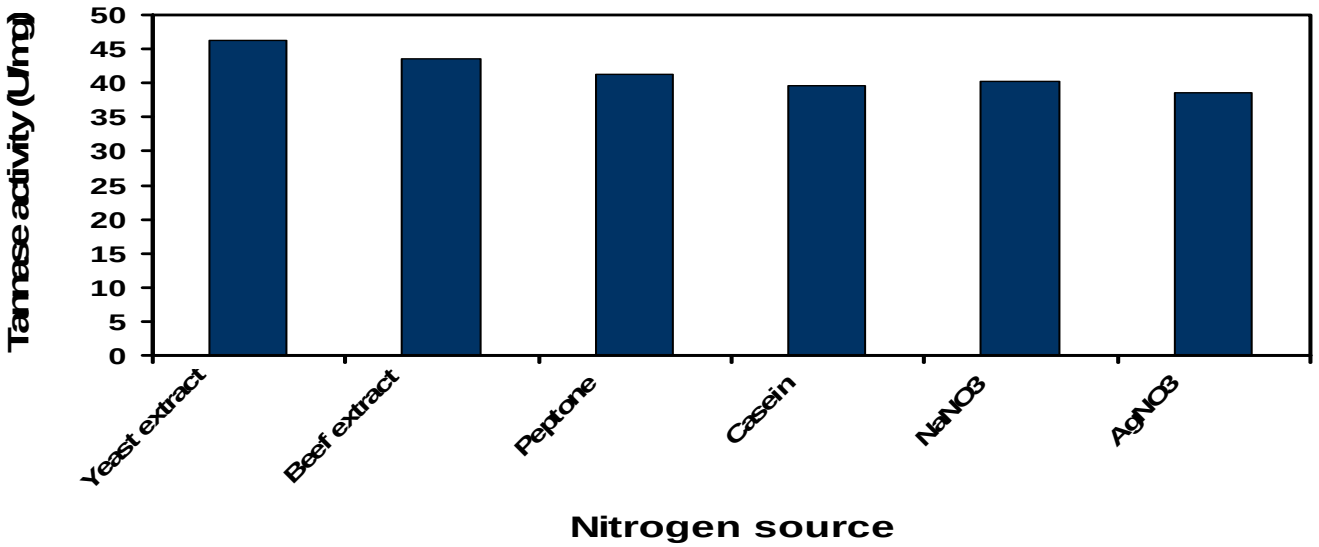

Fig 11: Effect of different nitrogen sources on tannase production

9. Effect of salt solution level

To study the effect of salt solution level on the growth and tannase production, different volumes of salt solution (0.5 ml, 1 ml, 1.5 ml, 2 ml, 2.5 ml, 3 ml, 3.5 ml, 4 ml, 4.5 ml and 5 ml) were used. Maximum enzyme production (47.3 U/mg) was observed with addition of 1 ml of salt solution to the production medium (**Figure 12**).

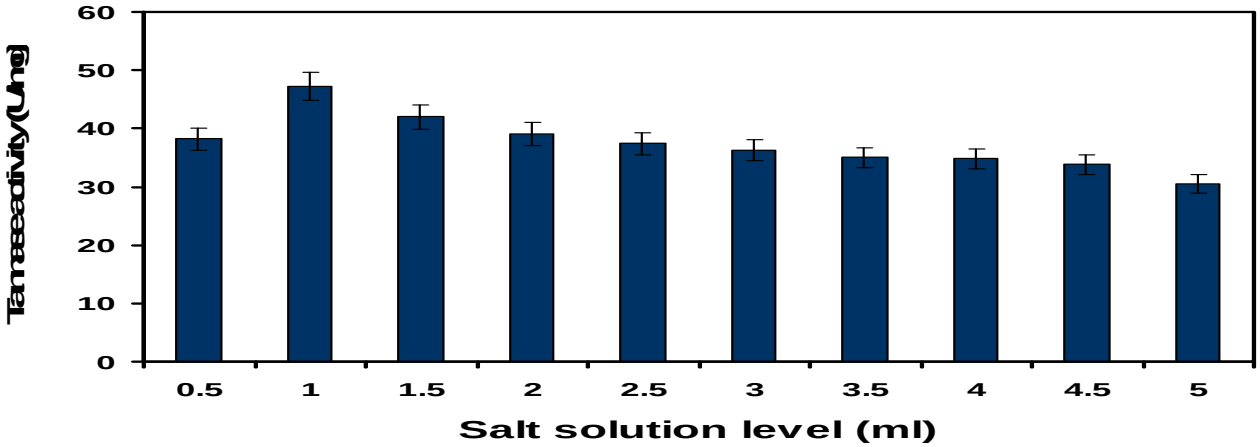

Fig 12: Effect of salt solution level on tannase production

Conclusion:

The present work has been taken up with a view of exploring the possibilities of using Wheat bran as a substrate and *Aspergillus terreus* as a microbial source for the production of tannase which can hydrolyze Tannic acid to gallic acid. Tannin acyl hydrolase is an industrially important enzyme that is mainly used in the food and pharmaceutical industry. As the range of applications of this enzyme is very wide there is always a scope for novel tannase with better characteristics, which may be suitable in the diverse fields of applications. Solid state fermentation technology using non pathogenic microorganisms which can produce hydrolytic enzymes such as tannase will be advantageous for the proper utilization of these residues. 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.

The culture conditions for the production of tannase enzyme from *Aspergillus terreus* was evaluated and standardized. These conditions were: solid state fermentation with, incubation temperature of 30 °C, fermentation time of 72 h, pH of 3.5, moisture content of 6ml, substrate concentration of 10 g/L wheat bran, inoculums of 3ml, sucrose as carbon source, yeast extract as nitrogen source and 1ml salt solution level used for the tannase production. The final yield of tannase obtained through solid state fermentation is 46.8 U/mg.

Tannase has been shown to be a very versatile enzyme. The enzyme finds application in the food, beverage, industrial and pharmaceutical industry.

References

1. Aguilar, C.N. and Gutiérrez-Sanchez, G. (2001) Review: sources, properties, applications and potential uses of tannin acyl hydrolase. *Food Sci. Technol. Int*. **7**: 373–382.
2. Aoki, K., Shinke, R. and Nishira, H. (1976a) Purification and some properties of yeast tannase. *Agric. Biol. Chem*. **40**: 79–85.

3. Bajpai, B. and Patil, S. (1996) Tannin acyl hydrolase (EC 3.1.1.20) activity in *Aspergillus, Penicillium, Fusarium* and *Trichoderma*. *World J. Microbiol. Biotechnol.* **12**: 217–220.
4. Barry, T.N., Manley, T.R., and Duncau, S. J. (1986) The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 4 Sites of charbohydrate and protein digestion as influenced by dietary reactive tannin concentration. *Br. J. Nutr*. **55**: 123–137.
5. Belmares, R., Contreras-Esquivel, J.C., Rodríguez-Herrera, R., Coronel, A.R. and Aguilar, C.N. (2004) Microbial production of tannase: An enzyme with potential use in food industry. *Lebensm.-Wissen. – Technol*. **37**: 857–864.
6. Butler, L.G. and Rogler, J.C. (1992) Biochemical mechanisms of the anti-nutritional effects of tannins in phenolic compounds in food and their effects on health. Chapter 23. In: Ho, C.T., Lee, C.Y. and Huang, M.T. (eds.), ACS Symposium Series 506, American Chemical Society, pp. 298–304.
7. Chung, K.-T., Wei, C.-I. and Johnson, M.G. (1998) Are tannins a double-edged sword in biology and health? *Trends Food Sci. Technol*. **9**: 1–8.
8. Daniel, E.M., Ratnayake, S., Kinstle, T. and Stoner, G.D. (1991) The effect of pH and rat intestinal content on the liberation of ellagic acid from purified and crude ellagitannins. *J. Nat. Prod*. **54**: 946–952.
9. Deschamps, A., Otuk, G. and Lebeault, J. (1983) Production of tannase and degradation of chestnut tannin by bacteria. *J. Ferment. Technol.* **61**: 55–59.
10. Haslam, E. (1989) Plant Polyphenol – Vegetable Tannins Revisited. Cambridge University Press, Cambridge, Cambridge, pp. 9, 122.
11. Iibuchi, S., Minoda, Y. and Yamada, K. (1968) Studies on tannin acyl hydrolase of microorganisms. Part III. Purification of the enzyme and some properties of it. *Agric. Biol. Chem*. **32**: 803–809.
12. Lee, K.H. (1992) Plant phenolics compounds as cytotoxic antitumour agents in phenolic compounds in food and their effects on health. II. Antioxidants and cancer prevention. Chapter 29, In: Ho, C.T., Lee, C.Y. and Huang, M.T. (eds.), ACS Symposium Series 506, American Chemical Society, pp. 367–379.
13. Lekha, P.K. and Lonsane, B.K. (1994) Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid-state, liquid surface and submerged fermentations. *Process Biochem*. **29**: 497–503.
14. Lekha, P.K. and Lonsane, B.K. (1997) Production and application of tannin acyl hydrolase: state of the art. *Adv. Appl. Microbiol*. **44**: 215–260.
15. Li, M., Yao, K., He, Q. and Jia, D. (2006) Biodegradation of gallotannins and ellagitannins. *J. Basic Microbiol*. **46**: 68–84.
16. Lowry, J. B., McSweeney, C. S. and Palmer, B. (1996) Changing perceptions of the effect of plant phenolics on nutrient supply in the ruminant. *Aust. J. Agric. Res*. **47**: 829–842.
17. Mizumo, T., Uchino, K., Toukairin, T., Tanabe, A., Nakashima, R., Yamamoto, N. and Ogawara, H. (1992) Inhibitory effect of tannic acid sulfate and related sulfates on infectivity, cytopathic effect and giant cell formation of human immunodeficiency virus. *Planta Med*. **58**: 535–539.
18. Okuda, T., Yoshida, T. and Hatano, T. (1992) Pharmacological active tannins isolated from medicinal plants. *Basic Life Sci*. **59**: 539–569.
19. Rajakumar, G.S. and Nandy, S.C. (1983) Isolation, purification and some properties of *Penicillium chrysogenum* tannase. *Appl. Environ. Microbiol.* **46**: 525–527.
20. Serrano, J., Puupponen-Pimiä, R., Dauer, A., Aura, A.-M. and Saura-Calixto, F. (2009) Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. (Review) *Mol. Nutr. Food Res*. **53**: S310–S329.
21. Sharma, S., Bhat, T.K. and Dawra, R.K. (2000) A spectrophotometric method for assay of tannase using rhodanine. *Anal. Biochem*. **279**: 85–89.
22. Skene, I.K. and Brooker, J.D. (1995) Characterization of tannin acyl hydrolase activity in ruminal bacterium, *Selenomonas ruminantium. Anaerobe* **1**: 321–327.