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Research Article

TANNASE PRODUCTION BY *ASPERGILLUS FLAVUS* ON DIFFERENT TANNIN RICH SUBSTRATES

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ABSTRACT

Utilization of agricultural residues through Biotechnology is becoming more and more significant with the dual goal of waste disposal and value addition. The present research was undertaken to compare the influence of different substrates on tannase activity by Aspergillus flavus by utilizing agro wastes such as tea, coffee, tamarind and white popinac leaves in a submerged fermentation system. To achieve this goal, an in vitro investigation was carried out to assess the enzyme production by the efficient tannolytic fungus Aspergillus flavus isolated from tealeaf waste disposal soil. Among the number of mycoflora Aspergillus flavus showed maximum hydrolyzing zone for tannolytic activity, it was selected as a candidate for enzyme study. Among the different substrates of 0.5g tannase activity by A. flavus was found to be significantly enhanced to a maximum level of 8.96from7.14Umg-1enzyme protein in tealeave satan intracellular level, upto 7th day of incubation and the least tannase activity of was observed in tamarind leaves.

INTRODUCTION

Tannins are naturally occurring plant phenolic compounds that are widelydistributed in different parts (bark, needles, hardwood, graces, seeds & flowers) of vascular plants (Waterman and Mole, 1994). Tannins are a group of complex oligomeric chains substances with high molecular weight (500-300Da), water soluble and polyphenolic secondary metabolites of plants.Tannins are complex organic, non-nitrogenous plant products, having astringent properties and are capable of combining with proteins, cellulose, gelatin and pectin to form insoluble complexes (Paranthaman*et al.,* 2009).

Tannase (tannin-acyl hydrolase) catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins, as tannic acid, resulting in the production of gallic acid and glucose. Tannase has a wide range of applications in industrial, biotechnological, environmental, pharmaceutical and other important fields such as instant tea production, clarifying coffee flavored soft drinks, removing haze, bitterness, astringency and improving the colors, enzymatic treatment for nutritive use of protein and carbohydrates from peas, enhancing the antioxidant activity of green tea and improving the quality of forages used in animal and bird feeds (Lokeswari*et al.*, 2010). The source of tannase production is mainly by the microorganisms like bacteria, yeast and fungi. *Aspergilli*were reported as the besttannase 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 aremicroorganisms, because the enzymes produced by them are more stable than that obtained from other sources.

Agro industrial residues are generally considered the best substrates for the process of enzyme production. The major crop wastes like cotton stalks, jute sticks, and coconut shell and coir pith are used in enzyme production. Several naturally occurring agricultural by-products such as wheat bran, coconut oil cake, 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).The microbial tannase are commercially important as they are more stable than the similar ones obtained from other sources like plant and animal sources and can withstand extreme conditions of temperature and pH. The microorganisms are potential sources for the production of tannase in submerged and solid state fermentation (Gonzalez *et al.*, 2012).

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MATERIALS AND METHODS

Tea leaf waste soil sample was collected from the disposal yard of tea industry, Hubbathalai, The Nilgiri District, Tamil Nadu. The sample was serially diluted and plated on potato dextrose agar medium and incubated for seven days at 30°C. After incubation, the plates were observed for fungal growth and were sub-cultured and maintained on PDA slants at 4°C. The fungal isolates were identified based on their morphology, mycelia structure and spore formation (Domsch and Gams1972).

Primary Screening of Tannolytic Fungi

The isolateswere grown on one per cent tannic acid agar plates and incubated at 28°C. After 5 days of growth, 0.1 per cent congo red solution was added and counter stained with 1M NaCl for 15-20 min. A clear zone of tannase hydrolysis gave an indication of tannase producing microorganism. The diameter of the clear zone was measured to provide a quantitative comparison of tannolytic activity. The fungal strain showing largest zone of decolorization was selected for enzyme production.

Enzyme Production

Erlenmeyer flasks containing 100ml of tannin containing Liquid Medium was sterilized at 1 atm for 15 minutes. After cooling, one ml of Streptomycin sulphate (10,000 ppm) was added and incubated for 3, 5, 7,9 and 11 days at 30°C under static conditions. The mycelium was filtered through Whatman No. 40 filter paper using a Buchner funnelunder suction. The clear filtrate obtained was used as a source of extracellular enzyme. A quantity of 5.0 g of the washed mycelia mat was macerated in five ml of citrate buffer of pH 5.0 in a pre-chilled mortar and pestle with a pinch of acid washed sand. The homogenate was centrifuged and the supernatant was used as crude source of intracellular enzyme.

Tannase Assay (Mondal and Pati, 2000)

The enzyme solution (0.1 ml) was incubated with 0.3 ml of 1.0% tannic acid in0.2M citrate buffer (pH 5.0) at 400 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 and then centrifuged for 10min. The precipitate was dissolved in 2ml of SDS-triethanolamine 1% w/v, and the absorbency was measured at 550 nm after addition of 1ml of $FeCl_3$(0.13 M). A standard graph was prepared with gallic acid in the concentration range of 50µg/ml.The protein concentration was estimated according to the method Lowry *et al* (1951).

Effect of Tannase on Different Substrates

To assess the enhanced enzyme production by the selected fungal strain, differenttannin rich substrates like tea, coffee, tamarind and white popinac leaf powder (0.5g)were supplemented in the fermentation medium for an incubation period of 3, 5, 7, 9 and 11 days.

RESULTS

Screening of Fungi for Tannolytic Activity (Hydrolyzing Zone)

Among the number of mycoflora like *Rhizopusstolonifer, Aspergillusniger, A. flavus, A. fumigatus, Geotrichum*sp., *Penicillium*sp., *Trichodermaviride,* isolatedfrom tea industry waste disposal area soil only 4 fungal strains *Aspergillus flavus, A. fumigatus, Penicillium*sp., *and Aspergillus*sp., showed maximum hydrolyzing zone.A significantly highest hydrolyzing zone (clear zone) of 36 mm out of colonydiameter of 65 mm was shown by *Aspergillusflavus* followed by *Aspergillus fumigatus* (30 mm out of colony diameter of 60 mm). Bradoo *et al*. (1996) carried out tannase activity on tannic acid and agar plates (TAA) containing 1% tannic acid and 3% agar. They observed clear zone of hydrolysis of tannic acid around the fungal colony after 72 h incubation. Batra and *Saxena (2005) observed a good hydrolytic zone*on*tannic acid on agar plates by Aspergillus acolumaris*.

Table 1 The Diameter of Colony and Hydrolyzing Zone

S.NO	Fungal strains	Colony diameter(mm)	Hydrolyzing Zone(mm)
1.	*Aspergillus fumigatus*	60	30
2.	*Penicillium*sp.	35	16
3.	*Aspergillusflavus*	65	36
4.	*Aspergillus*sp.	40	18

Assessing Tannase Production on Different Substrates

At an Intracellular and Extracellular level

There was an increasing trend in the tannase activity by *Aspergillu sflavus* up to 7th day of incubation and after that a decreasing trend was observed. Among the different tannin rich leaves used as substrates (tea leaf –*Camelliasinensis* L., coffee leaf –*Coffea Arabica* L., white popinac –*Leucanaleucocephala* Lam. and tamarind leaf-*Tamarindusindica* L.), the tannase activity by *Aspergillus flavus*was enhanced to a maximum level of 8.96 from 7.14 Umg^{-1} enzyme protein in tea leaves up to 7^{th} day of incubation and its activity declined on the 11^{th} day of incubation gradually to 4.63 Umg^{-1} enzyme protein protein compared to control (from 4.17 to 5.67 Umg^{-1} enzyme protein up to 7^{th} day and declined to 4.67 Umg^{-1} enzyme protein). The tannase activity of white popinac and coffee leaves as substrates were on par with each other. Their values ranged to 8.40 from 6.54 Umg^{-1} enzyme protein and to 8.27 from 5.15 Umg^{-1} enzyme protein up to 7^{th} day of incubation and decreased to 4.66 and 4.43 Umg^{-1} enzyme protein. The least tannase activity of 6.72 from 4.77 Umg^{-1} enzyme protein up to 7th day and decline in its activity to 3.26 Umg^{-1} enzyme protein on the 11^{th} day of incubation compared to control from 4.07 to 4.71 Umg^{-1} enzyme protein up to 7^{th} day and decline its activity 2.87 Umg^{-1} enzyme protein. At an extracellular level, tannase activity by *Aspergillus flavus* showed increased activity from 3.86 to 7.84 Umg^{-1} enzyme protein up to 7^{th}day of incubation and declined to 4.27 Umg^{-1} enzyme protein compared to control from 2.34 to 5.07 Umg^{-1} enzyme protein in white popinac as substrate. The enhanced tannase activity in tea and coffee leaves as substrate by *A. flavus* were on par with each other from 5.46 to 7.57 Umg^{-1} enzyme protein and from 4.65 to 7.55 Umg^{-1} enzyme protein up to 7th day of incubation and after that its activity decreased to 4.37 Umg^{-1} enzyme protein and 3.82 Umg^{-1} enzyme protein in tea and coffee leaves as

substrates compared to control on the 11th day of incubation. The minimal activity of 6.44 from 4.73 Umg^{-1} enzyme protein to 5.84 was recorded on the 7th day and after that its activity decreased to 3.14 Umg^{-1} enzyme protein. It may be inferred from the statistical scrutiny (2 way ANOVA) of the data that the production of tannase activity was found to be significantly enhanced up to 7th day at an intra and extra cellular level ($p<0.05$).Similar view was expressed by Kumar *et al*., (2007) who reported highest yield of tannase in in jamun leaves as substrate by *A. rubber*. Banerjee *et al*. (2007) found maximum tannase production at an extracellular level by *Aspergillusaculaetus* after 72 h incubation. The present result is on par with the report of Murugan*et al.* (2009). The present result is related to the findings of Shanmugapriya *et al*. (2014). They reported that the fungal cultures *Aspergillus flavus* and *Aspergillusniger* produced high yield of extracellular tannase (1.32 U/ml and 1.43 U/ml) under solid state fermentation using *Syzygiumcumini* seed powder as substrate. Handy and Fawzy (2012) reported highest tannase activity of 9.14 Uml-1 by *Aspergillusniger* in *Ficusnitida* leaves as substrate at 30°C and at pH 5.0. The present result is in accordance with the view of Iqbal and Kapoor (2012) who observed that the strain T16 *Trichodrermaharizanum* MTCC 10841 showed highest tannase activity of 16.36 U/ml in amla fruit as substrate compared to tannic acid. Jana *et al*. (2012) used tannin rich plant residues (haritaki, pomegranate, tea leaf waste, tamarind and arjun fruit) as substrate for tannase production by *Penicilliumpurpuogenum* PAF6. Among them, tamarind seed powder showed the maximum tannase activity.

Table 2 Tannase Activity (Umg^{-1}Protein) of *Aspergillusflavus*on Tea Leaf as Substrate

	Tea leaf as substrate	3rd day	5th day	7th day	9th day	11th day	SED	CD (p<0.0 5)
ntracellular	Control	5.64	6.67	4.17	5.27	4.67		
	with substrate	7.14	8.66	7.77	5.59	4.63	0.2	0.4
Extracellular	Control	4.83	8.13	5.88	4.14	3.94		
	with substrate	5.46	7.52	4.49	4.81	3.37	0.2	0.3

Values of mean of three triplicates
Umg^{-1} = 1µ mol of gallic acid released min^{-1} mg^{-1} protein

Table 3 Tannase Activity (Umg^{-1}Protein) Of *Aspergillusflavus*onCoffee Leaf as Substrate

	Coffee leafas substrate	3rd day	5th day	7th day	9th day	11th day	SED	CD (p<0 .05)
Intracellular	Control	5.15	7.83	5.24	4.43	3.71		
	with substrate	4.89	8.27	6.18	4.43	4.43	0.16	0.32
Extracellular	Control	3.22	7.55	5.64	3.61	3.32		
	with substrate	4.65	5.83	5.49	5.41	3.82	0.09	0.2

Values are mean of three triplicates
Umg^{-1}=1µ mol of gallic acid released $min^{-1}mg^{-1}$ protein

Table 4 TannaseActivity (Umg^{-1} Protein) of *Aspergillusflavus*on TamarindLeaf as Substrate

	Tamarind leafas substrate	3rd day	5th day	7th day	9th day	11th day	SED	CD (p<0. 05)
ExtracellularIntracellular	Control	4.77	6.72	4.64	4.27	2.87		
	with substrate	4.07	5.71	4.84	3.66	3.26	0.08	0.18
	Control	3.56	6.44	4.34	4.05	2.96		
	with substrate	4.73	6.14	5.05	3.48	3.41	0.13	0.3

Values are mean of three triplicates
Umg^{-1}=1µ mol of gallic acid released min^{-1} mg^{-1} protein

Table 5 Tannase Activity (Umg^{-1}protein) of *Aspergillus flavus*in White Popinac Leaf as Substrate

	White Popinacas substrate	3rd day	5th day	7th day	9th day	11th day	SE D	CD (p<0. 05)
Intracellular	Control	4.48	5.14	5.91	4.67	3.48		
	with substrate	6.54	7.67	8.40	5.83	4.66	0.13	0.26
Extracellular	Control	2.34	4.29	5.07	4.91	3.73		
	with substrate	3.86	6.53	7.84	6.40	4.27	0.1	0.20

Values are mean of three triplicates
Umg^{-1}= 1µ mol of gallic acid released min^{-1} mg^{-1} protein

CONCLUSION

Thus, it can be deduced from the present findings that, the industrially important enzyme, tannase can be produced in an cost effective manner by utilizing tannin rich cheap substrates like leaves, seed powders, palm kernel cake, coffeehusk, tea waste, wheatbran, etc. Tannase or tannin acyl-hydrolase (E.C.3.1.120) catalyzes the hydrolysis of ester bonds present in gallotannins, complex tannins and produce gallic acid and glucose. The enzyme has been used in food and beverages, pharmaceuticals and chemical industries. Thus, this technology would not only reduce the production cost of tannase but also promote the effective utilization of agricultural residues as substrates.

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