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RESEARCH ARTICLE

TANNASE PRODUCTION FROM AGRO-WASTES AS SUBSTRATE BY *TRICHODERMA VIRIDE*

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ABSTRACT

Research on utilization of agro waste as raw materials for the production of industrially important products are gaining importance these days. Present study was focused on exploring the abilities of laboratory isolates of *Trichoderma viride* to produce a value added product, tannase by utilizing agro wastes such as Guava leaves, Eucalyptus leaves ,Rice husk and Sorghum husk. 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 *Trichoderma viride* isolated from tea waste decomposed soil. Among the number of mycoflora *Trichoderma viride* showed maximum hydrolyzing zone for tannolytic activity, it was selected as a candidate for enzyme study. Among the different agro wastes as substrates in different concentration (1%, 2%, 3% and 4%) maximum tannase activity of 4.34±0.004 Umg^{-1} protein was observed in 3% Guava leaves. The least tannase activity of 0.06±0.005 Umg^{-1} protein was recorded at 1% Eucalyptus leaves.

Key words: Agro wastes, Tannase, Trichoderma viride, Guava leaves, Rice husk.

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INTRODUCTION

Agro waste is the waste produced from the agricultural activities. Agro-industrial waste and by-products such as orange bagasse, sugarcane bagasse, wheat bran and other food processing waste are effective substrates for de polymerizing enzyme production by submerged fermentation which proved to be highly efficient technique in the production of tannase. Some natural tannin sources proved to be better substrates than commercial tannic acid for production of tannase. These agro-residues substrates can be substituted for costly tannic acid in the production medium for economic production of the enzyme at commercial level. Thus, there is a growing interest on basic and applied aspects of tannase. The antioxidant and anti-inflammatory activities of both guava fruit and leaf extracts were attributed to flavonoids, tannins (up to 12%) and triterpenes. *Eucalyptus* species have attracted attention from horticulturists, global development researchers, and environmentalists because of desirable traits such as being fast-growing sources of wood, producing oil that can be used for cleaning and as a natural insecticide, or an ability to be used to drain swamps and thereby reduce the risk of malaria.

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Rice bran is a by-product of the rice milling process (the conversion of brown rice to white), and it contains various antioxidants that impart beneficial effects on human health. It also contains a high level of dietary fibers (beta-glucan, pectin and gum). In addition, it also contains ferulic acid, which is also a component of the structure of non-lignified cell walls. Sorghum bran is a mixture of grain pericarp and of variable amounts of grain fragments (endosperm, germ). It is usually a by-product of the dry milling in sorghum flour manufacturing, but the manufacture of other sorghum –waste food product may also require a dehulling step. This is because the pericarp contain tannin that decrease food value and organoleptic qualities (Lazaro *et al*, 2000) Thus by realizing the importance of the enzyme tannase, the present study aimed to isolate and screen high tannase-producing fungi from various environmental sources. Submerged fermentation is widely used for enzyme production because it offers many advantages like uniform process conditions namely concentration, temperature, pH, aeration and agitation in the bioreactors. Tannins, naturally occurring secondary metabolites found in plants, are water soluble polyphenolic compounds which are widespread in bark, galls, leaves, and fruits and wood of plants. They are the fourth most abundant plant constituents after cellulose, hemicelluloses and lignin (Rana and Bhat, 2005). 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. Therefore, attempts have been made to find out some of the suitable tannin rich agro residues for the enzymatic conversion of their tannin content to gallic acid. Tannase (tannin acyl hydrolase) is a commercially important enzyme that catalyses the hydrolysis of ester bond and depside bond present in hydrolysable tannins to form glucose and gallic acid (Aguilar *et al*., 2007). It belongs to the family of hydrolases, specifically those acting on carboxylic ester bonds. In addition to catalyzing the hydrolysis of the central ester bond between the two aromatic rings of digallate (depsidase activity), tannase may also have an esterase activity. Tannase is a key enzyme in the degradation of gallotannins, a type of hydrolysable tannins.

Microorganisms, especially filamentous fungi, are the main sources of tannases with biotechnological potential. The genus *Aspergillus* has been mentioned as an important producer of enzymes for industrial application. Then, Emericela is a teleomorphic genus associated with genus *Aspergillus*. The production of tannase by filamentous fungi can be achived by using Submerged fermentation. Our aim to this study to isolate tannase producing fungi from tea leaf waste decomposed soil collected from the disposal yard of tea industry and to determine the tannase activity on different substrates (Guava leaves, Eucalyptus leaves, Rice Bran, and Sorgam Bran).

MATERIALS AND METHODS

Tea leaf waste decomposed soil was collected from the disposal yard of Tea industry, Ooty, The Nilgiri District, Tamil Nadu. The decomposed tea leaf waste soil was serially diluted and plated on potato dextrose agar medium and incubated for five days at 30˚C. After incubation, the plates were observed for fungal growth and were sub-cultured and maintained on PDA slants. The fungal isolates were identified based on their morphology, mycelia structure and spore formation (Barron, 1968; Ellis, 1976 and Domsche).

Primary Screening of Tannolytic Fungi

The identified fungal strains like, *Aspergillus flavus, Trichoderma viride, Rhizopus spp., Aspergillus niger* were grown individually 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 5, 7, 9 and 11 days under static conditions. The mycelium was filtered through Whatman No. 1 filter paper using a Buchner funnel under suction. The clear filtrate was used as a source of extracellular enzyme. 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 centrifuge at 5,000g for 10 minutes 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 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 5,000 x g, for 10min. The precipitate was dissolved in 2ml of SDS-triethanolamine 1% w/v, solution and the absorbency was measured at 550 nm 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. 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, different tannin rich substrates, like Guava leaves, Eucalyptus leaves, Rice Bran, and Sorghum Bran (0.5g)were supplemented in the fermentation medium at different concentration (1%, 2%, 3% and 4%) for an incubation period of 5, 7, 9 and 11 days.

RESULTS

Screening of fungi for Tannolytic Activity (Hydrolyzing zone)

Among the number of mycoflora like *Aspergillus niger, A. flavus, A. fumigatus, Rhizopus spp., Rhizopus stolonifer, Geotrichum spp., Penicillium sp., Trichoderma viride,* isolated from tea industry waste disposal area soil only 4 fungal strains *Aspergillus flavus, Trichoderma viride, Rhizopus spp., and Aspergillus niger.,* showed the maximum hydrolyzing zone. A significantly highest hydrolyzing zone (clear zone) of 54 mm out of colony diameter of 66 mm was shown by *Trichoderma viride* followed by *Aspergillus flavus* (46 mm out of colony diameter of 58 mm).

Table 1.The Diameter of the Colony and Hydrolysis Zone

S.No	Fungal stain	Colony diameter(mm)	Hydrolytic Zone(mm)
1.	*Aspergillus flavus*	58	46
2.	*Trichoderma viride*	63	54
3.	*Rhizopus spp.*	34	30
4.	*Aspergillus niger*	44	41

Batra and Saxena (2005) observed a good hydrolytic zone on tannic acid on agar plates by *Aspergillus acolumaris.* Similar view was reported by Hamada *et al.* (2013) who observed maximum hydrolytic clear zone (53mm±0) in *Aspergillus niger* Van Tighem isolated from tannery soil sample. According to Melo *et al.,* (2013) the largest zone of 38.33±0.21mm was observed in *Aspergillus japonicas* strain 238.The present study was related to the findings of Mahdi *et al.,* (2014) who reported that in their study isolated thirty *Aspergillus niger* isolates, produced tannins with different diameter of tannic acid hydrolyzing zone which ranged between 18-16mm at 28˚C for 72 hr.

Table 2. Tannase activity (Umg^{-1} protein) of *Trichoderma viride* on leaves waste as Substrates

Leaves waste As Substrate		Concentration	Days				SEd CD(p<0.05) CD(p<0.01)
			5	7	9	11	
Guava Leaves	Intra	1%	0.55±0.004	0.63±0.002	0.65±0.005	0.45±0.004	**0.03232 0.07316 0.90123**
		2%	0.64±0.001	0.90±0.003	0.93±0.004	0.53±0.002	
		3%	0.55±0.008	0.65±0.005	1.13±0.001	0.42±0.002	
		4%	0.33±0.002	0.39±0.004	0.65±0.003	0.23±0.001	
		Control	0.26±0.004	0.34±0.004	0.45±0.005	0.21±0.003	
	Extra	1%	1.24±0.007	2.029±0.003	2.97±0.003	1.20±0.002	
		2%	1.27±0.003	2.69±0.005	2.85±0.003	0.60±0.005	
		3%	1.75±0.004	2.99±0.008	4.34±0.004	1.34±0.002	
		4%	1.53±0.005	2.01±0.005	3.34±0.003	1.26±0.004	
		Control	0.98±0.004	1.06±0.002	2.04±0.002	0.23±0.005	
Eucalyptus Leaves	Intra	1%	0.38±0.003	0.49±0.005	0.92±0.002	0.06±0.005	**0.02236 0.03967 0.07202**
		2%	0.63±0.002	0.83±0.001	0.89±0.006	0.53±0.003	
		3%	0.76±0.003	0.87±0.005	1.67±0.007	0.55±0.003	
		4%	0.60±0.007	0.72±0.003	0.76±0.004	0.44±0.005	
		Control	0.26±0.004	0.34±0.004	0.45±0.005	0.21±0.003	
	Extra	1%	1.77±0.005	3.26±0.004	3.62±0.002	0.88±0.001	
		2%	1.02±0.005	1.69±0.003	2.98±0.004	0.93±0.009	
		3%	2.04±0.006	3.16±0.004	4.33±0.006	0.53±0.002	
		4%	2.03±0.003	2.15±0.004	2.96±0.002	1.76±0.003	
		Control	0.98±0.004	1.06±0.002	2.04±0.002	0.23±0.005	

Results are the mean ± standard deviation of triplicates
Umg-1 = µ mol gallic acid released min-1 mg-1 protein

Table 3. Tannase activity (Umg^{-1} protein) of *Trichoderma viride* on *Bran waste* as Substrates

Bran Waste As Substrates		Concentration	Days				SEd CD(p<0.05) CD(p<0.01)
			5	7	9	11	
Rice Bran	Intra	1%	1.74±0.003	3.49±0.003	3.94±0.002	1.33±0.002	**0.00325 0.00543 0.00847**
		2%	2.26±0.004	2.32±0.004	3.43±0.003	0.48±0.003	
		3%	1.70±0.006	2.87±0.003	3.32±0.005	1.67±0.005	
		4%	1.50±0.003	1.64±0.003	1.89±0.005	0.95±0.004	
		Control	0.03±0.004	0.07±0.005	0.75±0.003	0.07±0.004	
	Extra	1%	1.30±0.002	1.72±0.006	2.23±0.004	1.18±0.003	
		2%	1.25±0.001	1.44±0.001	3.29±0.002	0.80±0.005	
		3%	1.38±0.002	1.65±0.002	2.05±0.008	0.70±0.002	
		4%	0.67±0.006	0.73±0.001	0.93±0.002	0.49±0.005	
		Control	0.24±0.003	0.29±0.007	0.55±0.003	0.32±0.001	
Sorghum Bran	Intra	1%	1.68±0.002	1.90±0.003	2.15±0.002	1.23±0.004	**0.00261 0.00304 0.00676**
		2%	0.88±0.003	0.97±0.009	1.17±0.003	0.50±0.004	
		3%	0.75±0.002	1.08±0.002	1.12±0.003	0.60±0.006	
		4%	0.57±0.003	1.06±0.004	1.44±0.005	0.57±0.002	
		Control	0.03±0.004	0.07±0.005	0.75±0.003	0.07±0.004	
	Extra	1%	0.66±0.004	0.71±0.003	0.85±0.004	0.42±0.004	
		2%	1.45±0.003	1.48±0.001	1.99±0.006	1.40±0.005	
		3%	1.12±0.001	1.20±0.009	1.70±0.004	1.06±0.005	
		4%	0.94±0.005	1.28±0.004	1.46±0.003	0.41±0.004	
		Control	0.24±0.003	0.29±0.007	0.55±0.003	0.32±0.001	

Results are the mean ± standard deviation of triplicates
Umg-1 = µ mol gallic acid released min-1 mg-1 protein

Effect of Tannase on Leaf Waste as Substrates

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 at both extra and intracellular level Among the leaf waste substrates the maximum tannase activity of production 4.34±0.004 Umg^{-1} protein and 4.33±0.006 Umg^{-1} protein at an extracellular level in 3 per cent guava and eucalyptus leaves were on par with each other respectively on the 9th day of incubation when compared to control (2.04 ±0.002Umg^{-1} protein). The least activity of 1.20±002 Umg^{-1} protein and 0.53±0.002 Umg^{-1} protein were exhibited in 3 per cent guava and Eucalyptus leaves on the 11th day of incubation compared to the control.

At an intracellular level, tannase activity by *Trichoderma viride* showed increased activity of 1.67±0.007 Umg^{-1} protein and 1.13±0.001 Umg^{-1} protein at 3 per cent eucalyptus and guava leaves up to 9th day of incubation and the activity declined to 0.06±0.005Umg^{-1} protein and 0.23±0.002 Umg^{-1} protein at 1 and 4 per cent eucalyptus and guava leaves when compared to the control. The present result is related to the findings of Kumar *et al* (2007) who repored that *Aspergillus ruber* produced highest tannase (69 U/g dry substrate) with jamun leaves as substrate .Similar results were obtained by Handy and Fawzy (2012) who revealed that the maximum activity of 81.5 U 50 ml was obtained in *Ficus nitida* leaves as substrate. The present result is in accordance with the view of Kapoor and Iqbal, (2012) who reported that the enzyme

showed appreciable activity with Eucalyptus bark (88%) extract as substrate.

Effect of Tannase on barns as substrate

An increasing trend in enzyme activity at both intra and extracellular level was observed in brans (rice and sorghum) in different concentrations (1%, 2%, 3% and 4%) upto9 days of incubation and after that, a decreasing trend was observed. Among the different concentrations, highest tannase activity of 3.94±0.002 Umg^{-1} protein and 3.29±0.002 Umg^{-1} protein was recorded in 1 and 2 per cent in rice bran as substrate when compared to the sorghum bran (2.15±0.002Umg^{-1} protein and 1.99±0.006Umg^{-1} protein) in 2 and 1 per cent at an intra and extracellular level at 9th day of incubation. The least value of 0.48±0.003 Umg^{-1} protein and 0.49±0.005 Umg^{-1} protein was recorded in 2 and 4 per cent rice bran and 0.57±0.002 Umg^{-1} protein and 0.41±0.004Umg^{-1} protein in 4 per cent sorghum bran at an intra and extracellular level on the 11th day of incubation. 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 on the 9th day at an intra and extra cellular level ($p<0.05$). Reddy and Rathod (2012) recorded tannase production level in different materials as red-gram husk (34.0 U/ml) > acacia pod (33.5 U/ml) > sorghum husk (33.1 U/ml) > spent tea powder (30.0 U/ml) at 30°C and 5.5 pH which was similar to our findings. The result coincides with the result of Malgireddy and Nimma (2015) who recordered the maximum enzyme activity in wheat bran (41.6 U/mg) by *Aspergillus niger.* Similar results were observed by Battestin and Macedo (2007) who reported that maximum tannase synthesis was recorded for five days with 15% (w/w) tannic acid concentration, ammonium nitrate as additional nitrogen and 50:50 of coffee husk: rice bran as residual substrate by *Paecilomyces variotii*.

Conclusion

Thus, the present work has been taken up with a view of exploring the possibilities of using, Guava and Eucalyptus leaves, Rice and Sorghum Bran as a substrates and *Trichoderma viride* 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, 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. 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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