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Effects of Fungal Co-Culture for the Biosynthesis of Tannase and Gallic Acid from Grape Wastes under Solid State Fermentation

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Abstract: Modified solid-state fermentation (MSSF) of tannin-rich substrates for production of tannase and gallic acid was carried out using fungal co-cultures. Tannin acyl hydrolase or tannase is an enzyme produced extracellularly in solid state fermentation by various fungi. The solid-state fermentation offers a number of advantages over submerged and liquid conventional fermentation for the enzyme production. The production is simple, using vegetable residues such as coffee wastes, grape, cashew or by-products as wheat bran, rice or oats, to which tannic acid is added. The use of residues is an alternative to solve pollution problems that can be caused by an incorrect environmental disposal. Tannase enzyme has several applications on food, juices and pharmaceutical industries. The objective of this work was to select tannase producing fungi such as *Aspergillus niger, Penicillium chrysogenum, Trichhoderma* viride and the effect of Co-culture (*Aspergillus niger* + *Pencillium chrysogenum*, *Pencillium chrysogenum* + *Trichoderma viride*, *Aspergillus niger* + *Trichoderma viride, Aspergillus niger* + *Pencillium chrysogenum* + *Trichoderma viride*) were studied in optimized conditions for the production of this enzyme using grape's peel as a substrate. The Co-culture *Penicillium chrysogenum*+ *Trichhoderma viride* produced highest activity of 84±2 U/g/min than other organisms. Optimized incubation period for the production of enzyme was found to be 96 h. In purification step, 60-80% ammonium sulphate saturation and DEAE A-50 Column Chromatography 50-60 fractions were found to be suitable giving maximum Tannase activity.

Key words: Tannase · Tannic acid · Fermentation · Purification · Grape's peel

INTRODUCTION

Grape is the most widely cultivated fruit crop in the world. From the world's total production of 60 million tonnes, about 68% of grapes are used for winemaking. Grape pomace constitutes about 16% of the original fruit. The average composition of this medium includes carbohydrates, fibre, fats, proteins and mineral salts [1]. The main component of the fibre is lignin and then hemicelluloses, cellulose and pectin. This by-product, with scanty economic profitability and pollutant, can be reduced using SSF and relevant value-added products (as enzymes) can be obtained using an economic technology. India is the second major producer of fruits and vegetables and ranks next to Brazil and China respectively, in the world. It contributes 10 percent of world fruit production and 14 per cent of world vegetable production. Fruits and vegetables are more prone to spoilage than cereals due to their nature and composition and this spoilage occurs at the time of harvesting, handling transportation, storage, marketing and processing resulting in waste. Processing fruit produces two types of waste: a solid waste of peel/skin, seeds, stones, etc and a liquid waste of juice and wash waters. In some fruits the discarded portion can be very high (e.g mango 30-50%, banana 20%, pineapple 40-50% and orange 30-50%). Therefore, there is often a serious waste disposal problem, which can lead to problems with flies and rats around the processing room, if not correctly dealt with. If there are no plans to use the waste it should be buried or fed to animals well away from the processing site. Efficient management of these wastes can help in preserving vital nutrients of our foods and feeds and bringing down India produces a huge quantity of grape and food products using grapes. A substantial quantity of grapes becomes spoiled and ultimately thrown out while processing. This grape waste gets contaminated by environmental bacteria and thus forms a foul mass.

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According to India Agricultural Research Data Book 2004, the losses in fruits and vegetables are to the tune of 30 per cent. Taking estimated production of fruits and vegetables in India at 150 million tones, the total waste generated comes to 50 million tones per annum. The post-harvest technologies for perishable horticultural produce serve as an effective tool for getting better return to the produce and also help in avoiding wastage both at production site and distribution centers, which will help in regulating the market infrastructure. Recycling of fruit and vegetable waste is one of the most important means of utilizing it in a number of innovative ways yielding new products and meeting the requirements of essential products required in human, animal and plant nutrition as well as in the pharmaceutical industry. Microbial technology is available for recycling and processing of fruit and vegetable waste and following products can be made out of the different processes.

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 microorganisms, especially enzymes or secondary metabolites. Agro-industrial residues are generally considered the best substrates for the process, including enzyme production, based on SSF [2]. Compared with submerged fermentation, the use of SSF presents advantages such as lower power requirements, smaller reactor volume and high productivity [3]. Castilho *et al.*, [4] state that the conditions in solid-state fermentation were closer to those found in the natural habitat of filamentous fungi, which were, thus, able to grow better and excrete larger quantities of enzymes.

Tannin acyl hydrolase commonly called tannase is produced by a number of microorganisms like fungi-*Aspergillus, Penicillium, Rhizopus* sp, Yeast-*Candida* sp. and bacteria - *Bacillus* sp. [5,6] Several agro-industrial waste and by-products such as orange bagasse [7], Sugar cane bagasse [8] wheat bran [9] and other food processing waste [10] are effective substrates for depolymerizing enzyme production by solid-state fermentation, which proved to be highly efficient technique in the production of tannase. The major commercial application of this enzyme is in the hydrolysis of gallotannin to gallic acid, which is an intermediate required for the synthesis of an antifolic antibacterial drug trimethoprim [11]. Tannase is extensively used in the preparation of instant tea, wine, beer and coffee – flavored soft drinks and also as additive for detannification of food [12].

This study was taken up with the objective to produce tannase and gallic acid through bioconversion of grape's peel by using co-culture fungal organisms.

MATERIALS AND METHODS

Raw Material: In this study, the peel of black grapes was used as a substrate and it was obtained from the fruit market in Thanjavur, Tamilnadu, India. The grape was first washed and the peel was obtained by simple pressing of the fruit. The pulp will come out and the peel was collected. The collected peel was used as a substrate for tannase production under solid state fermentation.

Microorganisms: *Aspergillus niger, Trichoderma viride* and *Penicillium chrysogenum* were isolated by primary selection from naturally contaminated grapes by serial dilution and pour plate techniques. The pure cultures were identified by their morphology and colony characteristics. The organisms were maintained on PDA slants and stored at 4°C. The slants were freshly made once a month.

Preparation of Spore Inoculums: Aspergillus niger, Trichoderma viride and *Penicillium chrysogenum* spore inoculums were prepared by adding 2.5mL of sterile distilled water containing 0.1 % Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions and number of viable spores in the suspension was determined using the plate count method. A volume of 1 mL with concentration of $36x10^9$ spores was used as inoculums.

Production of Tannase under SSF: A five gram substrate of grape peel was taken in 250-mL Erlenmeyer flask and moistened with 5 mL of salt solution. The composition of the salt solution was NH_4NO_3 0.5 %, NaCl 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.1 % and Tannic acid 4% at pH =5.5. The contents were sterilized by autoclaving at 121°C; 15lbps for 20 min. The cooled sterilized solid substrate was inoculated with 1 ml of the spore inoculums, the spore inoculum was prepared in different combinations for co-culture method, after inoculation the substrate with organisms were mixed properly and incubated at 30 °C for 168 h.

The individual and co-culture organisms used (Table 1) in this study are as follows. The two microorganisms were used together where the ratio of the organisms 1:1

Table 1:

Treatment Name	Microorganisms
A	*Aspergillus niger*
B	*Pencillium chrysogenum*
C	*Trichoderma viride*
D	*Aspergillus niger* + *Pencillium chrysogenum*
E	*Pencillium chrysogenum* + *Trichoderma viride*
F	*Aspergillus niger* + *Trichoderma viride*
G	*Aspergillus niger*+*Pencillium chrysogenum*+*Trichoderma viride*

Extraction and Analysis of Crude Enzyme: A 10% mycelial suspension mass from the fermented substrate was prepared in 0.05 M citrate buffer, pH 5.0 and frozen overnight. Acid washed sand, four times the weight of the mycelium, was added and the mixture was ground in a chilled Pestle&Mortar kept in an ice bath. Crude enzyme was separated from the fermented matter by centrifugation at 8000 rpm at 4°C for 20 min. The filtrate was collected in bottles and preserved for further studies. The supernatant (mycelia extract) was used for tannase assay.

Purification and Characterization: A volume each of 100 ml of crude tannase was taken, added slowly with the various concentration levels (0–40, 40–60 and 60–80 %) of ammonium sulphate. The addition of ammonium sulphate was done under constant stirring at 4 °C for 30 min and then stirring was continued for another 30 min and then allowed for settlement for 3 h at 4°C. The precipitated protein was separated by centrifugation at 8000 rpm at 4°C for 20 min. The separated proteins were then dissolved in minimum amount of 0.05 M citrate buffer (pH-5) and refrigerated for further analysis. Precipitated proteins were transferred into a dialysis tube using a micropipette and dialyzed against citrate buffer (0.05 M, pH-5) at 4 °C. The buffer was stirred gently using a magnetic stirrer to enhance solute exchange. Dialysis was conducted over night and the buffer was changed several times to increase the efficiency of the dialysis.

DEAE Sephadex A-50 Chromatography: A Glass column was packed with DEAE Sephadex A-50 and was equilibrated with 0.05 M citrate buffer (pH 5.0). One ml of the dialyzed sample was applied on the column and the elution was done using 0.05 M citrate buffer (pH 5.0). The fractions were monitored and collected. The fractions corresponding to tannase activity were pooled and used for estimation.

Tannase Assay: Tannase was assayed following Sharma *et al.,* [13] method using gallic acid as a standard. The pink colour developed was read at 520 nm using a spectrophotometer (Shimadzu UV-160A). The enzyme activity was calculated from the change in absorbance. One unit of tannase activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under defined reaction conditions. Enzyme yield was expressed as units/gram dry substrate (U/gms/min).

$$\Delta A520 = (Atest - Ablank) - (Acontrol - Ablank)$$

Effect of Incubation Time: After inoculation the flasks were incubated at 30°C and the enzyme activity was measured after different time periods ranging from 48 h to 168 h.

Estimation of Protein: During incubation period the protein was estimated following Lowry's method [14] using bovine serum albumin as a standard.

Estimation of Tannin: The estimation of tannin content was done following the protein precipitation method of Haggerman *et al.* [15]. Dried grape's peel was ground finely in methanol and kept overnight at 4 °C. One ml of extract was taken in a tube and 3 ml of BSA solution was added and kept for 15 min at room temperature. The tubes were centrifuged at 5000g for 10 min, supernatant was discarded and pellet was dissolved in 3ml of SDS-triethanolamine solution. One ml of $FeCl_3$ solution was added and tubes were kept for 15 min at room temperature for color stabilization. Color was read at 530 nm against the blank.

HPTLC Analysis of Gallic Acid in Fermented Substrate Chromatographic Parameters and Conditions: Standard zones were applied as bands on 20 x 10 cm aluminum silica gel 60 F254 HPTLC plates by means of (Qualigens) Linomat V automated spray-on band applicator equipped with a 100-μl syringe and operated with the following settings: band length 6 mm, application rate 15 s GL-1, distance between bands 4 mm, distance from the plate side edge 1.2 cm and distance from the bottom of the plate 1.5 cm. Plates were developed to a distance of 6.5 cm beyond the origin with toluene-acetone-methanol-formic acid (46: 8: 5: 1) in a vapour equilibrated Camag HPTLC twin trough chamber. (The development time was about 17 min). After development, the plates were air dried for

15 min. Densitometric evaluation of the chromatograms: Camag TLC scanner 3 was used to densitometrically quantify the bands using WINCATS software (version 4 X).and the scanner operating parameters were: (mode: absorption / reflection; slit dimension: 5 x 0.1 mm scanning rate: 20 mm s-1 and monochromator bandwidth: 20 nm at an optimized wavelength 254 nm [5].

RESULTS AND DISCUSSION

Tannase Production in Solid State Fermentation Using Grape's Peel: In the present study co-culture method is very effective in the production of enzyme under SSF at 30°C. Tannase produced by most of the potent strains like *A.niger, P. chrysogenum, A.oryzae* also showed temperature optima at 30°C [6]. Tannase production by the organisms (A,B,C,D,E,F,G) at 30°C were reported in Table 2.

From the above results the *P. chrysogenum +T.viride* (E) *had* a highest tannase activity of 39.70±2 U/g/min in the crude form when it was subjected to ammonium sulphate precipitation (60-80%) the activity was increased to 53.11±3 U/g/min. Tannase from *Paecilomyces variotii* was precipitated using 50%saturation of ammonium sulphate, some of the nonenzymatic proteins were shown to be removed and at 70% saturation of ammonium sulphate, a yield of 78.7% was reported by Mahendran *et al*., [16]. After dialysis the activity of the tannase was enhanced. The dialyzed enzyme was further purified through DEAE-Sephadex A-50 and the eluted fractions showed 84±2 U/g/min. Mitchell and Lonsane, [17] reported that the Production enzyme is often simple, when agro-industrial by-products like wheat bran, rice bran or wheat straw are used as substrate, weight of substrate is low. Hence, enzyme activity is usually very high.

Table 2: Tannase production in Grape's peel

Tannase activity (U/g/min)				
		Ammonium		Column
Treatment	Crude	sulphate precipitation	Dialysis	Chromatography
A	29.70±2	32.11±3	42.92±2	51±2
B	21.23±3	33.76±2	56.48±3	61±2
C	24.91±3	39.14±1	52.76±1	63±1
D	22.02±1	30.64±1	41.50±2	53±2
E	39.70±2	53.11±3	72.92±1	84±2
F	28.70±1	42.11±2	62.92±1	74±3
G	30.70±2	40.11±3	52.92±2	64±1

Effect of Incubation Period: In our study all the organisms produced more tannase in 96th hours of incubation. The same results were reported by Nisha K. Rana *et al*., [18], that the total tannase yield was maximum at 120 h for SmF and LSF, whereas it was at 96 h of growth for SSF process. Previously maximum extracellular tannase and gallic acid production was recorded in 96 h and 120 h by *A.niger* and *Rhizopus oryzae* [9,19,7]. The enzyme production started after 48 h of incubation and increased with time reaching a maximum at 96 h.This might be the fungi entered in to its exponential phase. Thereafter, the enzyme production started decreasing (Fig. 1).

Estimation of Protein: The protein content of all the organisms showed maximum at 96 hrs (Fig.2) after that it was decreased.It might be the production of tannase was more at 96 hrs of incubation, so the intracellular protein also comes out from the organisms in that time.The enzyme production when started to decrease, the protein content of the substrate also decreased. After studying the extracellular protein content it was found that the organism produced maximum tannase in its exponential phase of growth [20].

Estimation of Tannin: In the present study the tannin content in the fermented substrate was high level in 48 hrs (Fig.3) of incubation afterwards it was reduced, it could be the tannin was degraded by the tannase which was produced by the organisms. Generally tannins are toxic as well as bacteriostatic compounds and have non-reversible reaction to protein [21]. Nevertheless, some microorganisms degrade this compound by producing tannase and play an active role in the soil for nutrient recycling through decomposition of tannin-containing plant materials [22]. Tannic acid is a polyphenolic compound, because of which it is generally considered an antinutrient and antimicrobial agent [22]. But a large number of fungi have been reported to degrade tannins by producing tannase [6,23]. The enzyme degrades the tannic acid into gallic acid and glucose, which are ultimately utilized by the organism for growth [6].

Determination of Gallic Acid by HPTLC: TLC separation of phenolic acids from methanolic extracts of fermented grape's peel by *P.chrysogenum+T.viride* (E) at 96 hrs indicated the presence of gallic acid. Banerjee *et al*., stated that the organism produced maximum amount of enzyme within 72–84 h and gallic acid in 60 h. In HPTLC the conditions used led to a good separation of the peaks which could be identified in the chromatogram of Gallic

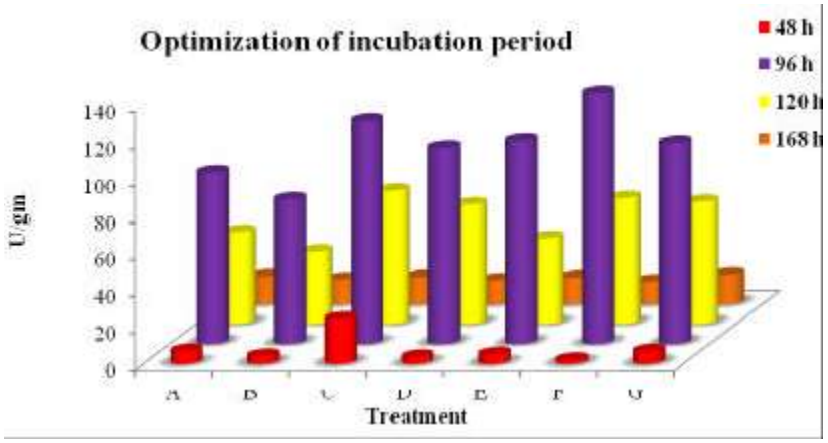

Fig. 1: Tannase activity in 48 h-168 h of incubation

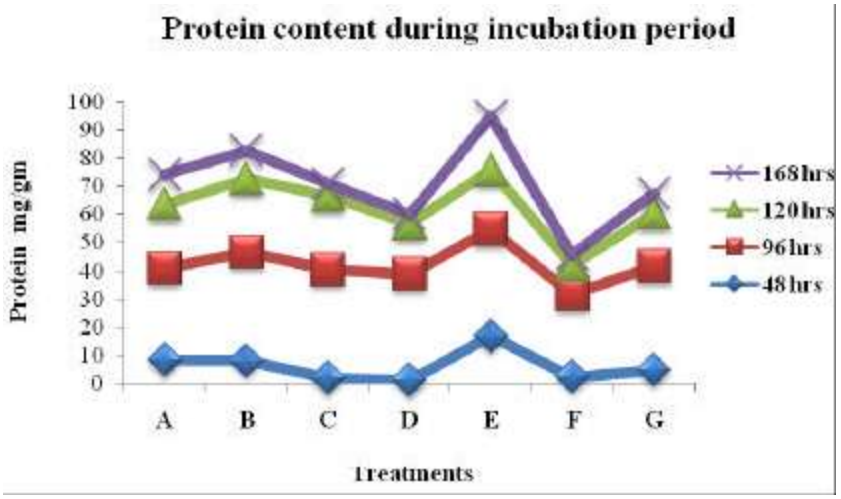

Fig. 2: Protein content of the fermented substrate

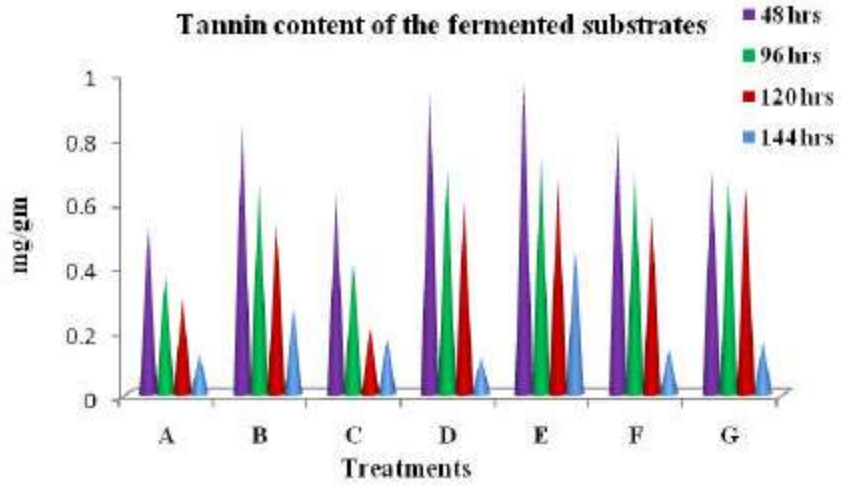

Fig. 3: Tannin content of the fermented substrartes

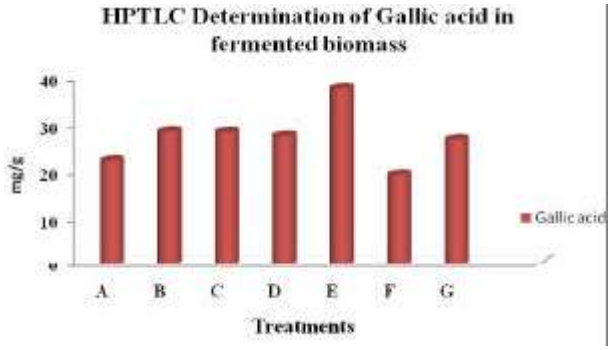

Fig. 4: HPTLC analysis of Flavonoids

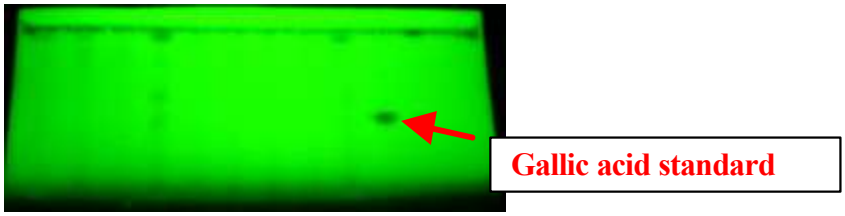

Fig. 5: Gallic acid separation in aluminum silica gel 60 F254 HPTLC plates

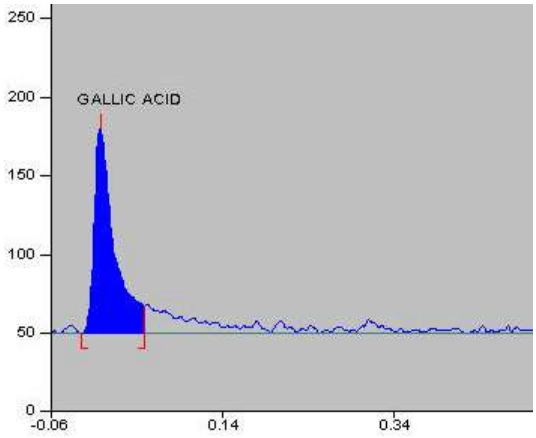

Fig. 6: HPTLC Chromatogram of gallic acid standard

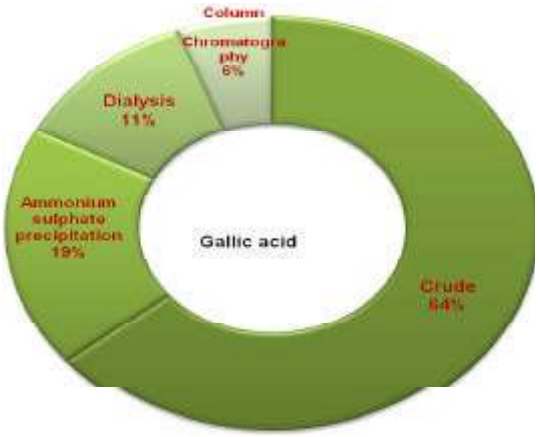

Fig. 7: Estimation of Gallic acid in different purification steps

acid 37.7 mg/g (Rt=0.07). It was identified by comparison with the standard flavonoids chromatogram (Fig. 4, 5 & 6). In optimum conditions the gallic acid production in different purification steps shown in Figure 7.

CONCLUSION

Tannase from *Pennicilium chrysogenum +Trichoderma viride* was purified by DEAE sephadex and it was used to hydrolyse tannic acid for production of gallic acid. From this study it is concluded that the co-culture method was suitable to produce a large amount of Gallic acid in SSF. This co-culture was most suitable for bioconversion of grape's peel to obtain increased quantities of gallic acid.

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