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Screening and characterisation of tannase producing fungi from soil near tannery Industry

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Abstract
Tannase is an industrially important enzyme and has several applications in various industries such as foods, animal feeds, cosmetics, pharmaceutical, chemical, leather industries etc. The enzyme has potential uses in the treatment of tannery or industrial effluents. Despite the long history and numerous publications, tannase is still considered as one of the costly industrial enzymes. The aim of this study was to isolate a tannase-producing fungus from industrial effluents and evaluate its tannin degrading properties. In the present study tannase producing fungi were isolated from soil nearby tannery industries. Twenty-six isolates of tannase-producing fungi were isolated and were screened using tannic acid agar plate method. Zone of hydrolysis confirmed their Tannin degrading ability. Ninteen isolates were screened as tannase producers. Accordingly, Four Isolates, which exhibited high tannase activity, were chosen to undergo final screening. Study suggested that the tannase isolated from fungi can for tannin biodegradation in the industrial effluents.

Keywords: Tannase, tannin degrading activity, fungi, industrial effluents

Introduction
Tannin acyl hydrolase (E.C. 3.1.1.20) is commonly referred as tannase, it catalyses the hydrolysis reaction of the ester bonds present in the gallotannins, complex tannins, and gallic acid esters. The enzyme is used in food and beverage processing; however, the practical use of this enzyme is at present limited due to insufficient knowledge about its properties, optimal expression, and large-scale application (Tieghem, 1867) [19]. The major commercial applications of the tannases reside in the elaboration of instantaneous tea or of acorn liquor and in the production of gallic acid, the latter being an important intermediary compound in the synthesis of the antibacterial drug, trimethroprim, used in the pharmaceutical industry and also in the food industry; gallic acid is a substrate for the chemical or enzymatic synthesis of propyl gallate, a potent antioxidant. Moreover, tannase is used as a clarifying agent in some wines, beers, fruit juices, and in refreshing drinks with coffee flavour. Its commercial production is through microorganisms using submerged culture (SmC), where it is intracellularly expressed, implying additional costs during its production (Belmares *et al*. 2004; Belur 2011; Chae and Yu 1983; Lekha and Lonsane 1997) [4, 5, 8, 14]. An excellent review about the production and applications of tannase was reported by Lekha and Lonsane (1997) [14], summarizing a lot of useful information generated in almost 130 years of research. During the last 10 years, novel and interesting information has been published.

Tannins are naturally occurring polyphenolic compounds with varying molecular weights that occur naturally in the plant kingdom. These phenolic compounds differ from others by having the ability to precipitate proteins from solutions. In the plant kingdom, these tannins are found in leaves, bark, and wood (Lewis, 1969) [15]. Tannins are considered to be plants' secondary metabolic products because they play no direct role in plant metabolism. After lignin, tannins are the second most abundant group of plant phenolics (Bhat *et al.*,1998; Aguilar *et al.*, 2001) [6, 1]. One of the major characteristics of tannins is their ability to form strong complexes with protein and other macromolecules such as starch, cellulose, and minerals. It is widely accepted that tannins are divided into four major groups: gallotannins, ellagitannins, condensed tannins, and complex tannins (Banerjee *et al.*, 2001) [2].

The tannase enzyme has potential uses in the treatment of tannin containing effluents and pre-treatment of tannin containing animal feed (Belur and Mugeraya 2011) [5]. Biodegradation technology is a recent method most successfully employed in the treatment of several kinds of industrial wastes. Biological treatment is also recognized as most desirable, easiest and cost effective in pollution abatement programmes. Moreover, it is free from sludge accumulation a serious problem of disposal as in the case of chemical treatment (Pandey et. al. 2017) [18]. However, further studies on the subject are required to understand the microbial degradation process of such molecules and to develop system for treatment of tannin containing effluents. Recently, the application of enzyme technology to treat effluents has been garnering a great deal of attention because of its potential to easily treat wastewater without causing secondary pollution upon the removal of pollutants (Durai and Manivasagar, 2011; Zhao *et al.*, 2017) [9, 20].

Due to rapid industrial development in the last two decades in India, the disposal of industrial effluents has become a serious problem. Tanneries, textiles, breweries and food processing industries generate effluents having a high BOD value and which contain tannins that are not easily degradable (Gupta, 2014) [11]. Therefore, the main problem is about how to treat the tannin containing effluents. In order to develop a more effective and eco-friendly treatment technology, a wastewater treatment plant with an enzyme system would be developed for safe disposal of these tannin containing effluents.

Materials and Methods

Collection of Soil Samples

Soil samples of nearby local tannery industries in Mumbai were collected and enriched in Tannin containing medium. The enriched soil samples were diluted and procced for isolation and characterisation using selective culture medium.

Isolation of tannase producing fungi

Each isolation source sample was serially diluted (10^{-1} to 10^{-4}) with sterile distilled water. One milliliter from each dilution was plated into TAA medium using pour plate technique. Total of 26 fungal isolates were obtained and they were screened for the production of tannase by spot inoculating the culture to a tannin agar medium, containing 1gm yeast extract, 0.5 gm Nacl, 0.5 gm tannin, 1 gm sucrose, 3gm Agar- agar, D/W 100 ml. The inoculated plates were incubated for 72 hours at 30°C. The plates were flooded with 0.01 M FeCl3 which reacts with tannic acid and forms a brown colour (R. Kumar et. al., 2010). The tannase producing fungal isolates were subjected to quantitative secondary screening by performing tannase assay by Mondals colorimetric method. The cultures obtained were grown on PDA, supplemented with 0.01 tannic acid, slants and maintained at 4º C (working cultures).

Characterisation of isolates

Morphological character were observed according to their growth pattern and zone formations by the fungal isolates. Microscopic examination of fungi was done with lacto phenol cotton blue Production of tannase Enzyme production was carried out in 100 mL Erlenmeyer flask containing 50 mL tannic acid broth medium (yeast extract 1 gm, Nacl 0.5 gm, tannic acid 0.5 gm, sucrose 1 gm, D/W 50ml). The medium was sterilized at 121°C at 15 lbs for 15 min, Tannic acid was filter sterilized and added after sterilization.

Preparation of Spore suspension

The fungal growth from the Potato Dextrose slants was scrapped off and suspended into 10 mL sterile 0.1 % Tween 80 solution. Suspension was mixed using cyclomixer to break cell aggregates. The spore suspension was serially diluted and plated on PDA for determination of colony forming unit.

Inoculum Preparation

5% v/v spore suspension of the isolates was used as inoculum in 50 mL tannic acid medium. Cells were removed by filtration through Whatman filter paper No. 1. Cellfree broth containing crude enzyme i.e. filtrate was used for estimating the tannase activity.

Tannase Assay

Standard graph of tannic acid was prepared by making the various concentrations of 1% tannic acid ranging from 1mg/ml to 10mg/ml, from every concentrations of tannic acid 0.5 ml of tannic acid, in 0.2 M acetate buffer (pH 5.0) mix with 2 ml of Bovine Serum Albumin (1 mg/ml) which precipitate the tannic acid. A control reaction was also carried out with heat denatured enzyme. The tubes were then centrifuged (5,000 x g, 10 min) and the precipitate was dissolved in 2 ml of SDS triethanolamine (1% w/v, triethanolamine) solution and the absorbance was measured at 530 nm after addition of 1 ml of FeCl3 (0.13 M). (Mondal *et.al.,* 2001) [2] One unit of the tannase was defined as the amount of enzyme, which is able to hydrolyse 1 mole of ester linkage of tannic acid per min at specific condition.

Results and Discussion

Isolation of tannase producing fungi

A total of 26 (FH1 to FH26) fungal isolates were obtained which exhibited zone of tannin hydrolysis, thus confirming their ability to degrade tannin. The fungal isolates were preserved on Potato Dextrose Agar slants. Microbial tannase production at industrial level using tannic acid as a substrate is fairly expensive. Therefore, it was trusted that agricultural wastes containing tannin could be considered as a alternative source of tannic acid for producing this enzyme. One hundred and five isolates of tannase producing fungi were isolated from nineteen samples collected from the environment at Alexandria and Beheira Governorates, Egypt. Source, number, scientific name and code number of isolates are presented in Table 1. Selection of the isolation sources was based on the fact that the presence of tannase producing microorganisms may fairly exist in tannins containing environments. Many authors used similar isolation sources for isolating tannase producing fungi. Tannery effluent samples were used for isolating tannase producing fungi (Batra and Saxena, 2005; Manjit *et al.,* 2008; Murugan *et al.,* 2007) [3, 16, 17]. Tannase producing Aspergillus tamarii was isolated from soil inundated by effluent of a tannery at Oji River local Government Area of Enugu State, Nigeria (Enemuor and Odibo, 2009) [10]. Tannase-producing Rhizopus oryzae was isolated from soil sample of Indian Institute of Technology campus (Hadi *et al.,* 1994) [12].

Characterisation of fungal isolates

Morphological features (colony characteristics) based on size, shape, margin, texture, opacity; elevation and pigment were observed. Zone of tannin hydrolysis was measured after 72 hours incubation. Microscopic examination of fungal isolates revealed that the isolates FH3 and HF 9 belonged to Aspergillus species, while FH5 and FH 23 belonged to Penicillium, species. Gustavo *et.al.* 2001 report the isolation of tannin hydrolyzing *Aspergillus niger.*

Secondary Screening of potential tannase producing fungal isolates

Twenty-six fungal isolates were screened on the basis of their tannase producing efficiency under stationary condition. The results are as shown in Fig. 3. The isolates FH3, FH 9, FH5 and FH 23 exhibited maximum tannase activity viz. -54.6 U/ml 76.4U/ml, 60.5 U/m 53.4U/ml respectively. On the basis of morphology and microscopic examination FH3 and FH5 as *Aspergillus spp* and *Aspergillus flavus respectively*. FH9 were identified as *Penicillium spp.* while FH23 as *Trichoderma spp.* The fungal isolate FH23 exhibiting maximum tannase activity was selected for further studies. Similarly, Hamdy H.S.and Fawzy E.M. (2011) reported economic production of tannase by *Aspergillus niger*.

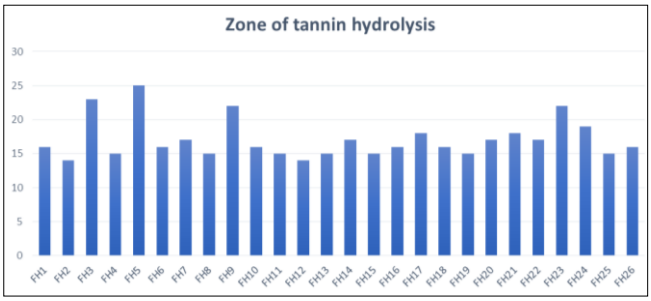

Fig 1: Screening of fungal isolates for tannin hydrolysis

Table 1: Screening of fungal isolates for tannin hydrolysis

Sr.no.	Fungal isolates	Zone of tannin hydrolysis
1.	FH1	16
2.	FH2	14
3.	FH3	23
4.	FH4	15
5.	FH5	25
6.	FH6	16
7.	FH7	17
8.	FH8	15
9.	FH9	22
10.	FH10	16
11.	FH11	15
12.	FH12	14
13.	FH13	15
14.	FH14	17
15.	FH15	15
16.	FH16	16
17.	FH17	18
18.	FH18	16
19.	FH19	15
20.	FH20	17
21.	FH21	18
22.	FH22	17
23.	FH23	22
24.	FH24	19
25.	FH25	15
26.	FH26	16

Conclusion

Thus, in the present study, various fungal tannase producers were isolated from various tannin rich sites, screened on the basis of tannase producing efficiency and their morphological, and microscopic characters were studied. The potential isolate FH 5, identified as *Aspergillus flavus*, will be used for tannase production and purification and its application will be exploited in food industry for debittering of fruit juices, clarification of wine etc. It will also be applied for treatment of tannery effluent.

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