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Isolation and Characterization of potential Tannase producing fungi from Mangroves and Tanneries

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Abstract: Tannase or tannin acyl hydrolase (EC 3.1.1.20) is one of the most versatile biocatalysts which plays significant role in different bioconversion reactions under protein-precipitating conditions. Considering the immense applications of tannase in several bio-based industries, the present study aims to isolate and characterize fungal strains which are potent tannase producers from tannin rich environments. Among the 56 fungal strains isolated, 40 isolates showed zone of hydrolysis in tannic acid agar plates. These tannase positive strains were subjected to tannic acid production under shake flask system and found that 5 strains viz, *Asp* TBG20(a) (5.21 U/mL), *Asp* TBG22(d) (7.11 U/mL), *Asp* TBG24(b) (6.14 U/mL), *Asp* TBG28(a) (6.32 U/mL) and Asp (8.20 U/mL) produced significant amount of tannase. Based on morphological and molecular characterization, the strains were identified as *Aspergillus niger* sp., *A. japonicus* sp. and *A. aculeatus* sp.

Keywords: Tannase, *Aspergillus*, characterization, screening

Introduction

Enzymes of microbial origin are having important applications in many areas of bio-based industries. Tannase (tannin acyl hydrolyse, E.C.3.1.1.20) is an inducible enzyme of high commercial value involved in the hydrolysis of tannins. It catalyzes the hydrolysis of ester and depside bonds of hydrolysable tannins (tannic acid, methygallate, ethylgallate, n-propylgallate and isoamylgallate) releasing glucose and gallic acid [1]. Tannase has wide applications in the food, feed, pharmaceutical and chemical industries, however the principal uses are in the elaboration of instantaneous tea and the production of gallic acid ester [2, 3]. Tannase has been

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widely used to hydrolyze tea cream in the processing of tea [4]. The enzymatic treatment of tea beverage improves its quality, taste and increases the antioxidant activity [5, 6, 7]. Gallic acid (3, 4, 5-trihydroxybenzoic acid) and related compounds possess many potential therapeutic properties including anticancer and antimicrobial properties [3].

Materials and Methods

Sample Collection and Isolation of Fungal strains

Soil and effluent samples were collected aseptically from different mangrove areas in Kerala and Tanneries of Calcutta and fungal strains were isolated by Standard dilution plate technique. The fungal colonies obtained made into pure cultures and stored at 4° C for further studies.

Qualitative Screening for Tannase Activity

The fungal strains isolated were subjected to primary screening for tannase activity by the method described by Bradoo *et al*. [8] using Tannic acid agar (TAA) medium which contained (g/L): $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; Tannic acid, 10; Agar, 30; pH 4.5. The medium was autoclaved at 121 °C for 15 min. The solution of tannic acid was sterilized separately by filtering through 0.45 μm nitrocellulose filter (25 mm diameter, Genetix Biotech Asia Pvt. Ltd.) and then added to the medium. The TAA plates were point inoculated with the fungal strains and incubated at 30 °C. After incubation for 96 h, diameter of clear zone formed around the colonies were measured.

Quantitative Screening for Tannase Activity

Czapek Dox agar medium supplemented with tannic acid as sole carbon source was used for secondary screening of the fungal isolates [8]. The ingredients of medium include (g/L): $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; tannic acid, 10; pH 5. To the sterilized media fungal spores were inoculated at a concentration of 1×10^7spores/mL. The inoculated culture medium was incubated for 5 days at room temperature with constant agitation at 120 rpm in an orbital shaker.

Harvesting of Tannase

After incubation the mycelial suspension was filtered through Whatman No.1 filter paper, and the culture filtrate was centrifuged at 12,000 g for 30 min at 4 °C.

Tannase Assay

Tannase was assayed using the following method described by Sharma *et al*. [9] based on the formation of chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2-thio-4-ketothiazolidine). A standard curve was prepared using gallic acid. One unit of tannase activity was defined as the amount of enzyme required to liberate 1 μM of gallic acid/min under defined conditions.

Morphological Characterization of fungal strains

Morphological characters were studied by inoculating the fungal isolates onto Czapek Solution Agar (CZA) which contained (g/L): Sucrose, 30.0; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; Agar, 15.0; pH 7.3 ± 0.2 and incubated for 5 to 7 days. Every 24 h plates were examined and the colony characteristics like surface and reverse colony colour, colony margins, elevations, growth rate etc were noted.

Micro-morphological characters were studied by staining the 5 day old fungal colonies with lactophenol cotton blue and examined under low, high & oil immersion objectives. The isolates which were morphologically similar to *Aspergillus* were selected. The selected isolates were maintained on Sabouraud Dextrose Agar (SDA) slants and stored at 4 °C for further study.

Molecular Characterization of Selected Strains

Molecular characterization was performed by isolating genomic DNA followed by PCR amplification and sequencing.

DNA Isolation

DNA was extracted by modified CTAB method described by Möller *et al*. [10].

PCR Amplification

PCR amplification of the ITS Region was carried out in 25 µl reaction mixture containing 2.5 µl of 10X amplification buffer (100 mM Tris HCl, pH-8 at 25 °C, 15 mM $MgCl_2$, 500 mM KCl and 10% Triton X-100), 0.2 µl of 25 mM dNTP mixture, 0.74 U of Taq polymerase (Finzyme, Finland), 1 µl each of the primer pair ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) and ITS5 (5’-GGAAGTAAAAGTCGT AAC-3’) (Integrated DNA Technologies, Inc., USA) and 40 ng of genomic DNA.

Bio-Rad thermal cycler (S 1000^{TM}) was used for amplification with the following PCR profile: an initial denaturation for 5 min at 97 °C, followed by 40 cycles of 1 min at 97 °C, 1 min at 48 °C and 2 min at 72 °C and a final extension at 72 °C for 5 min.

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

Phylogenetic Analysis: Homology search of the ITS sequence obtained was performed using BLAST search algorithm. Alignment of similar sequences was done using CLUSTAL W multiple alignment software and the phylogenetic tree was constructed using MEGA4 software. Distance estimation was done following maximum composite likelihood method by Tamura *et al*. [11]. The stability of relationship was assessed from bootstrap analysis of the neighbour-joining data.

Result and Discussion

Studies on tannase production by microbes reveal that fungi are predominant tannase producers and the ones which are extensively studied [8]. Among different habitats explored for tannase

producers, the least ones exploited are the mangrove ecosystems and tanneries. The mangrove ecosystem harbours several tannin rich plant species. Due to this specific nature of the ecosystem, the microbial flora of these ecosystems has the ability to degrade tannin as their energy source [12]. During the initial days of mangrove leaf decomposition the tannin content was high. At that time the fungal count was also high after that the count declined with lowering of tannin [13, 14]. The reason for the early colonization of fungi in tannin-rich mangrove leaves is due to its ability to produce tannase enzyme to degrade tannin [15]. Tannery effluents are also rich in phenolic and tannin rich compounds which act as natural substrates for tannase.

56 fungal strains were isolated from tannin rich regions such as mangrove soils and tannery effluents and screened for tannase production by tannic acid plate assay. Out of 56 screened, 40 isolates utilized tannic acid as sole source of carbon. Tannase utilization was determined by measuring the colony diameter and zone of clearance surrounding the colony after 96 h of incubation. Zone of clearance is due to the hydrolysis of tannic acid present in the media to gallic acid and glucose by tannase leading to a decrease in opacity of the media (Figure 1). Zone of diameter obtained for the fungal isolates in primary screening was shown in Table 1. Among the mangrove soil isolates, largest zone was recorded for *Asp* TBG24(b) (44 ± 2 mm) and *Asp* TBG28(a) (43 ± 2 mm) after 96 h of incubation. The isolate *Asp* TBG20(a) (42 ± 2 mm) showed maximum zone among the tannery effluent isolates. The fungal strains which showed zone of diameter above 30 mm were subjected to quantitative screening by submerged fermentation.

Figure 1: Primary screening for tannase producers on tannic acid agar plates

Sl. No.	Strain no.	Zone of diameter after 96 h (mm)
Mangrove soil isolates		
1	*Asp* TBG1	Nil
2	*Asp* TBG2	Nil
3	*Asp* TBG3	33 ± 2
4	*Asp* TBG6(a)	29 ± 1
5	*Asp* TBG6(b)	25 ± 1
6	*Asp* TBG6(c)	32 ± 0
7	*Asp* TBG6(d)	36 ± 3

8	*Asp* TBG6(e)	40 ± 2
9	*Asp* TBG6(f)	25 ± 1
10	*Asp* TBG10	Nil
11	*Asp* TBG11(b)	18.3 ± 1
12	*Asp* TBG22(a)	35 ± 1
13	*Asp* TBG22(b)	38 ± 2
14	*Asp* TBG22(c)	36 ± 2
15	*Asp* TBG22(d)	38 ± 2
16	*Asp* TBG22(e)	35.5 ± 1
17	*Asp* TBG23(a)	Nil
18	*Asp* TBG23(b)	Nil
19	*Asp* TBG23(c)	Nil
20	*Asp* TBG24(a)	26 ± 2
21	*Asp* TBG24(b)	44 ± 2
22	*Asp* TBG24(c)	33 ± 1
23	*Asp* TBG24(d)	28 ± 2
24	*Asp* TBG24(e)	26 ± 1
25	*Asp* TBG24(g)	30 ± 1
26	*Asp* TBG25(a)	Nil
27	*Asp* TBG25(b)	Nil
28	*Asp* TBG27(a)	25 ± 3
29	*Asp* TBG27(b)	27 ± 2
30	*Asp* TBG27(e)	Nil
31	*Asp* TBG28(a)	43 ± 2
32	*Asp* TBG28(b)	Nil
33	*Asp* TBG28(c)	33.7 ±2
34	*Asp* TBG29	27.5 ± 2

35	*Asp* TBG29(b)	25 ± 3
36	*Asp* TBG30	40 ± 2
37	*Asp* TBG30(b)	33 ± 1
38	*Asp* TBG31	Nil
39	*Asp* TBG32(b)	35.5 ± 2
40	*Asp* TBG32(c)	38.4 ± 1
41	*Asp* TBG33(a)	27.5 ± 1
42	*Asp* TBG33(b)	17.7 ± 3
43	*Asp* TBG33(c)	24 ± 2
44	*Asp* TBG34(a)	29 ± 2
45	*Asp* TBG34(b)	32.4 ± 2
Tannery effluent isolates		
46	*Asp* TBG12(a)	Nil
47	*Asp* TBG17(a)	Nil
48	*Asp* TBG17(b)	33 ± 2
49	*Asp* TBG18(a)	31 ± 3
50	*Asp* TBG18(b)	40 ± 1
51	*Asp* TBG20(a)	42 ± 2
52	*Asp* TBG20(b)	34 ± 2
53	*Asp* TBG20(c)	Nil
54	*Asp* TBG21(a)	33 ± 3
55	*Asp* TBG21(b)	Nil
56	*Asp* TBG21(c)	Nil

Table 1: Preliminary screening for tannase producing fungi

Secondary Screening for Tannase Activity

Secondary screening for tannase production by the fungal isolates was performed by submerged fermentation using Czapek Dox minimal medium supplemented with tannic acid as sole carbon source. Pre-induced fungal spores (1×10^7 spores/mL) were inoculated onto sterilized media and

incubated for 5 days. After incubation the culture filtrate was centrifuged at 12,000 g for 30 min at 4 °C and assayed for tannase activity. The isolate *Asp* TBG30 showed maximum tannase activity of about 8.20 U/mL after 5 days of incubation followed by *Asp* TBG22(d) (7.11 U/mL), *Asp* TBG28(a) (6.32 U/mL), *Asp* TBG24(b) (6.14 U/mL), and *Asp* TBG20(a) (5.21 U/mL). These five isolates were selected for further studies. Tannase activities obtained for the fungal isolates in secondary screening is shown in Table 2.

Table 2: Tannase activity produced by various fungal isolates in Secondary screening

Sl No.	**Strain no.**	**Activity (U/mL)**
Mangrove soil isolates		
	Asp TBG3	2.11 ± 0.16
	Asp TBG6(c)	2.04 ± 0.09
	Asp TBG6(d)	3.58 ± 0.18
	Asp TBG6(e)	2.33 ± 0.16
	Asp TBG22(a)	4.33 ± 0.11
	Asp TBG22(b)	3.79 ± 0.15
	Asp TBG22(c)	4.64 ± 0.17
	Asp TBG22(d)	7.11 ± 0.19
	Asp TBG22(e)	4.45 ± 0.16
	Asp TBG24(b)	6.14 ± 0.13
	Asp TBG24(c)	4.25 ± 0.06
	Asp TBG24(g)	3.78 ± 0.20
	Asp TBG28(a)	6.32 ± 0.08
	Asp TBG28(c)	3.58 ± 0.10
	Asp TBG30	8.20 ± 0.34
	Asp TBG30(b)	4.86 ± 0.06
	Asp TBG32(b)	4.12 ± 0.1
	Asp TBG32(c)	3.87 ± 0.18
	Asp TBG34(b)	2.77 ± 0.06
Tannery effluent isolates		

	Asp TBG17(b)	3.55 ± 0.09
	Asp TBG18(a)	2.14 ± 0.10
	Asp TBG18(b)	4.72 ± 0.07
	Asp TBG20(a)	5.21 ± 0.06
	Asp TBG20(b)	4.23 ± 0.06
	Asp TBG21(a)	2.87 ± 0.11

Morphological Characterization of Aspergillus Isolates

The colony morphology of the fungal isolates were studied by inoculating onto Czapek solution Agar (CZA) and incubated for 5 to 7 days. The microscopic features were examined by staining the 5 day old fungal mycelium with lactophenol cotton blue and observed under microscope. The colony morphological features of the selected strains were shown in table 3.

Strain No	Colony Characteristics				
	Surface colour	Margins	Reverse	Elevations	Growth
Asp TBG20(a)	Deep brown to black	Entire	Yellow	Umbonate	Rapid
Asp TBG22(d)	Light Brown	Entire	Straw colour	Umbonate	Rapid
Asp TBG24(b)	Light Brown	Entire	Cream	Umbonate	Rapid
Asp TBG28(a)	Black	Entire	Yellow	Umbonate	Rapid
Asp TBG30	Black	Entire	Hyaline	Umbonate	Rapid

Table 3: Colony morphology of the five *Aspergillus* isolates on Czapek solution agar

The micro-morphological features of the selected strains were as follows:

***Asp* TBG20 (a):** Conidia are globose, brown to black, very rough, with a diameter 4-5 µm. Conidiophores are hyaline, smooth-walled, with length ranging from 1-2 mm and diameter of 15-20 µm, and becomes darker at the apex and terminating in a globose vesicle with a diameter of 30-75 µm. Metulae and phialides (biseriate) cover the entire surface of the vesicle, darkly pigmented and roughened spores. Hyphae are septate and hyaline and conidial heads are initially radiate and columnar with age (Figure 2).

***Asp* TBG22(d):** They were uniseriate with radiate conidia head and globose to ellipsoidal vesicle measuring 29-45 µm in diameter. The conidiophores were brown, very short which measured 0.5-1 mm and a diameter of about 5-10 µm. Conidia sizes ranged between 3- 4.5 µm, globose, smooth and brown. The phialides are formed directly on the vesicle (Figure 2).

***Asp* TBG24 (b):** They were uniseriate with very large conidia head; the vesicle measured 45-60 µm in diameter and bearing phialides only. The phialides cover three quarters of the vesicle surface. The conidia are ellipsoidal and split into columns upon age. The conidiophores were brown and measured 2-3 mm and a diameter of about 9-13 µm. Conidia sizes ranged between 3.5-4.5 µm. They showed very wide vesicle and short conidiophores (Figure 2).

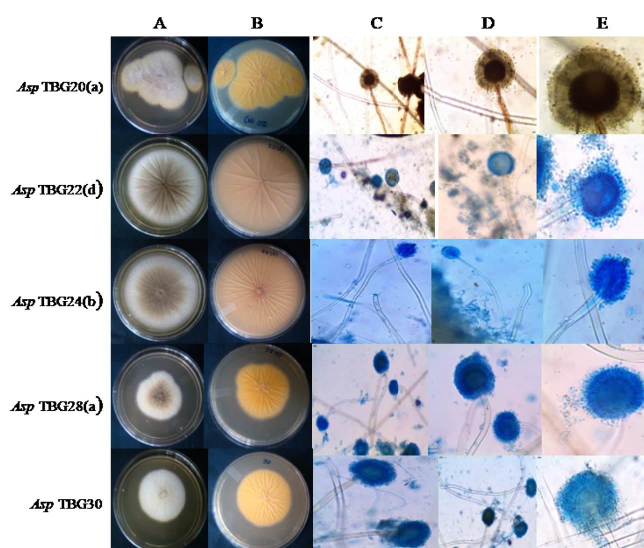
***Asp* TBG28 (a):** Conidia are globose, blackish brown, very rough, with diameter of 2.5-4 µm. Conidiophores are hyaline, smooth-walled, with length ranging from 0.8-2 mm and diameter of 7-10 µm, and becomes darker at the apex and terminating in a globose vesicle with a diameter of 20-50 µm. Metulae and phialides (biseriate) cover the entire surface of the vesicle, darkly pigmented and roughened spores. Hyphae are septate and hyaline and conidial heads are initially radiate and columnar with age (Figure 2).

***Asp* TBG30:** Conidia are globose, slightly brown, and rough, with diameter of 3-4.5 µm. Conidiophores are hyaline, smooth-walled, with length ranging from 0.4-2 mm and diameter of 8-13 µm, terminating in a globose vesicle with a diameter of 15-50 µm. Metulae and phialides (biseriate) cover the entire surface of the vesicle, darkly pigmented and roughened spores. Hyphae are septate and hyaline and conidial heads are initially radiate and columnar with age (Figure 2). Microscopic characters of the five *Aspergillus* isolates were shown in Table 4.

Table 4: Microscopic characteristics of five selected *Aspergillus* isolates

Strain No.	Microscopic Characters						
	Conidiophore		Vesicle		Conidia		Phialides
	Length	Diameter	Shape	Diameter	Heads	Diameter	
Asp TBG20(a)	1 - 2 mm	15 - 20 µm	Globose	30 - 75 µm	Black	4 - 5 µm	Biseriate
Asp TBG22(d)	0.5 - 1 mm	5 - 10 µm	Globose to ellipsoidal	29 - 45 µm	Purplish Brown	3 - 4.5 µm	Uniseriate
Asp TBG24(b)	2 - 3 mm	9 - 13µm	Ellipsoidal to columnar	45 - 60 µm	Purplish	3.5 - 4.5 µm	Uniseriate
Asp TBG28(a)	0.8 - 2 mm	7 - 10 µm	Globose	20 - 50 µm	Blackish brown	2.5 to 4 µm	Biseriate
Asp TBG30	0.4 - 2 mm	8 - 13 µm	Globose	15 - 50 µm	Slightly Brown	3 - 4.5 mm	Biseriate

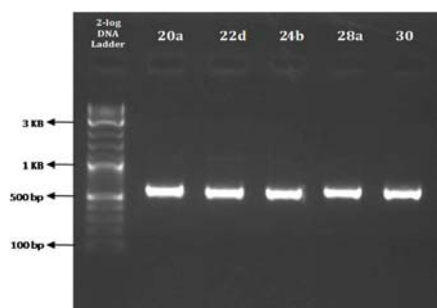
Figure 2: Morphological characters of the *Aspergillus* isolates on CZA (A-front & B-reverse) and microscopic characters after staining with lactophenol cotton blue



Molecular characterization of Aspergillus Isolates

Molecular characterization of the 5 *Aspergillus* isolates was done by extracting genomic DNA followed by amplification of ITS regions and sequencing. Amplification of ITS1-5.8S-ITS2 rDNA fragments were done using the primer pair ITS4 and ITS5 and the molecular size of the product were found to be 550 bp (Figure 3). The amplified product was sequenced using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

Figure 3: PCR amplification of the ITS1-5.8S-ITS2 rDNA gene of fungal isolates. Lane 1:100bp DNA ladder; Lane 2-6: Fungal isolates *Asp* TBG20 (a), 22(d), 24(b), 28(a) and 30 respectively.

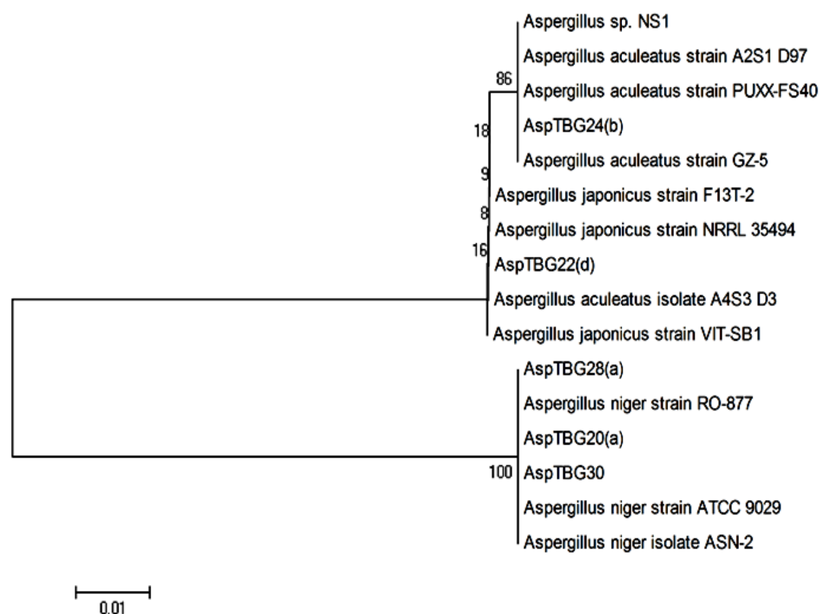


Identification of the 5 *Aspergillus* isolates were performed by comparing the sequence obtained with already available sequence in NCBI Genbank database using BLAST search tool. The BLAST analysis showed that isolates *Asp* TBG20(a), *Asp* TBG28(a) and *Asp* TBG30 showed 100% similarity with *Aspergillus niger*, the isolate *Asp* TBG22(d) shared 100% similarity with *A. japonicus* and isolate *Asp* TBG24(b) shared 100% similarity with *A. aculeatus*.

Phylogentic Analysis

To confirm the relationships of 5 *Aspergillus* isolates with 11 other strains (sequences obtained from NCBI), a composite likelihood analysis was conducted with 1000 bootstrap replications and a phylogenetic tree was constructed using MEGA 4.0 (Figure 4). In this tree there are three clades, of which the isolated strain of *Asp* TBG24(b) was clustered in a strongly supported clade together with *A. aculeatus*; *Asp* TBG22(d) clustered with *A. japonicus* and the strains *Asp* TBG20(a), *Asp* TBG28(a) and *Asp* TBG30 clustered with *A. niger*. Based on the morphological characterization and results of the similarity comparison of the ITS-5.8S gene sequences, it is concluded that the isolate *Asp* TBG24(b) is closely related to *A. aculeatus*; isolate *Asp* TBG22(d) is closely related to *A. japonicus* and the isolates *Asp* TBG20(a), *Asp* TBG28(a) and *Asp* TBG30 closely relates with *A. niger*. The strains were therefore named as *A. niger* TBG20(a), *A. japonicus* TBG22(d), *A. aculeatus* TBG24(b), *A. niger* TBG28(a), *A. niger* TBG30.

Figure 4: Phylogenetic tree showing evolutionary relationships of 16 taxa based on the similarities of ITS1-5.8S-ITS2 Sequences



Conclusion

The study involves the isolation and screening of fungi capable of producing tannase from least explored areas like mangrove sediments of Kerala and tanneries of Kolkata. A total of 56 strains were isolated and qualitatively screened for the production of tannase based on their zone formation on tannic acid agar plates. The tannase activities of the positive strains were confirmed quantitatively by submerged fermentation on tannic acid broth. 5 strains which showed promising tannase activities were selected and identified by morphological and molecular methods. The morphological and molecular characterization revealed that the all the potent isolates belonged to the genus *Aspergillus viz*, *A. niger*, *A. japonicus* and *A. aculeatus*. These potent fungal strains can be utilized for the industrial production of tannase enzyme which has immense applications in various bio-based industries.

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Conflict of Interest

The authors declare no conflict of interest

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