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Screening and Identification of halotolerant protease producing fungi from mangrove sediments of Kerala

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Abstract

Protease is the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields. Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties. One hundred and ninety one fungal isolates were isolated from different mangrove sediments and preliminarily screened for their ability to produce protease based on the zone of clearance on skimmed milk agar plate. Among these isolates, ninety isolates were protease positive and produced a Relative Enzymatic index(REA) ranging from 1-2. On secondary screening in protease specific fermentation broth, five promising proteolytic fungi with enzyme activity above 35 U/ml were selected for further investigations. Based on the molecular characterization and phylogenetic studies, the selected isolates were identified as *Penicillium goetzii*TBG PayV (Accession no. MF151170), *Aspergillus aculeatus* TBG EkmII(Accession no. MF151167), *Penicillium expansum*TBG Ezh4 (Accession no. MF151168), *Penicillium oxalicum*TBG PayIV(b) (Accession no. MF151169) and *Aspergillus flavus* TBG D2 Azk(Accession no. MF151171).

Keywords: protease, *Penicillium*, *Aspergillus*, REA, ITS- rDNA

INTRODUCTION

Microorganisms are known to play an indispensable role in technology for the production of enzymes on an industrial scale. The protease enzyme constitutes about

two thirds of total enzymes used in various industries and this dominance in the industrial market is expected to increase over the years (Gupta *et al*., 2002). Proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment etc (Agarwal *et al*., 2004).

New protease-producing microorganisms and perfected fermentation technology are needed to meet the ever-growing demand for this enzyme. Bioprospecting for new protease producing microorganisms from less studied environments may help to this end. Mangroves are salt tolerant plants of tropical and subtropical inter-tidal regions of the world. The specific regions where these plants occur are termed as 'mangrove ecosystems' and are extremely sensitive. The mangrove sediments of Kerala have very broad microbial diversity richness yet little studied and exploited.

It has been well demonstrated that a large amount of commercially available proteases are derived from fungal strains. Morphological and molecular identification based on rDNA, especially 18S and 28S ribosomal genes are useful for identifying fungal strains (Korabecna, 2007). The present study is thus aimed to isolate, screen and characterize protease producing fungi, in the morphological and molecular level, from various undisturbed Mangrove sediment regions of Kerala.

MATERIALS AND METHODS

Sample collection: Sediment samples were collected at five sample points from undisturbed Mangrove regions of Payyannur(12°06'05.24"N, 75°13'06.68"E), Thalassery (11°45'57.76"N, 75°29'02.12"E), Ernakulam (9°53'41.28"N, 76°21'45.42"E), Kannur(11°51'51.56"N,75°24'15.91"E),Azheekkal (9°07'01.61"N, 76°29'05.07"E) and Ezhupunna (9°49'30.03"N, 76°19'06.37"E).

Isolation of proteolytic fungi: Sample suspensions were serially diluted with 0.5 M NaCl and spread plated onto PDA plates according to standard protocols. Distinct colonies were sub cultured and maintained on PDA slants and stored for 4°C for further studies.

Semi-quantitative screening of protease activity: The fungal isolates were screened for their ability to produce protease enzyme as described by Oyeleke *et al*.(2010) on skimmed milk agar plates and the relative enzymatic activity were calculated.

***Quantitative screening*:** The selected fungal isolates from initial screening were inoculated in protease specific fermentation broth containing (% w/v): yeast extract 1.0, $MgSO_4$ 0.02, glucose 2.0, K_2HPO_4 0.1, pH 7.0. The inoculated flasks were incubated at 28˚C for 5-6 days in a rotary shaker and supernatant was used as source of protease enzyme (Chandrasekharan *et al*, 2015).

Assay for protease activity: Protease activity in the culture supernatant was determined according to the modified procedure of Tsuchida *et al*. (1986) by using casein as substrate. A mixture of 750 µl of 0.65% (w/v) of casein in 50mM phosphate buffer, pH 7 and 250 µl crude enzyme extract were incubated at 55°C for 10 minutes. After 10 minutes, the enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA). Then, the reaction mixture was centrifuged to separate the unreacted casein at 10,000 rpm for 15 minutes. The supernatant is mixed with 2 ml of 0.4M Na_2CO_3 and 0.5 ml of 3-fold diluted Folin Ciocalteu phenol reagent. The resulting solution was incubated at room temperature in the dark for 30 minutes and absorbance of the blue colour developed was measured at 660 nm against a reagent blank using a tyrosine standard. One unit of protease is defined as the amount of enzyme that released one µmol of tyrosine per ml per minute under the standard conditions.

Estimation of total protein: Protein Assay based on the method of Bradford (1976) was used for estimating total protein from all samples.

Screening of potent isolates in CYG broth: The isolates which showed promising protease activity were inoculated in CYG broth(Casein enzyme hydrolysate- 5g, Glucose-5g, Yeast extract-5g, pH-7±0.2) for measuring the induced protease enzyme and assayed for protease enzyme activity and protein as described above.

Halotolerance test of the selected isolates: The halotolerance test was performed following a modified procedure of Moubasher *et al*. (1990). The isolates were inoculated in Malt Extract Agar supplemented with the different concentrations of NaCl concentrations and incubated at 28±2°C for 10 days.

Morphological identification of potent fungal isolates: Morphological identification were done by inoculating the fungi on four different medias viz., Potato dextrose agar(PDA), Malt extract agar(MEA), Sabourauds dextrose agar(SDA) and Czapek Dox agar (CDA) plates, and observing growth characteristics after 5-7 days of incubation.Morphological species characterization was determined following the key characters(A.Nagamani, 2006) after mounting with lactophenol cotton blue.

Biochemical characterization of fungal isolates: Gelatin liquefaction: Cultures were inoculated on nutrient broth tubes containing 15% gelatin and incubated for 2-3 days at 28°C. After incubation, the tubes were kept at 2-3°C for 1 h and liquefaction was observed.

Tyrosine utilization: The fungal cultures were inoculated in slants containing ISP medium no.7 and incubated for 2 weeks at 28°C.The presence of a brownish black/greenish brown diffusible pigment is noted.

Sugar utilization: Sugars at 1% level(Glucose, arabinose, cellulose, fructose, sucrose, raffinose and maltose) is added to ISP medium no. 9,cultures inoculated incubated

for 2 weeks at 28°C and their utilization noted.

Molecular characterization: The genomic DNA of the fungi was extracted using modified CTAB method (Moller *et al*., 1998) and ITS1-5.8S-ITS2 rDNA fragment region was amplified with primers, ITS1 (5'- TCCGTAGGTGAAC CTGCGG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'(White *et al*., 1990). Bio-Rad S1000 thermal cycler was used for amplification with the following PCR profile using Emerald master mix: an initial denaturation for 1 min at 98°C, followed by 38cycles of 10sec at 97°C, 30sec at 48°C and 2 min at 72°C and a final extension at 72°C for 10 min. The amplified product was eluted using Wizard® SV gel and PCR clean up system and sequenced in ABI 3500 DNA Analyzer using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequences were searched against those already known at NCBI GenBank using BLAST search option. The sequences of ITS region were aligned with sequences of similar fungi retrieved from databases using CLUSTAL W(Thompson *et al*., 1997) and a phylogenetic tree was constructed using the neighbour-joining method using MEGA 7.0 with a bootstrap analysis of 1000 replicates (Kumar*et al*., 2004).The fungal ITS ribosomal region gene sequences determined in this study were deposited in GenBank and accession numbers obtained.

RESULTS AND DISCUSSION

Isolation of fungi from sediment samples: A total of 191 morphologically different fungal strains were isolated from various mangrove sediment samples. Colony morphology of fungi varied from circular, punctiform, rough, opaque, white colonies to filamentous, magnified, elevated, smooth, dark, black, buff coloured colonies in plate. A predominance of the genera *Aspergillus* and *Penicillium* among the isolated fungal isolates were found. Shweta *et al*. (2011) has already reported the presence of diverse genera of filamentous fungi and the predominance of these species among the mangroves and solar salterns of Goa.

Semi-quantitative screening for protease activity: About 48% of the total strains were found to produce protease enzyme in skimmed milk agar plate. Table 1 summarizes the results of primary screening for protease producing ability of the isolated strains. The relative enzyme activity (REA) of the strains ranged from 1-2. Similarly, Rajamani and Hilda (1987) and Alpress *et al*. (2002) used skimmed milk as substrate, for the screening of protease activity. The protease positive strains were screened further by secondary screening.

Table 1: Semi-quantitative protease activity of fungal isolates

Sl No.	Strain no.	Colony diameter (mm)	Zone Diameter(mm)	Relative Enzymatic Index(REA)
1	VI-d	57.67±1.53	71.16±1.60	1.23±1.04
2	VI-e	70.67±2.08	81.16±1.61	1.15±0.77
3	12-b	51.67±1.53	66.5±1.32	1.28±0.862
4	VI-g	62±2	76.66±2.08	1.23±1.04
5	VI-c	74.67±2.52	81.83±1.25	1.09±0.49
6	17-a	52.67±2.52	60.83±1.04	1.15±0.41
7	II-a	52.67±3.06	66.83±1.89	1.27±0.62
8	I	56.67±2.08	64.83±2.25	1.14±1.08
9	III	62±2	79.5±1.32	1.28±0.66
10	V	64±2	77.16±2.56	1.20±1.28
11	VI-a	64.67±4.51	74.83±2.25	1.16±0.49
12	VI-b	42.67±3.06	58.16±2.36	1.36±0.77
13	Pay6	9.5±0.86	11±3.61	1.15±4.19
14	Pay 4	15.33±1.53	21.16±0.76	1.38±0.5
15	Pay IV	17±2	24.16±2.25	1.42±1.12
16	Pay 5	21.33±1.53	31.5±1.5	1.47±0.98
17	Pay III	10.50±0.50	26.83±2.84	2.55±5.68
18	Pay 1	28±2	30.5±1.32	1.089±0.66
19	Azk 8	50.83±1.04	56.83±2.02	1.12±1.94
20	Azk 2	35.5±2.29	51.67±1.53	1.45±1.49
21	Azk 5	50.51±0.5	59.33±2.08	1.17±0.24
22	D5 Azk	31.5±1.32	34.33±2.08	1.08±0.63
23	A1 Azk	60.83±1.04	66.33±1.53	1.09±0.67
24	C1Azk	Negligible zone		
25	B1 Azk	52.83±1.89	60.33±1.53	1.14±0.81
26	D6 Azk	Negligible zone		
27	D3 Azk	54.5±1.80	61.17±1.61	1.12±0.89
28	D2Azk	32.16±1.44	32.17±1.76	1.00±1.22
29	D4 Azk	17.5±1.5	20.50±1.32	1.171±0.88
30	Ezh 4	21±1	37.16±2.02	1.76±2.02
31	Ezh 3	Negligible zone		
32	Tly 2	30.17±1.76	43.83±1.25	1.45±0.71
33	Tly 9	32.33±2.52	34.83±2.25	1.07±0.89
34	Tly 1	21.33±1.53	23.16±0.76	1.08±0.49
35	Tly 7	48±2	64.16±1.75	1.34±0.87
36	Tly 10	77.67±2.08	81.83±1.25	1.05±0.60
37	Tly 6	27±2	41.5±1.32	1.53±0.66
38	Tly 3	20.83±1.89	29.83±1.25	1.43±0.66
39	Tly 8	24.67±1.53	52.53±2.24	2.13±1.46
40	Tly 4	46.67±2.08	60.83±1.04	1.30±0.5
41	Tly 5	34±2	39.66±1.04	1.16±0.52
42	Ekm VI	28.66±2.25	36.67±3.06	1.28±1.36
43	Ekm VIII	13.83±1.25	18±2	1.30±1.6
44	Ekm IX	23.16±0.76	24.67±1.53	1.06±2.01

45	Ekm X	31.16±1.04	33±3	1.05±2.88
46	Ekm XI	20.16±0.76	20.50±1.32	1.01±1.73
47	Ekm IV	51.17±2.47	33.5±1.32	1.52±1.87
48	Ekm III	24.16±1.75	28±1	1.15±0.57
49	Ekm 11	71.5±0.86	73±3	1.02±3.48
50	Ekm 6	32.16±1.75	40.50±1.32	1.25±0.75
51	KV1	31.5±1.5	40.33±2.52	1.28±1.68
52	KV3	22.5±2.29	30.33±1.53	1.35±0.67
53	KV10	45.83±1.04	53±2.65	1.15±2.54
54	CLT-4	8.83±0.28	12±1.73	1.36±6.17
55	TV2(c)	8.5±0.5	12.17±1.76	1.43±3.52
56	Myr CF4	20±1.32	21.50±1.32	1.07±1
57	Chi 4	26.5±1.32	31.50±1.50	1.18±1.13
58	Chi 5	30.83±1.04	35.30±1.57	1.14±1.51
59	Chi 6	60.83±1.04	69.50±2.78	1.14±2.67
60	Chi 7	41.83±1.89	46.63±1.52	1.11±0.80
61	Chi 8	61.83±1.25	67.17±1.76	1.08±1.41
62	Chi 9	31.5±1.32	46.33±1.53	1.47±1.15
63	Chi 10	45.5±2.5	56.17±2.47	1.23±0.98
64	Nem 6	24.16±2.02	26.17±1.76	1.08±0.87
65	Nem 19	21.83±1.25	21.83±2.02	1±1.61
66	Nem 20	51.5±0.86	64.50±1.80	1.25±2.09
67	Nem 21	46.5±1.5	65.83±3.55	1.41±2.36
68	Nem 22	67.5±1.32	73.83±1.89	1.09±1.43
69	Nem 23	31.5±0.86	40.17±0.76	1.27±0.88
70	Nem F6	29.38±1.25	30.17±1.76	1.02±1.41
71	Nem F8	59.83±1.25	72.50±2.50	1.211±2
72	Nem F9	32.67±2.25	34.67±2.52	1.06±1.12
73	Nem F10	27.5±1.80	33.83±1.89	1.23±1.05
74	Nem F17	32.63±3.25	42.83±1.26	1.31±0.38
75	Mu3	26.86±1.89	28.83±2.57	1.07±1.36
76	Ana 2	39.83±1.25	42±2	1.05±1.6
77	Ana 5	68.5±1.32	70.50±2.29	1.03±1.73
78	Ana 6	23.82±1.25	26.50±1.32	1.11±1.05
79	Ana 7	31.77±1.89	52.83±2.25	1.66±1.19
80	Ana 9	31.66±1.25	32.50±2.29	1.03±1.832
81	Ana 10	31.45±1.25	33.50±3.04	1.06±2.43
82	Ana 11	44.16±1.75	45.17±2.75	1.02±1.57
83	PP6	11.16±0.76	12.17±2.02	1.09±2.65
84	D1Azk	42.17±2.57	50.65±1.25	1.20±0.48
85	PAY 9	51.17±1.61	59.58±1.27	1.16±0.78
86	Ekm 13	63.50±2.78	68.5±1.5	1.08±0.54
87	Ekm I	42.50±2.50	56.5±1.32	1.33±0.53
88	PAY IV(b)	52.50±2.29	67.5±1.32	1.28±0.57
89	PAY 4(c)	29.33±3.06	44.33±1.75	1.51±0.57
90	PAY IV(c)	44.50±2.29	59.5±1.32	1.33±0.57
91	PAY V	62.50±1.80	78.83±1.04	1.26±0.57

Values represent mean of three replicates ±SD

Fig 1: Plate assay for protease showing clear zones in Skimmed milk agar plate

Quantitative screening of Protease Production in Liquid Culture: The strains which showed significant zone of hydrolysis in the primary screening were confirmed for its protease activity by submerged fermentation. The culture filtrate with high protease activity were designated as TBG Pay V, TBG Pay IV(b), TBG Ezh 4, TBG D2 Azk and TBG Ekm II. The isolate TBG Pay V showed the highest protease activity level in broth culture media (81.60± 0.99 $Uml^{-1)}$. This is in close agreement with Palanivel *et al*.(2013) which reported a maximum enzyme activity of 81U/ml from an extremophilic *Aspergillus sps* .The protease activity of isolates TBG EkmII (64.53± 1.06 $Uml^{-1)}$, TBG D2 Azk(53.22± 0.60$Uml^{-1)}$ followed by TBG Ezh 4 (44.39± 0.56 $Uml^{-1)}$ and TBG Pay IV(b)(39.53±0.82$Uml^{-1)}$ aslo recorded. The protease activity of the isolates as calculated from the standard tyrosine curve is shown in Fig 2.

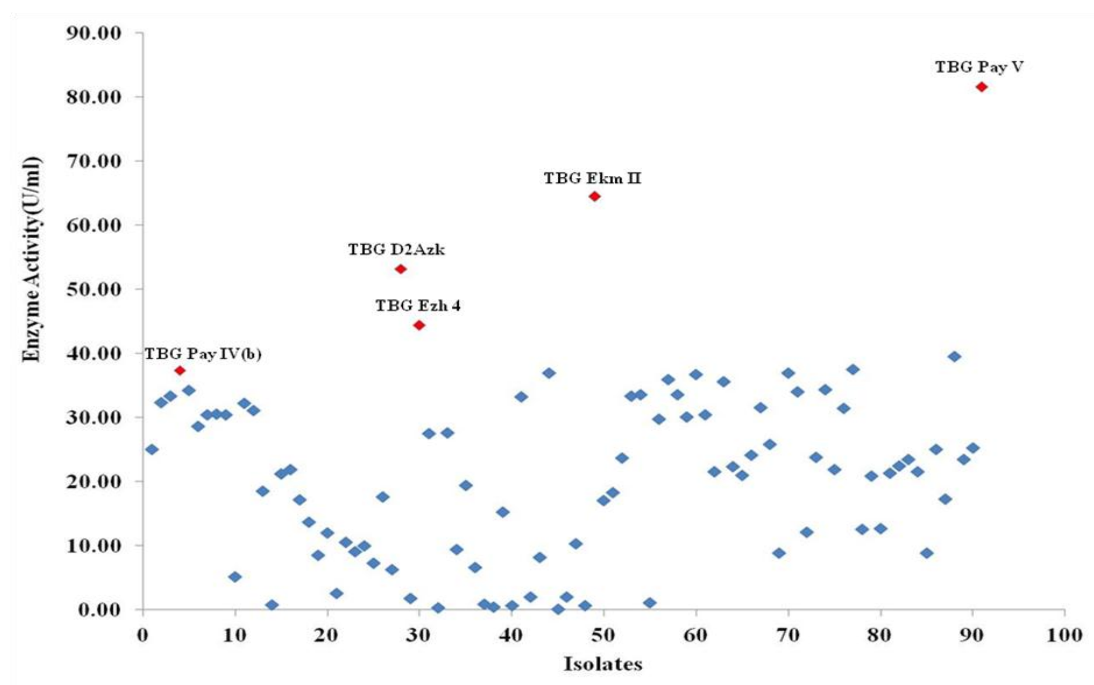

Fig 2: Enzyme activity of isolates calculated from standard Tyrosine graph (Those marked in red denotes isolates with highest activity)

Screening of potent isolates in CYG broth: In CYG broth containing casein enzyme hydrolysate the highest activity was again confirmed for TBG PayV(181.57± 2.74Uml^{-1}) followed byTBG Ekm II(172.34± 2.22 Uml^{-1}), TBG D2 Azk(147.14 ± 3.21Uml^{-1}), TBG Ezh 4(131.03±2.15 Uml^{-1}) and TBG Pay IV(b)(123.45±2.33 Uml^{-1}). Vaishali *et al.* (2012) has reported an increased protease production of upto 112Uml^{-1} in an isolate of *Aspergillus versicolor* when grown in casein containing medium.

Estimation of total protein and biomass: The highest protein content was obtained for TBG Ekm II (89.29 ±0.75mg/ml), followed by TBG PayV(86.81±1.45mg/ml), TBG Ezh 4 (61.88±1.38 mg/ml), TBG D2 Azk (56.85±1.24mg/ml) and TBG Pay IV(b) (56.34±0.80). TBG Pay V showed a higher biomass of 5.82g/L followed by TBG Ekm II (4.86g/L), TBG Ezh 4 (3.12g/L), TBG D2 Azk (2.96g/L) and TBG Pay IV(b) (2.58g/L).

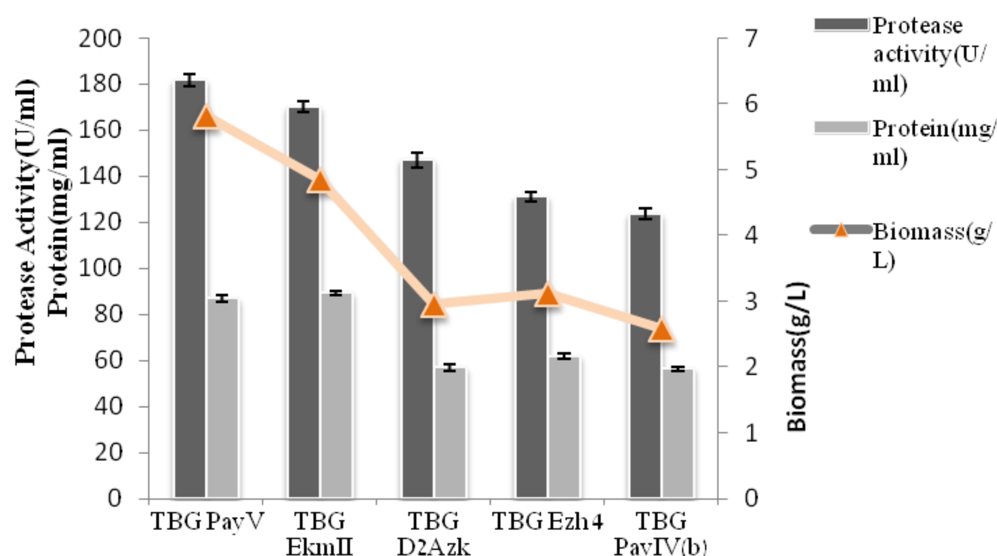

Fig 3: Graph showing protease activity, protein content and biomass of the promising isolates in CYG broth

Halotolerance test of the selected isolates: All the five potent isolates grew in malt extract agar (MEA) media supplemented with 0-15% NaCl. TBG Pay IV(b), TBG Pay V and TBG D2 Azk could survive NaCl upto 20% whereas TBG Pay V and TBG Pay IV(b) could tolerate upto 25% NaCl (Table 3).The results obtained in the present study are also in good agreement with similar studies conducted on fungal diversity from hypersaline environment by Zalar *et al*. (2007).

Table 2: Halotolerance test of potent isolates on MEA with different NaCl concentrations(Values ± SD)

Fungal isolate	0% NaCl	5% NaCl	7% NaCl	10% NaCl	15% NaCl	20% NaCl	25% NaCl
TBG Ekm II	75±1.22	72±0.74	61±2.00	41±1.54	19±0.68	-	-
TBG Pay V	71±0.6	70±0.35	72±1.45	53±2.02	29±1.24	14±0.67	10±0.66
TBG Pay IV(b)	72±0.98	71±1.32	62±0.64	54±1.65	29±1.78	15±1.58	12±1.34
TBG Ezh 4	72±1.53	72±1.05	60±0.56	35±0.26	14±0.69	-	-
TBG D2 Azk	72±0.66	72±1.46	61±1.08	40±1.71	31±2.07	19±1.05	-

Morphological and Microscopic identification of potent fungal isolates:

Table 3: Microscopical characteristics of the fungal isolates

Microscopical characteristics of Fungal isolates					
Strains	**TBG Ekm II**	**TBG Ezh 4**	**TBG D2 Azk**	**TBG Pay V**	**TBG Pay IV(b)**
Structure	Uniseriate	Biseriate	Biseriate	Biseriate	Biseriate
Shape of conidia	Globose	Ellipsoidal	Globose and radiate	Ellipsoidal	Ellipsoidal
Shape of vesicle	Spherical-globose	Ellipsoidal	Globose	Ellipsoid	Ellipsoid
Diameter of vesicle	48 - 74 µm	Metulae- 3.5 µm x4.5 µm Phialide-6-10 no	Metulae 3.8 x 5 µm Philaide-8-10 no	Metulae-4.24 µm x 3.02 µm Phialide-6-8 in no(Biverticilliate)	Metulae-2.50 µm x 3.5 µm Phialide-6.9 in no (Terverticilliate with 1-2 rami)
Conidiophore	Short	Long	Long	Long	Long
Colour of conidia	Brown	Green	Brownish yellow	Green	Green

Table 4: Morphological characteristics of fungal isolates in different media

Cultural characteristics of fungal isolates					
Strains	**TBG Ekm II**	**TBG Ezh 4**	**TBG D2 Azk**	**TBG Pay V**	**TBG Pay IV(b)**
Malt Extract Agar(MEA)					
Colony diameter	37mm	47mm	47mm	25mm	30mm
Appearance	Black	Dark green	Dark green	Dark green centre with white margins	Radially sulcate floccose centrally
Texture	Floccose	Velutinous	Umbonate	Powdery	Floccose
Mycelium	White	Inconspicuous	Initially hairy then to dry powdery	Inconspicuous	Inconspicuous but dull green
Furrows	Inconspicuous but grey apperance	Nil	Nil	Present	Nil
Exudates	Nil	Nil	Nil	Nil	Nil
Pigment production	Nil	Nil	Nil	Nil	Nil
Reverse colour	Berge/Cinnamon	Orange	Yellow	Light yellow	Yellow
Reverse morphology	plane	Plane	Smooth	Plane	Smooth
Potato Dextrose Agar(PDA)					
Colony diameter	35mm	39mm	49mm	40mm	40mm
Appearance	Black-dark brown	Whitish green	Dark olive green	Dark greenish	Dark green with white margins
Texture	Rough	Velutinous	Umbonate	Velutinous-floccose	Floccose
Mycelium	White	White	Nil	White	Greenish white
Furrows	Radial	Nil	Nil	Nil	Nil
Exudates	Nil	Nil	Nil	Nil	Nil
Pigment production	Lemon yellow soluble pigment	Nil	Nil	Nil	Nil
Reverse colour	Yellow	White	Milky white	Beige	Brownish yellow
Reverse morphology	Distinct radial furrows	Plane	Plane	Plane	Plane
Czapek Dox Agar(CDA)					
Colony diameter	40mm	45mm	42mm	40mm	30mm
Appearance	Whitish yellow	Whitish green	Greenish yellow	Radially convolute green	Radially sulcate centrally umbonate
Texture	Rough	Plane floccose	Hairy with abundant conidia	Lightly floccose	Floccose
Mycelium	White with black scattered conidia	Inconspicuous	Submerged	Inconspicuous	White at the centre and glaucous blue-green
Furrows	Nil	Nil	Nil	Nil	Nil
Exudates	Nil	Limited	Nil	Nil	Nil
Pigment production	Nil	Nil	Nil	Nil	NIl
Reverse colour	Yellow	Orange	Colourless	Yellow brown	Yellow
Reverse morphology	Plane	Plane	Plane	Undulate	Plane
Sabourauds Dextrose Agar(SDA)					
Colony diameter	51mm	34mm	50mm	35mm	30mm
Appearance	Black	Dark	Yellow brown	Dark greenish white	Radially sulcate floccose

		greenish white			centrally
Texture	Floccose	Velutinous	Smooth	Smooth with narrow ridges	Floccose
Mycelium	Light yellow	White inconspicuou s	Hairy	White	White-buff coloured
Furrows	Radial	Nil	Nil	Nil	Nil
Exudates	Nil	Nil	Nil	Nil	Nil
Pigment production	Nil	Nil	Nil	Nil	Nil
Reverse colour	Deep yellow	Green	Yellow	Lemon yellow	Yellow
Reverse morphology	Distinct furrows	Plane	Plane	Plane	Smooth

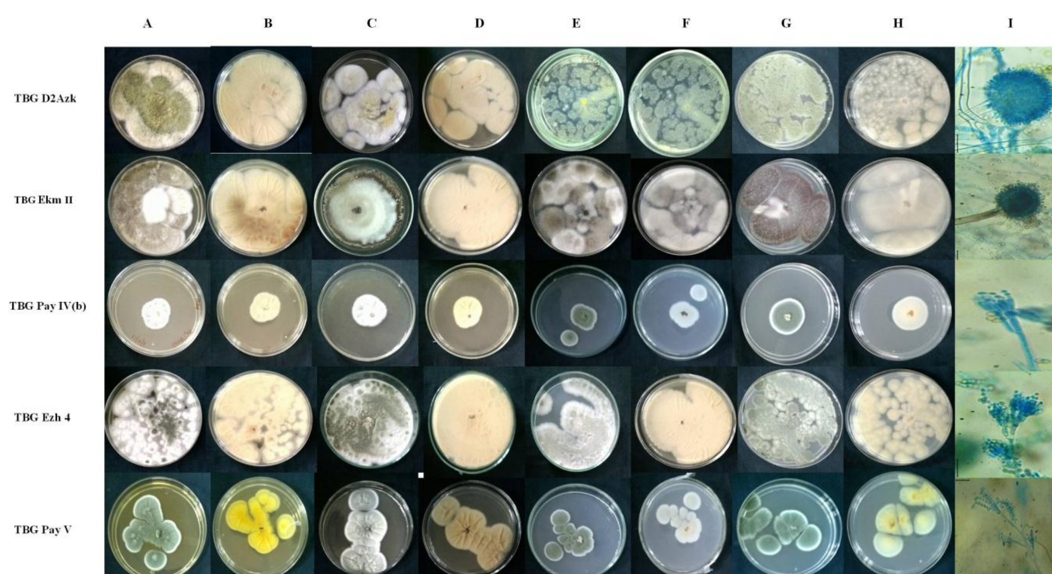

Fig 4: Colony morphology of isolates in different medium and their microscopical images(A-MEA,B-Reverse side of MEA,C-SDA,D-Reverse side of SDA,E-CDA, F-Reverse side of CDA,G-PDA,H-Reverse side of PDA,I-Microscopic image of the strains)

Biochemical characterization of fungal isolates: Gelatin liquefaction: Except the strain TBG Pay IV(b), no other isolate liquefied gelatin after keeping for 2-3°C for 1 h.

Tyrosine utilization: A brownish diffusible pigment was produced by TBG D2 Azk when inoculated in tyrosine utilization media. The other isolates did not produce any pigment and are considered to be negative.

Sugar utilization: Sugar utilization by each isolate is represented in the Table 5.

Table 5: Sugar utilization of the isolates

Sugar	TBG Ezh4	TBG PayV	TBG PayIV(b)	TBG Ekm II	TBG D2 Azk
Cellulose	++	+	+	+	+
Raffinose	++	-	-	++	++
Sucrose	++	++	++	++	+
Inositol	+	±	±	++	+
Mannitol	++	±	±	++	+
Fructose	+	++	±	+	+

(++ - strongly positive utilization,+ - positive utilization, ± - Utilization doubtful, - -utilization negative)

Molecular characterization of the isolates: Genomic DNA was extracted from the isolates and PCR was carried out using ITS primers. All the five isolates showed an amplified product size ranging from 540-600 bp. The amplified ITS1-5.8S-ITS2 fragment was sequenced and compared with sequences in the BLAST alignment program of the Genbank database. The phylogenetic tree was constructed using MEGA 7.0 (Fig.6). Based on the results of similarity comparison of the ITS-5.8S gene sequences it is concluded that the isolate TBG D2 Azk is closely related to *Aspergillus flavus* (Accession no.MF151171), the isolate TBG Pay V is closely related to *Penicillium goetzii*(Accession no.MF151170),TBG Pay IV(b) is closely related to *Penicillium oxalicum*(Accession no.MF151169), the isolate TBG Ekm II is closely related to Aspergillus aculeatus(Accession no.MF151167) and the isolate TBG Ezh 4is closely related to *Penicillium expansum* (Accession no.MF151168).

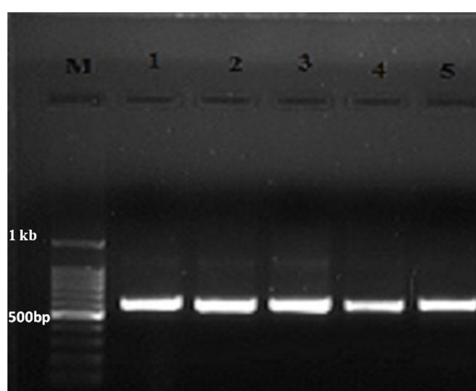

Fig 5: Agarose gel pic showing DNA bands of the isolates.Lane1-100bp ladder,1-TBG EkmII,2-TBG Pay V,3-TBG Pay IV(b),4-TBG Ezh 4,5-TBG D2Azk

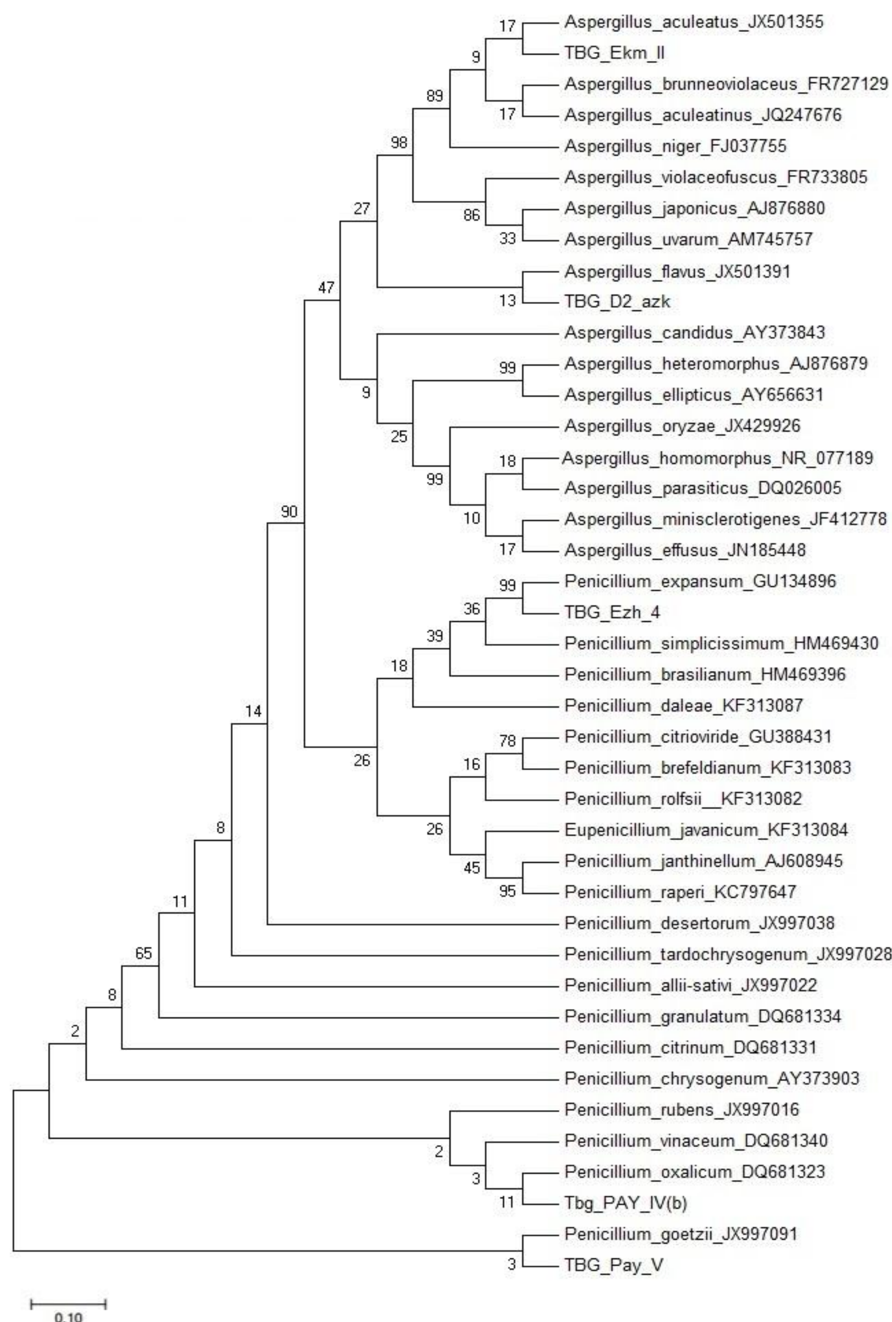

Fig. 6: Phylogenetic tree showing evolutionary relationships of 30 taxa based on the similarities of ITS1-5.8S-ITS2 Sequences. The evolutionary history was inferred using the Neighbour joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Phylogenetic analyses were conducted in MEGA 7.

CONCLUSION

The study includes the isolation and screening of halotolerant fungi capable of producing protease enzyme from less explored sediments of five different mangrove sites of Kerala. A total of 191 isolates were obtained and all of them were screened for the production of protease enzyme qualitatively based on their zone formation in Skimmed milk agar plates. The positive strains were screened for the enzyme quantitatively in protease specific fermentation broth without the substrate. Five potent isolates obtained from secondary screening were rechecked for their protease production in broth containing casein as substrate for measuring the induced protease enzyme. These strains were found to be halotolerant and they were identified by morphological, microscopical and molecular methods. All the potent isolates belonged to the genus *Aspergillus* and *Penicillium* and the identification was done upto the species level and deposited in the GenBank database. The identification of halotolerant fungi of mangrove origin and the potential of these strains to produce protease enzyme makes these fungi promising candidates for industrial application.

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