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Salt-tolerant chitin and chitosan modifying enzymes from *Talaromyces stipitatus*, a mangrove endophyte

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Abstract

In this paper, we show that a *Talaromyces stipitatus* isolated as an endophyte from the root of the mangrove tree *Avicennia marina*, produces salt-tolerant chitinase and chitosanases. The endophyte is halotolerant and produces these chitin modifying enzymes even in the presence of a high concentration of NaCl in the growth medium. The chitosanases produced could act on chitosans of low, medium and high degrees of acetylation. The presence of NaCl influenced the production of isoforms of chitinase and chitosanase by the endophyte. The chitinase activity was not altered much by NaCl concentration.

Key words – chitinase – chitosanase – halotolerant enzymes – fungal enzymes

Introduction

Chitin is made of repeating units of β-1,4 linked N-acetyl-D-glucosamine and is an essential part of fungal cell walls, shell of crustaceans and exoskeleton of insects. Fungi produce nearly 25 different chitinases (Seidl 2008) to facilitate restructuring of their cell walls during growth, branching, fusion of hyphae and also to aid in parasitism (Adams 2004). Fungal chitinases belong to glycosyl hydrolase (GH) 18 family. Fungi also produce chitin deacetylases which deacetylate chitin to chitosans, which are further degraded by chitosanases. Chitin and its derivatives have many industrial and pharmaceutical uses (Howard et al. 2003). They could be used for biological control of parasites, drug delivery, wound healing, and waste water treatment (Dahiya et al. 2006, Aoyagi et al. 2007, Nam et al. 2010). Likewise, chitooligosaccharides have many pharmaceutical applications including in tumour and asthma inhibition, in treatment of osteoporosis and wounds, as antibacterial, anti fungal and anti malarial agents and as vectors for gene delivery (Aam et al. 2010). Purification and modification of chitin using chemical methods are difficult and hence, chitin-modifying enzymes of microbial source are explored for these activities (Muzzarelli 1999). In this regard, chitin modifying enzymes from fungi have attracted much attention (Gortari & Hours 2008).

In the recent times, fungi from little-explored habitats such as endophytes of terrestrial plants (Govinda Rajulu et al. 2011, Cord-Landwehr et al. 2016), marine algae and seagrasses

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(Venkatachalam et al. 2015), fungi from soils exposed to chitin products (Malathi et al. 2015) and thermophilic fungi (Li et al. 2010) have been explored with the aim of finding novel chitin modifying enzymes. Considering the roles of chitin modifying enzymes in generating products of biotechnological potential, it is essential that several fungi from unique habitats are studied for elucidation of these enzymes. It is also of much importance to study them in detail since several aspects of these enzymes including their regulation and specific roles are not yet clearly understood (Seidl 2008, Govinda Rajulu et al. 2011).

In the present study, we screened an endophytic fungus isolated from the root of a mangrove tree for its chitinase and chitosanase activities. Endophytes are mostly ascomycete fungi which are non-disease causing endosymbionts of plants (Hyde & Soytong 2008).

Materials & Methods

Fungal source

In an initial study, we screened for chitinase several endophytes from Vivekananda Institute of Tropical Mycology (VINSTROM)'s culture collection and those we isolated from the roots of mangroves of Pichavaram (11.4226° N, 79.7748° E), Tamilnadu state. An endophyte isolated from the root of the mangrove *Avicennia marina* showed the maximum chitinase activity and was selected for further study. Based on culture and molecular characteristics (explained in the next sections), it was identified as *Talaromyces stipitatus* (Thom) Benjamin (≡ *Penicillium stipitatum* Thom) and was screened for the production of salt-tolerant chitinase and chitosanases.

Test for salt-tolerance

The fungus was grown on Czapek Dox Agar medium (CDA) for 5 days and the margin of the colony was cut with a sterile cork borer (5mm dia) and this mycelial plug was placed (mycelial surface down) at the center of a Petri dish (9cm dia.) containing 20ml of CDA amended with different concentrations of NaCl (0, 1.5%, 2.5%, 3.5%, or 4.5%) (Cantrell et al. 2006). Petri dishes were incubated at 26 ±2ºC and the colony diameter was measured every day with calipers. Replicates were maintained for each treatment.

Preparation of samples for detecting chitinase and chitosanases

The endophyte was grown in Potato Dextrose medium [potato 200 g, dextrose 20 g, distilled water 1l, pH 6.0 with different concentration of NaCl (0, 1.5%, 2.5%, 3.5%, or 4.5%)] for 5 days as static culture at 26 ±2° C. The mycelium was filtered and 100ml of the culture filtrate was dialyzed for 15 h against distilled water. The dialyzed culture filtrate was lyophilized and used as crude enzyme source for detecting chitin modifying enzymes.

Spectrophotometric assay for chitinase (Govindarajulu et al. 2011)

A reaction mixture made of 0.6 ml of 0.1 M sodium acetate buffer (pH 5.2), 0.2 ml of CM-chitin-RBV (Remazol brilliant violet-dye labeled chitin, Löwe Biochemica, Germany), and 0.02 ml of enzyme preparation (5 mg of freeze dried powder mixed in buffer) was prepared. The blank tube contained all the reagents except the enzyme which was replaced with 0.02 ml of buffer. The reaction mixture was incubated at 37°C for 60 min allowing the substrate to be digested. The reaction was terminated by adding 0.2 ml of 2 N HCl, incubated at 0°C for 10 min, and centrifuged at high speed for 5 min. The supernatant was assayed for enzyme activity by measuring the absorbance at 550 nm (Wirth & Wolf 1990, Govindarajulu et al. 2011). The enzyme activity is expressed as ΔA550nm/h × mg × protein.

Dot blot assay for chitinase and chitosanases (Govindarajulu et al. 2011)

A composite gel consisting of stacks of glycol chitin or chitosans of 1.6, 38 or 56% degree of acetylation (DA) was layered as follows. A gel was prepared by mixing a solution of 1 ml of 30% Acrylamide/Bisacrylamide, 0.3 ml substrate (glycol chitin or one of the chitosans), 1.7 ml of

Sodium acetate buffer (pH 5.2), 0.003 ml of 40% Ammonium persulphate and 0.003 ml of 100% TEMED. The surface of this gel was layered with butanol to aid polymerization. Accordingly, a compound gel consisting of glycol chitin or chitosan (of 56, 38 or 1.6% DA) was obtained.

10 mg of the lyophilized culture filtrate of the fungus (grown in different NaCl concentrations as mentioned above) was mixed with 1 ml of 50 mM sodium acetate buffer (pH 5.2) and centrifuged at high speed for 5 min; 5 µl of the supernatant was spotted on the gel and incubated at 37˚ C for 12 h. The gels were stained with 0.01% calcofluor white for 5 min, washed with distilled water for 1 h and observed under UV transilluminator to detect zones of darkness which indicated enzyme activity.

Zymography for chitinase and chitosanases (Govindarajulu et al. 2011)

A separating gel containing 4 ml of 30 % Acrylamide/Bisacrylamide, 1 ml of substrate [1 % glycol chitin or 0.1 % chitosans of 1.6, 38 or 56 % (DA)], 2.5 ml of 1.5 M Tris HCl buffer (pH 8.8), 2.5 ml of deionized water, 0.010 ml of 40 % ammonium persulphate and 0.010 ml of 100 % TEMED was prepared, poured into a gel cassette and left for 20 min for polymerization. After polymerization, the stacking gel was prepared by mixing 0.85 ml of 30 % Acrylamide/Bisacrylamide, 1.25 ml of 0.5 M Tris HCl buffer (pH 6.8), 2.9 ml of deionized water, 0.005 ml of 40 % Ammonium persulphate and 0.005 ml of 100 % TEMED and layered on the separating gel in the cassette. A comb was inserted between the gel cassettes.

The sample was prepared as mentioned under the dot blot assay. The supernatant (25 µl) was mixed with 25 µl of sample buffer without β-mercaptoethanol and subjected to SDS-PAGE. After electrophoresis (50 mA for 5 h), the gel was washed twice for 20 min in 50 mM Na-acetate buffer (pH 5.2) with 1 % Triton X-100. It was then washed twice in the same buffer (without 1 % Triton X-100) for 20 min to remove the Triton X-100. The gel was incubated at 37 °C for 12 h under shaking in 50 mM Na-acetate buffer (pH 5.2) solution, and then stained with 0.01 % calcofluor in 0.5 M Tris-buffer (pH 8.9) solution for 5 min; finally, the gel was washed with deionized water for 1 h and visualized under UV transilluminator.

Genomic DNA extraction

The mycelium from a 6-day old PDA grown culture was treated with 500 µl of DNA extraction Lysis buffer (1M Tris, 5M NaCl, 0.5M EDTA, 10 % SDS) for overnight at room temperature. After incubation, equal volume of phenol was added, mixed briefly and centrifuged at 10600 g for 15 min at 4°C (Eppendorf 5810R). The upper clear layer was transferred to a fresh tube and to this, equal volumes of chloroform: isoamylalcohol (24:1) was added and centrifuged at 10600 g for 15 min at 4°C. The upper layer was then precipitated using 0.3M Sodium acetate and absolute alcohol by incubating for 3 h at -80°C. The tubes were then thawed and centrifuged at 10600 g for 15 min at 4°C. The supernatant was discarded and 100 µl TE-Buffer and 15 µl of RNase (10 mg/ml) were added and the tubes were incubated for 45 min at 37°C. The genomic DNA was again precipitated by adding 0.7 volume of isopropanol and 0.1 volume 0.3 M Sodium acetate and centrifuged at 10600 g for 15 min at 4°C. The pellet was washed with 70 % ethanol and the genomic DNA obtained was run in a 0.8 % agarose gel.

PCR amplification and sequencing of ITS region

The amplification of the ITS region was carried out using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTA1TTGATATGC-3') (White et al. 1990). PCR was carried out in a 25 µL reaction mix consisting of 10X PCR buffer, forward and reverse primers (10 µM each), 4mM dNTPs, 1 Unit of Taq DNA Polymerase, 1% DMSO, 25mM $MgCl_2$ and ~50 ng of fungal genomic DNA as template. The cycling reactions were performed in a Master Cycler Thermocycler (Eppendorf, USA) under the following conditions: 95ºC for 10 min, followed by 30 cycles of 95ºC for 60 s, 55ºC for 60 s and 72ºC for 90 s, and finally at 72ºC for 10 min. The amplicons were checked on 1.5 % agarose gel to determine product size and purity. The amplicons were purified using gel elution technique and then sequenced using ABI 3130 Genetic

Analyzer using both the forward and reverse primers. The sequences were manually edited and aligned using BioEdit software and then searched for similarity using NCBI Blast.

The ITS1-5.8S-ITS2 sequence fragment of *Talaromyces* sp. was aligned with published sequences from the type specimens of *Talaromyces* and *Penicillium* (anamorph) available from GenBank database. The sequences were downloaded and aligned using ClustalW (Thompson et al. 1994) with default settings. In the final analysis a total of 76 nucleotide sequences were included (Table 1). The aligned sequences were then manually adjusted and evolutionary tree of the datasets were inferred using Maximum Likelihood method (Tamura & Nei 1993) by using MEGA version 6.06 (Tamura et al. 2011) to identify closely related sequences. The branch support was assessed by performing a bootstrap analysis with 1000 replicates (Felsenstein 1985). All ambiguous positions containing gaps and missing data were eliminated and a total of 437 positions were included in the final dataset. A culture of this fungus has been deposited with National Fungal Culture Collection of India, Pune (Accession No. 4222) and the ITS sequence has been deposited in GenBank (Accession No. MG996147).

Results

The endophyte did not have an obligatory requirement for salt to grow indicating that it is not halophilic. It grew on the highest concentration of NaCl tested (4.5 % = 0.8M) proving that it is halotolerant (Fig. 1). A spectrophotometric assay showed that the endophyte produced chitinase in NaCl-amended growth medium as well as in control medium lacking NaCl. The activity of chitinase was slightly more when the fungus was grown on medium having 1.5, 2.5 or 3.5% of NaCl than that on control medium (Fig. 2); however, chitinase activity was marginally low in the medium amended with 4.5% NaCl. A dot blot test also confirmed that the fungus produced chitinase in all the concentrations of NaCl tested (Fig. 3). This test also revealed that the endophyte produced chitosanases acting on chitosans with high (56%), medium (38%) and low (1.6%) degrees of acetylation (Fig. 3); furthermore, the production of these enzymes was not affected by NaCl concentration used (Fig. 3). A sensitive polyacrylamide gel electrophoresis for visualizing isoforms of chitinase and chitosanases showed that new isoforms of the enzymes were induced by the presence of NaCl in the growth medium (Fig. 4).

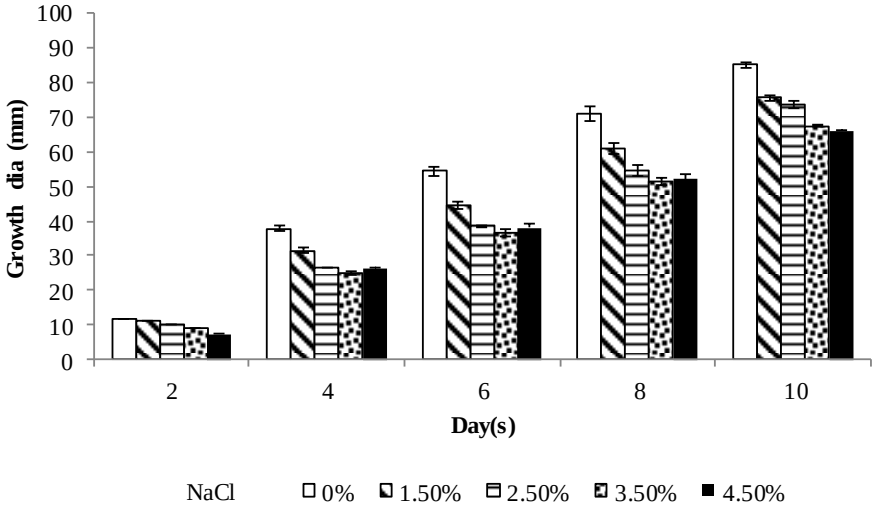

Figure 1 – Growth of *T. stipitatus* in the presence of different concentrations of NaCl in the growth medium. Bars represent Standard Error.

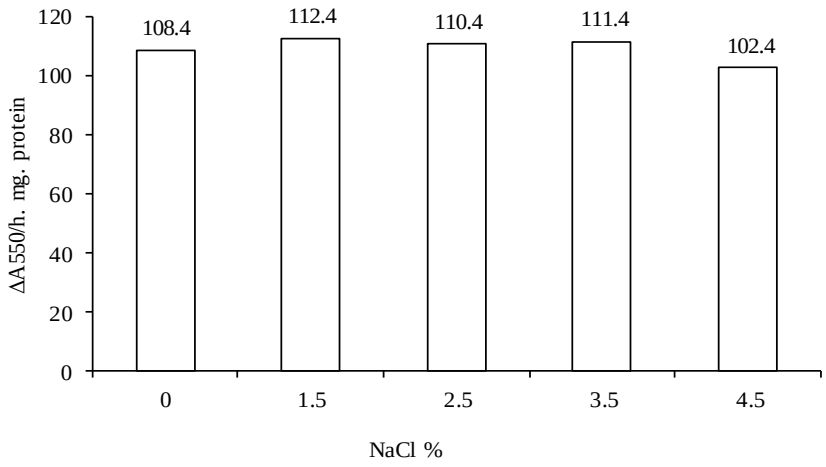

Figure 2 – Chitinase activity of *T. stipitatus* as influenced by different NaCl concentrations in the growth medium.

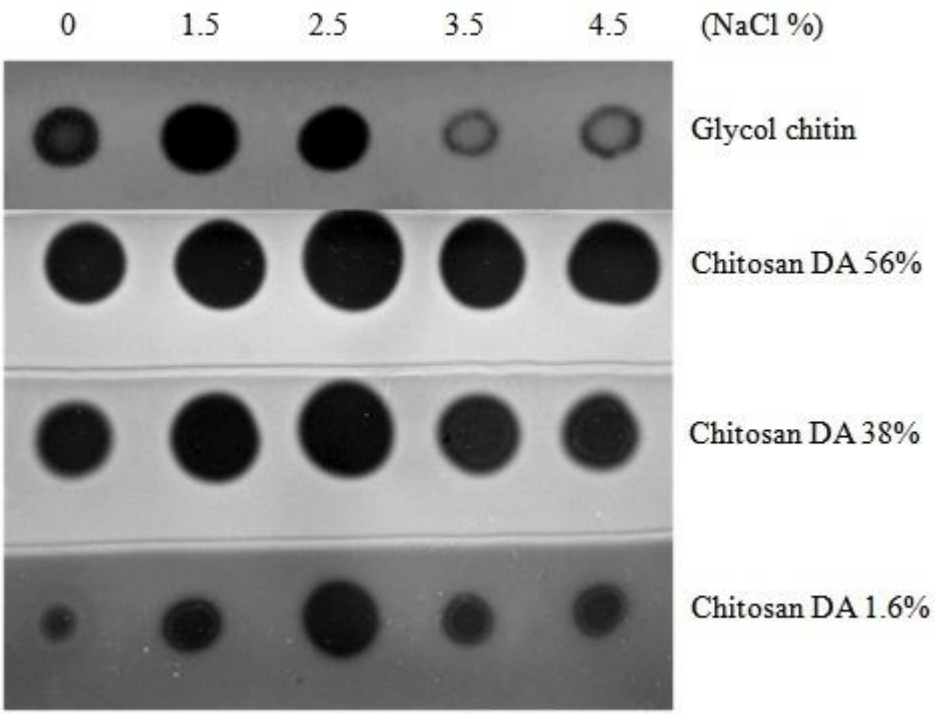

Figure 3 – Dot blot assay for **c**hitinase and chitosanases of *T. stipitatus.*

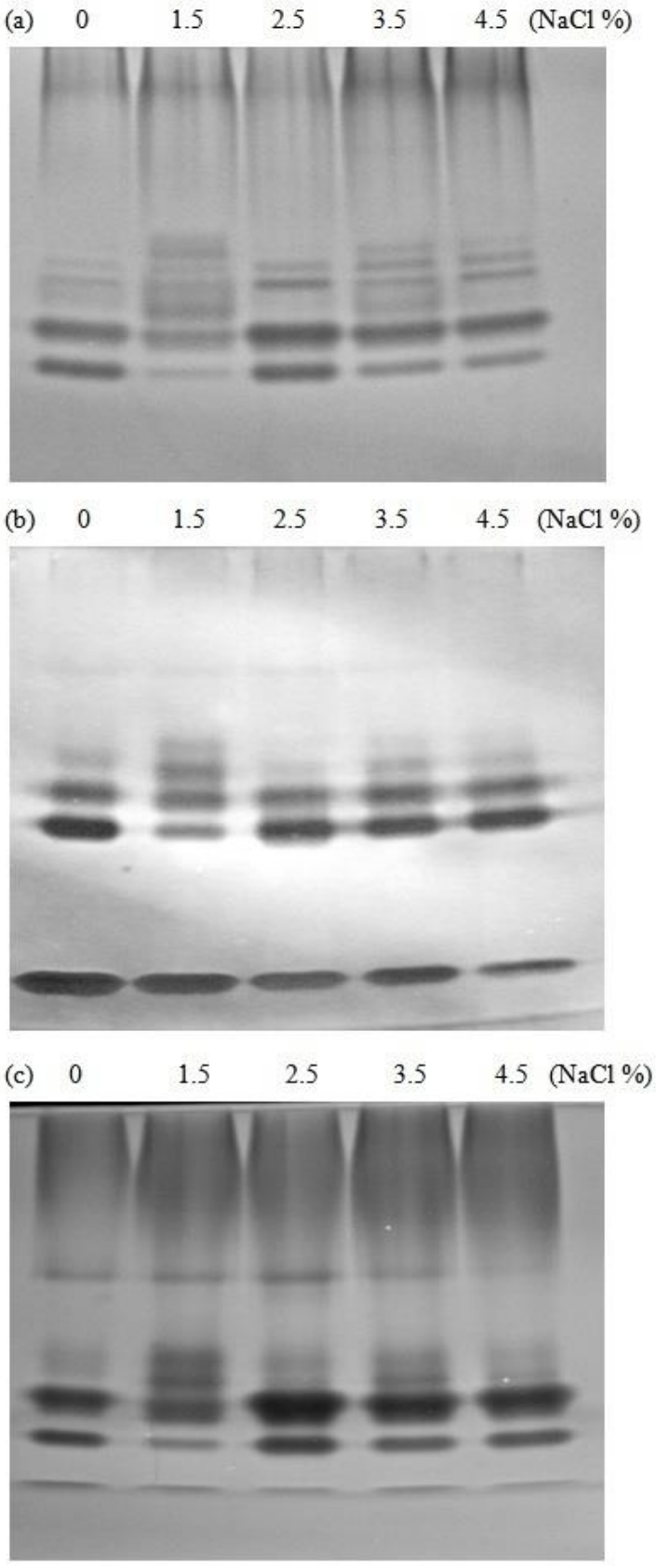

Figure 4 – Isoforms of chitinase (a) and chitosanases (b-Chitosan DA 56% and c-Chitosan DA 38%) of *T. stipitatus* as influenced by different concentrations of NaCl in the growth medium.

Table 1 Accession number of isolates used for phylogenetic analysis.

Sl. No.	Accession Number	Isolate (Type material)
1	MG996147	Our isolate
2	NR103661	*Penicillium adametzii*
3	NR138263	*Penicillium brefeldianum*
4	NR121299	*Penicillium brevicompactum*
5	NR111551	*Penicillium carneum*
6	NR077145	*Penicillium chrysogenum*
7	NR121224	*Penicillium citrinum*
8	NR121317	*Penicillium clavigerum*
9	NR138293	*Penicillium coeruleum*
10	NR121312	*Penicillium coffeae*
11	NR144827	*Penicillium consobrinum*
12	NR138349	*Penicillium fuscum*
13	NR138342	*Penicillium fusisporum*
14	NR103692	*Penicillium griseofulvum*
15	NR137913	*Penicillium hoeksii*
16	NR121311	*Penicillium indicum*
17	NR103693	*Penicillium kewense*
18	NR138336	*Penicillium kongii*
19	NR121222	*Penicillium lividum*
20	NR138339	*Penicillium ludwigii*
21	NR137878	*Penicillium mexicanum*
22	NR137129	*Penicillium monsserratidens*
23	NR138270	*Penicillium montanense*
24	NR121518	*Penicillium nothofagi*
25	NR121232	*Penicillium oxalicum*
26	NR111816	*Penicillium persicinum*
27	NR121258	*Penicillium pimiteouiense*
28	NR121230	*Penicillium raperi*
29	NR121239	*Penicillium restrictum*
30	NR121231	*Penicillium reticulisporum*
31	NR103621	*Penicillium roqueforti*
32	NR111494	*Penicillium roseopurpureum*
33	NR138351	*Penicillium roseoviride*
34	NR111815	*Penicillium rubens*
35	NR121243	*Penicillium rubidurum*
36	NR137849	*Penicillium salmoniflumine*
37	NR077157	*Penicillium sclerotiorum*
38	NR111819	*Penicillium sinaicum*
39	NR077158	*Penicillium spinulosum*
40	NR119812	*Penicillium sumatrense*
41	NR111508	*Penicillium svalbardense*
42	NR111486	*Penicillium tropicoides*
43	NR111485	*Penicillium tropicum*
44	NR138355	*Penicillium trzebinskii*
45	NR153221	*Penicillium tubakianum*
46	NR121251	*Penicillium tularense*

Table 1 Continued.

Sl. No.	Accession Number	Isolate (Type material)
47	NR121257	*Penicillium turbatum*
48	NR137907	*Penicillium vagum*
49	NR119495	*Penicillium verrucosum*
50	NR077137	*Penicillium virgatum*
51	NR121209	*Penicillium viticola*
52	NR111491	*Penicillium waksmanii*
53	NR153225	*Penicillium wisconsinense*
54	NR111501	*Penicillium zonatum*
55	NR120179	*Talaromyces amestolkiae*
56	NR121530	*Talaromyces apiculatus*
57	NR147431	*Talaromyces australis*
58	NR147433	*Talaromyces columbinus*
59	NR103669	*Talaromyces diversus*
60	NR121528	*Talaromyces echinosporus*
61	NR147430	*Talaromyces kendrickii*
62	NR103671	*Talaromyces marneffei*
63	NR103672	*Talaromyces muroii*
64	NR145151	*Talaromyces primulinus*
65	NR121529	*Talaromyces purpureogenus*
66	NR145153	*Talaromyces purpureus*
67	NR147439	*Talaromyces qii*
68	NR111780	*Talaromyces ruber*
69	NR103682	*Talaromyces rubicundus*
70	NR103683	*Talaromyces siamensis*
71	NR147424	*Talaromyces stipitatus*
72	NR111781	*Talaromyces stollii*
73	NR147428	*Talaromyces thailandensis*
74	NR145156	*Talaromyces udagawae*
75	NR153228	*Talaromyces veerkampii*
76	NR103675	*Talaromyces verruculosus*

The ITS1-5.8S-ITS2 sequence fragment of the endophyte isolate showed a 100% match with *Talaromyces stipitatus* HF05001 (Acc. No. KU057945). A Maximum Likelihood analysis was performed to compare our sequence with other related sequences belonging to genera *Talaromyces* and *Penicillium* (anamorph state of *Talaromyces*) available in GenBank database. For this analysis, we included only sequences obtained from type material. A total of 76 sequences (23 sequences belonging to *Talaromyces* and 53 belonging to *Penicillium*) were used for this analysis. The maximum likelihood tree showed two well separated clades, one belonging to all *Talaromyces* isolates and the other consisting of isolates belonging to *Penicillium* sp. Our sequence formed a tight clade with very high bootstrap support with sequences belonging to *T. stipitatus* (Fig. 5).

Discussion

Mangroves are intertidal habitats where wood and other substrates are decayed by salt tolerant fungi (Hyde & Jones 1998, Hyde & Lee 1998) and there has been much research on the biodiversity of both saprobes and endophytes in this milieu (Suryanarayanan et al. 1998, Kumaresan & Suryanarayanan 2001, 2002). In this study we used an endophyte isolate from mangroves to determine the salt-tolerant chitinase and chitosanases, which facilitate life in such

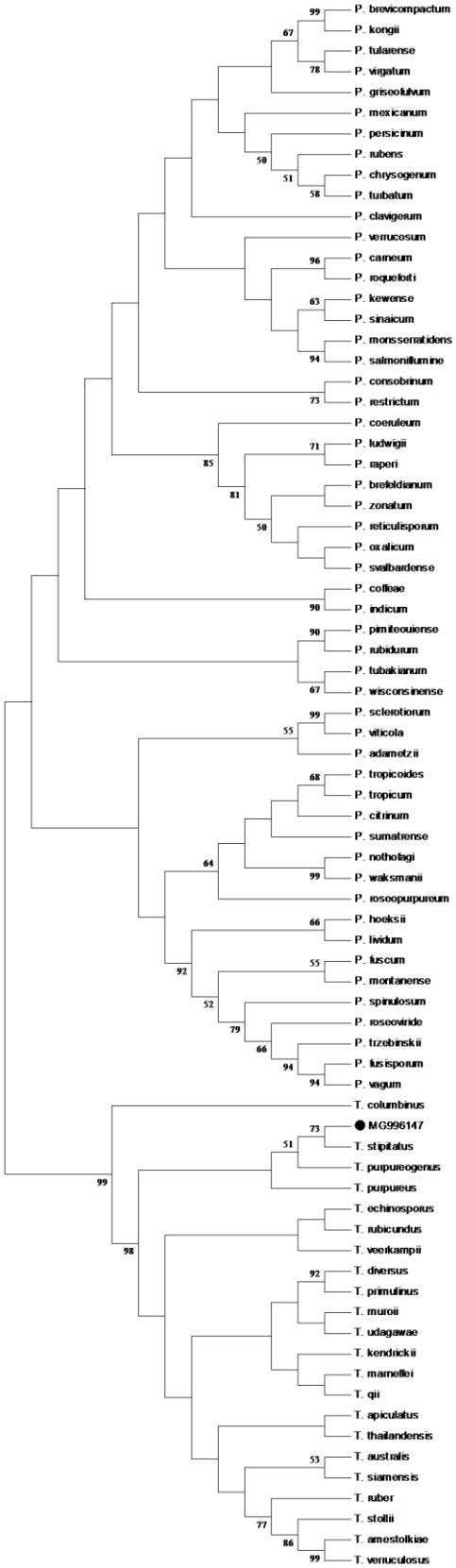

Figure 5 – Bootstrap consensus tree inferred using maximum likelihood method based on the Tamura-Nei model for aligned ITS rDNA sequences of *Talaromyces* (T) and *Penicillium* (P) species. Bootstrap values above 50% from 1000 replications are shown next to the branches. MG996147 is the fungus used in the current study.

saline environments (Venkatachalam et al. 2015, Thirunavukkarasu et al. 2017). We have limited information regarding chitin and chitosan modifying enzymes of fungi than those of bacteria (Malathi et al. 2015). It would therefore be useful to investigate fungi from different ecological niches for these industrially useful enzymes (Govinda Rajulu et al. 2011). *Talaromyces stipitatus* is known to produce several novel enzymes (Garcia-Conesa et al. 2004, Mandalari et al. 2008). Here we show that a *T. stipitatus* isolated as an endophyte in the roots of a mangrove produces salt tolerant chitinase and chitosanases and that NaCl induces the expression of new isoforms of chitinase and chitosanases. Earlier, we have reported that many endophytes isolated from leaves of terrestrial plants (Govinda Rajulu et al. 2011) and endophytes of marine algae and seagrasses (Venkatachalam et al. 2015) produce chitinases; it is interesting to note that the activity of chitinase observed from *T. stipitatus* (No. 4222) is five to twenty times more than that reported by us for the terrestrial (Govinda Rajulu et al. 2011) or marine plant endophytes (Venkatachalam et al. 2015). This increased activity of chitinase as well as the induction of its isoforms by salt in *T. stipitatus* warrant further studies. With reference to chitosanase, a strain of *T. stipitatus* (B8M2R4) has been previously reported to produce a chitosanase belonging to glycosyl hydrolase (GH) family 75 (Zhu et al. 2012). Interestingly, the endophyte in the present study produced salt-tolerant chitosanases which acted on chitosans with very low as well as high degrees of acetylation. The induction of isoforms of chitosanase by NaCl in this fungus indicates that the influence of salt on chitin modifying enzymes of halotolerant fungi has to be studied in detail. Being a mangrove tree, the roots of *Avicennia marina*, from where the endophyte was isolated in the present study, are subjected to wide fluctuations in salinity. Since high salt stress increases the levels of chitinases in salt-tolerant plants (Wang et al. 2015), further studies in this direction will provide clues on whether chitinases of endophytes of mangrove roots influence their host metabolism. Moreover, chitin and chitosans are known to stimulate defense responses in plants resulting in the accumulation of pathogenesis related proteins and phytoalexins (El Hadrami et al. 2010). Since endophytes are persistent members of the plant microbiome, it would be worthwhile to address the role of chitin and chitosan modifying enzymes of these endosymbionts in biotic stress tolerance of mangroves as well. Additionally, our results show that root endophytes of mangroves could be explored for novel salt-tolerant chitin modifying enzymes which could be exploited technologically.

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