

Screening marine-derived endophytic fungi for xylan-degrading enzymes

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Marine-derived fungi surviving as symptomless endophytes in seaweeds and seagrasses were screened for production of xylan-degrading enzymes. Of the eight endophyte isolates obtained from five different seagrasses and another eight from six different marine algae, half of them exhibited xylanase activity in an agar plate assay. Further examination of these lead candidates using spectrophotometric assays revealed that *Trichoderma harzianum*, endophytic in the brown alga *Sargassum wightii*, had the maximum secreted xylanase and xylosidase activity. Moreover, this fungus could grow in NaCl-containing media (up to 1.2 M NaCl), and inclusion of 0.26 M NaCl in the media elicited a two- and three-fold increase in extracellular xylanase and xylosidase activity respectively. These findings highlight the potential of prospecting marine-derived fungal endophytes to identify novel cell-wall degrading enzymes of value to the biofuel industry.

Keywords: Biomass deconstruction, marine-derived fungi, *Trichoderma harzianum*, xylan-degrading enzymes.

Introduction

LIGNIN, cellulose and hemicellulose are three polymers that make up the lignocellulosic biomass (LCB). While cellulose is the major component, the relative amount of the three polymers varies depending on the type of biomass. Food, fibre and fuel industries depend on the economical and efficient conversion of cellulose and hemicellulose polymers to their constituent monomers¹⁻³. However, deconstruction of LCB remains a challenging goal. For example, the cellulase-catalysed conversion of cellulose (a glucose homopolymer) to glucose is impeded by hemicellulose that acts as a physical barrier⁴⁻⁶. The focus of this study is the enzymatic hydrolysis of hemicellulose, an objective critical both for complete depoly-

merization of cellulose in LCB and for the generation of useful end-products from hemicellulose.

Hemicellulose refers broadly to branched non-cellulosic polymers and differs in make-up within and across higher plants/algae^{1,7-9}. Xylans, linear polymers of 1,4-linked β -D-xylopyranosyl residues, are the major constituents of hemicellulose in angiosperms contributing up to 30% of the cell-wall content. Xylans are typically heteropolymers with side-chain substitutions containing D-glucuronopyranosyl (with or without methylation), D-galactopyranosyl, L-arabinofuranosyl, acetyl, feruloyl, and *p*-coumaroyl groups, whose presence varies depending on the source. Given this heterogeneous composition, xylan degradation requires the concerted and synergistic action of main chain-deconstructing glycohydrolases and side chain-deconstructing esterases. However, xylanases and xylosidases are the principal enzymes in this repertoire.

Xylanases cleave xylan polymers to release short xylooligosaccharides (e.g. xylopentaose), which in turn are cleaved by xylosidases to release xylose. Although it has been appreciated for some time that xylanases are produced by a wide range of organisms spanning marine algae, protozoans, crustaceans, insects, plants and fungi⁸, bioprospecting for improved variants that fulfil a niche requirement has motivated studies of bacteria and fungi from deep-sea hydrothermal vents¹⁰, the Antarctic¹¹, marine sediments¹², insect gut¹³, alimentary canal of ruminant animals¹⁴, compost/soil^{15,16} and decaying wood/litter¹⁷. Because xylan-degrading enzymes from marine sources are less studied compared to those from terrestrial organisms and filamentous fungi produce xylanases at much higher levels than bacteria^{18,19}, we investigated xylanases and xylosidases produced by marine-derived fungi (MDF) surviving as endophytes in marine angiosperms and seaweeds.

There were two reasons that inspired our focus on MDF. First, they have not been studied for their cell wall-restructuring catalytic potential, although marine fungi have been investigated for salt-tolerant, extracellular enzymes²⁰. Fungi of the marine ecosystem are broadly

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grouped into obligate marine fungi and MDF. The former grow and sporulate in the marine environment, whereas the latter are either terrestrial or freshwater forms that may be present in the marine habitat²¹. The MDF exist either as saprotrophs in wood and marine sediments, or as parasites and symbionts of various marine organisms such as algae, fish, coral, molluscs, sponges and seagrasses. There are many studies that highlight the synthetic ability of endophytic MDF to generate bioactive secondary metabolites^{22–25}, but few address their potential for producing industrially important enzymes²⁶. This situation mirrors the fact that endophytes associated with terrestrial plants have been studied as a rich source of metabolites with bioactivities^{27–29}, and only recently been explored as an excellent source of enzymes^{3,30,31}. Second, since some seagrasses contain up to 40% xylan³², we postulated that endophytic fungi residing in seagrasses might have evolved efficient mechanisms for xylan degradation. We report here that indeed MDF isolated as endophytes from marine algae and seagrasses produce xylanases and xylosidases, thus underscoring the need to study these fungi for their biocatalyst inventory.

Materials and methods

Sources of endophytes

We screened the secretomes of 16 marine-derived endophytic fungi for xylanase activity. These fungi were isolated from algae and sea grasses^{33,34} growing in Kodyakkarai (10°17'0"N, 79°49'0"E), Keezhakkarai (9.2314°N, 78.7844°E) and Mandapam (9.2800°N, 79.1200°E), along the southeast coast of India. While the genera of these fungi were identified largely on the basis of their spore characteristics, two of them (644 and 516) were further identified at the species level by analysis of their ribosomal internal transcribed spacer (ITS) and one (562) was identified by analysing its translation elongation factor-1 α (EF1 α) DNA sequence; two of them (580 and 568) had been identified before using their ITS sequence (Table 1). Representative cultures of these fungi have been deposited with either the Microbial Type Culture Collection (MTCC), Chandigarh or the National Fungal Culture Collection of India (NFCCI) Pune, with the following accession numbers: *Curvularia tuberculata* 1 (NFCCI 2434), *Phomopsis* sp. 1 (MTCC10345), *Trichoderma* sp. 1 (MTCC10344). (Although brown algae do not belong to the plant kingdom, we use the term 'endophytes' in this study to denote the symptomless endosymbiotic fungi associated with them.)

DNA extraction, amplification and sequencing of ITS and EF1 α DNA

Using a standard phenol–chloroform-based extraction method, genomic DNA was isolated from mycelia grown

on potato dextrose agar (PDA) medium for a period of 7 days. While ITS1-5.8S-ITS2 regions of fungal rDNA were amplified by PCR using the fungal-specific primers ITS4 and ITS5 (ref. 35), the EF1 α -coding DNA was amplified using primers EF-728 and EF2 (refs 36 and 37). These amplicons (~500–600 bp in both cases) were electrophoresed on a 1% (w/v) agarose gel, excised and extracted, and subsequently sequenced using either ITS4 or EF728 in an automated sequencer (Applied Biosystems 3130 Genetic Analyzer). The sequences obtained were used as queries in BLASTn (default settings) to find the closest match in GenBank and in MycoBank databases; the same hits were obtained regardless of the database used. These ITS and EF1 α sequences were then submitted to GenBank and their accession numbers are listed in Table 1.

*Qualitative plate assay to assess secreted xylanase activity*³⁷

Endophyte isolates were grown in oat spelt xylan medium (OX; 9 g oat spelt xylan (Sigma), 5 g yeast extract, 1 g NaNO₃, 1 g KH₂PO₄, 1 g peptone, 0.3 g MgSO₄·7H₂O, 1000 ml water, pH 5.5) for 5 days at 31°C with shaking (120 rpm in a REMI orbital rotary shaker). The culture fluid was filtered through a Whatman #1 filter paper, centrifuged at 9600 g for 20 min at 4°C, and the supernatant was used as the enzyme input for the plate assay described below.

Oat spelt xylan (9 g) and agar (18 g) were dissolved in 1000 ml of 100 mM sodium phosphate (pH 4.5, 5.5 or 6.5), and autoclaved before being dispensed into petri dishes. After setting of the agar, a cork borer (5 mm diameter) was used to create the diffusion wells (Figure 1). To each well, 10 μ l of culture supernatant (crude enzyme) was loaded, and the agar plate incubated overnight at 31°C. Formation of a clear zone around the well was used to score a given endophyte secretome as positive for xylanase activity³⁸. Ten microlitres of uninoculated OX medium in lieu of a crude extract served as a negative control (Figure 1).

Preparation of crude secretome extracts for xylanase and xylosidase assays

Endophyte isolates were grown in birch wood xylan (BX) medium (10 g birch wood xylan, 5 g yeast extract, 1 g NaNO₃, 1 g KH₂PO₄, 1 g peptone, 0.3 g MgSO₄·7H₂O) for 5 days at 31°C in an orbital shaker (120 rpm). The culture fluid was then filtered through a Whatman #1 filter paper, and the filtrate centrifuged at 9600 g for 20 min at 4°C. The supernatant was dialysed against water (with three changes over 16–18 h) at 4°C and lyophilized. The protein amount in these dried secretomes was measured using either the Bradford or the bicinchoninic acid assay, with bovine serum albumin serving as the standard.

Table 1. Summary of endophytes displaying xylanase activity

Fungus (isolate number)	Isolate from seagrass (1–8) or algal (9–16) species	Xylanase activity (pH 4.5, 5.5, 6.5)	Accession numbers for ITS or EF1 α sequences
<i>Aphanocladium</i> sp. (508)	<i>Thalassia</i> sp.	–	
<i>Aspergillus terreus</i> (644)	<i>Cymodocea serrulata</i>	+	KP122964
<i>Cladosporium</i> sp.1 (505)	<i>Thalassia</i> sp.1	–	
<i>Curvularia tuberculata</i> (503)	<i>Thalassia</i> sp.1	–	
<i>Curvularia tuberculata</i> (516)	<i>Syringodium</i> sp.	+	KP122965
<i>Gonatophragmium mori</i> (622)	<i>Thalassia</i> sp.	+	
<i>Nodulisporium</i> sp. (679)	<i>Thalassia</i> sp. 3	+	
<i>Torulomyces</i> sp. (683)	<i>Syringodium</i> sp.	+	
<i>Cladosporium</i> sp.1 (547)	<i>Ulva lactuca</i>	–	
<i>Drechslera</i> sp. 1 (583)	<i>Sargassum wightii</i>	–	
<i>Drechslera</i> sp. 2 (610)	<i>Turbenaria</i> sp.	–	
<i>Fusarium oxysporum</i> (580)	<i>Sargassum wightii</i>	+	KC254033*
<i>Nigrospora</i> sp. (538)	<i>Jania adherens</i>	–	
<i>Phoma</i> sp. 1 (605)	<i>Sargassum</i> sp.	–	
<i>Phomopsis</i> sp. (562)	<i>Portieria hornemonii</i>	+	KP204449
<i>Trichoderma harzianum</i> (568)	<i>Sargassum wightii</i>	+	KC330218*

*These species were identified in an earlier study³³. +, Positive; –, Negative.

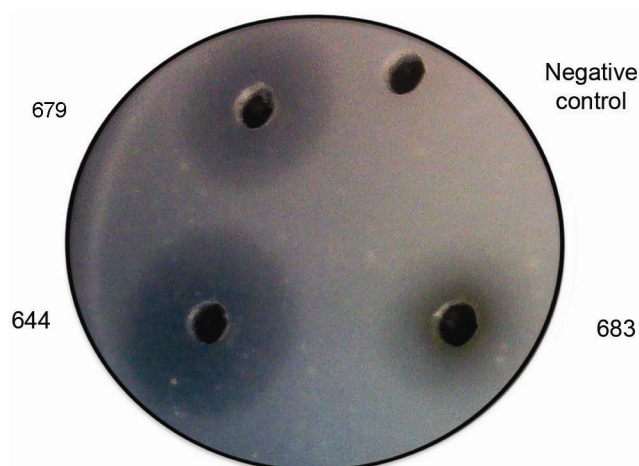


Figure 1. Agar plate assay for secreted xylanase activity. Culture filtrates of endophytes were tested for their ability to hydrolyze oat spelt xylan impregnated in an agar plate. Endophytes were scored as positive if their filtrates resulted in a zone of clearance, as observed here with *Aspergillus terreus* (644), *Nodulisporium* sp. (679) and *Torulomyces* sp. (683). Uninoculated culture filtrate served as the negative control. While three representative species are shown here, similar studies were conducted to obtain the data summarized in Table 1.

Enzyme assays

Xylanase assays: Xylanase activity was assayed as described elsewhere³⁹. The underlying principle is that xylan degradation releases xylose, which in turn can reduce 3,5-dinitrosalicylic acid (DNS) to 3-amino, 5-nitrosalicylic acid that absorbs at 540 nm. Using a standard curve generated with known quantities of xylose, the amount of xylose released in an enzymatic assay is easily determined.

We employed two variants of this xylanase assay. For the end-point measurements (Figure 2), we used a 2 ml assay mixture containing 1.8 ml of 1% (w/v) birch wood

xylan in 50 mM sodium citrate (pH 5.3) and 0.2 ml of secretome (crude enzyme). The reaction was initiated by incubation at 50°C for 5 min, and immediately added to 3 ml of 1% DNS prior to boiling for 10 min and subsequent addition of 1 ml 40% (w/v) potassium tartrate. The Abs₅₄₀ of these solutions was measured using a Unicop SpectroQuest 4802 spectrophotometer.

For the kinetic assays at 37°C (Figures 3 and 5), we used a final concentration of 50 mM sodium acetate (pH 5.5) and 0.5% (w/v) birch wood xylan. A 360 μ l reaction mixture contained 0.5% (w/v) birch wood xylan in 50 mM sodium acetate (pH 5.5). The substrate in buffer (310 μ l) and the secretome resuspended in phosphate-buffered saline (PBS; ~30 to 300 μ g in 50 μ l) were individually pre-incubated for 10 min at 37°C, before they were mixed to initiate the reaction. Five aliquots were withdrawn over 15 or 75 min (depending on strong or weak activity), and each 72 μ l aliquot was mixed with 72 μ l 1% (w/v) DNS and boiled for 10 min. The boiled samples were cooled and added to 72 μ l 40% (w/v) potassium tartrate. The final volume (216 μ l for each time point) was divided into two equal parts to enable two independent measurements at 540 nm by a Flexstation 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, USA).

For each secretome, a specific blank was generated to ensure that xylose carryover (if any) was accounted for. Rather than performing a time course after addition of a pre-incubated crude secretome to the substrate, the blanks entailed immediate boiling with 1% (w/v) DNS. The subsequent steps were the same as those described above for the corresponding test assays.

Xylosidase assays: β -Xylosidase activity was determined by measuring the amount of p-nitrophenol (pNP)

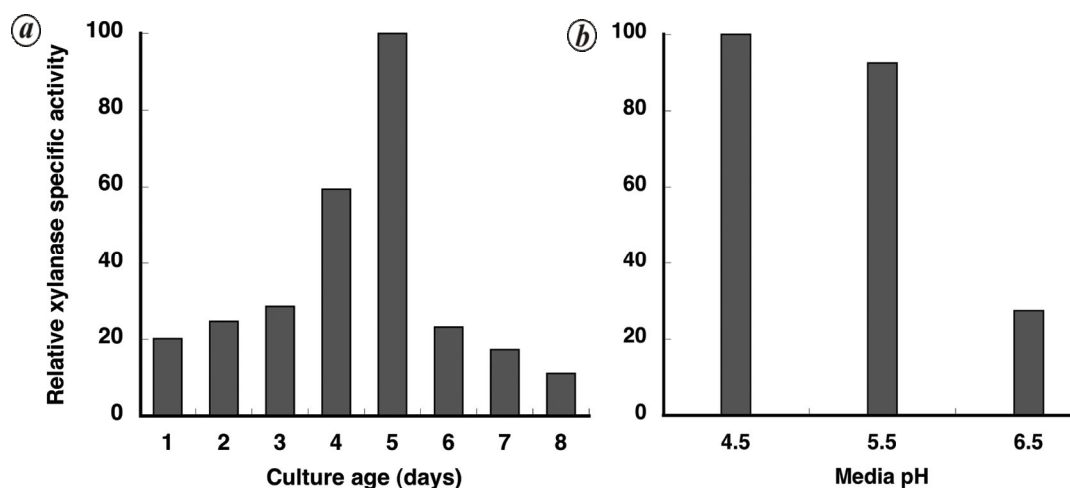


Figure 2. Effect of culture age and media pH on xylanase activity secreted by *T. harzianum*. Single time-point measurements were used to determine the xylanase activities in filtrates of cultures grown in the presence of 1% (w/v) xylan, either at pH 5.5 for 1–8 days (a) or at three different pH values for 5 days (b). The activities are normalized against the highest value observed in each comparison panel. From end-point measurements, we determined xylanase specific activities at 50°C to exhibit less than twofold variation and a maximum of 46 U/mg (e.g. pH 4.5). The standard deviation of the mean from three independent xylanase activity measurements did not exceed 24% (with the exception of the measurement for day 1, 44%).

released from *p*-nitrophenyl- β -D-xyloside (pNPX; Sigma, St Louis, USA). All kinetic assays were performed at 37°C in a final concentration of 33 mM sodium acetate (pH 5.2) and 3.3 mM pNPX. The substrate was pre-incubated at 37°C for 7 min in a volume of 696 μ l, and the reaction initiated by adding a defined amount of secretome (~25–65 μ g protein resuspended in 24 μ l PBS). Four aliquots were withdrawn every 15 min during a 1 h incubation (Figure 3); for the experiments examining effect of NaCl in the media (Figure 4), only three aliquots were withdrawn during a 45 min incubation. At each time point, the reactions were terminated by adding an equal volume of 1 M sodium carbonate. The amount of pNP formed was determined based on the absorbance at 405 nm. For each time point, the final quenched reaction (360 μ l) was divided into three equal parts to allow three independent measurements at 405 nm by a Flexstation 3 Multi-Mode Microplate Reader. As a negative control, we incubated the substrate without enzyme and subtracted the values for uncatalysed breakdown of pNPX in our calculations of xylosidase activities.

Additional comments related to enzyme assays: The dried secretomes were re-suspended in PBS and centrifuged at 16,000 *g* for 10 min to remove particulate material. The resulting supernatant was used in the enzyme assays. Although there was variability in the protein content of secretomes obtained from the different species, we used the same amount of total protein in our individual assays.

In all assays, one unit is defined as the amount of enzyme that produces 1 μ mol of product per minute. Linear regression (Excel) analysis of the product formed as a function of time was used to calculate the initial velocity

($r^2 \geq 0.96$ for xylanase and $r^2 \geq 0.98$ for xylosidase). Mean and standard deviation values were calculated from three independent assays, and the means used to calculate the relative specific activities (Figures 3 and 4).

Since we used two versions of the xylanase assay at the two research sites (in India and the US), there are some differences in the specific activities determined (Figures 2 and 3). These differences likely stem from end-point (Figure 2) versus kinetic assays (Figures 3 and 4), as well as the concentration of substrate and temperature of the assay. However, these variations do not influence the inferences drawn as each set of data was used to make comparisons only within that group.

Effect of culture age and pH on extracellular xylanase activity of Trichoderma harzianum

Trichoderma harzianum was inoculated in 10 ml BX medium and incubated at 31°C in an orbital shaker (120 rpm). For evaluating the effect of culture age, the filtrate was obtained from *T. harzianum* cultures grown for 1, 2, 3, 4, 5, 6, 7 and 8 days. For assessing the effect of media pH, *T. harzianum* was cultured in BX medium (pH 4.5, 5.5 or 7.5) for five days before the filtrate was collected. In these two instances, end-point assays at 50°C were used to determine the xylanase activity.

Effect of NaCl on the growth of T. harzianum

To study the effect of NaCl on the growth of *T. harzianum*, a plug of mycelium (5 mm diameter) was cut from the margin of a colony growing actively on Czapek–Dox agar (CDA) medium (2 g NaNO₃, 1 g KH₂PO₄, 0.5 g

MgSO₄, 0.5 g KCl, 0.01 g FeSO₄, 30 g sucrose, 1000 ml water). This mycelial plug was placed on CDA amended with 0%, 1.5%, 2.5%, 3.5% or 7% (w/v) NaCl and incubated at 26°C for 25 days with periodic measurements of the colony diameter. The mean values reported were calculated from data obtained in three replicates (Figure 5).

Effect of including NaCl on extracellular xylanase and xylosidase activity of *T. harzianum*

To examine the effect of including NaCl in the media, *T. harzianum* was cultured for five days in BX medium

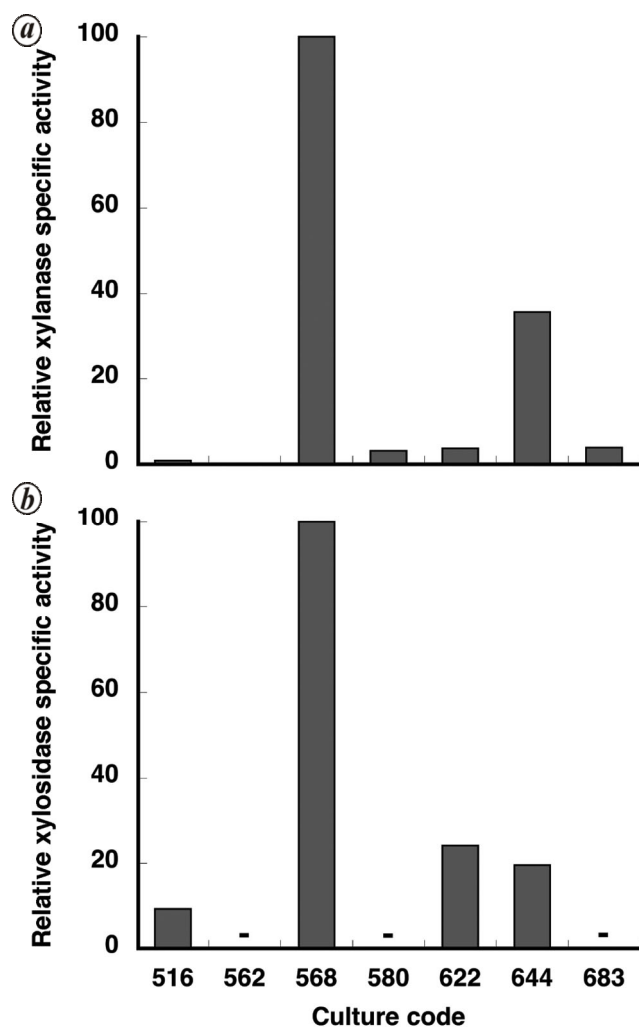


Figure 3. Comparison of secreted xylanase (a) and xylosidase (b) specific activity. Crude secretomes from the indicated endophytes were assayed for xylanase and xylosidase, and the experimentally determined initial velocities were converted to specific activities. To facilitate a comparison, all specific activities were normalized using *Trichoderma harzianum* as reference (100% corresponds to 2.2 U/mg for xylanase and 0.0024 U/mg for xylosidase, see text). The initial velocities at 37°C were determined in three independent trials, and the standard deviation of the mean did not exceed 26% for xylanase and 27% for xylosidase. Due to their very weak activities, reliable xylosidase initial velocities could not be determined for 562, 580 and 683, and are therefore indicated by ‘-’.

(pH 5.5) containing 0, 1.5, 2.0 or 2.5% (w/v) NaCl. The filtrates were collected as described above, and kinetic assays at 37°C were used to determine the xylanase and xylosidase activities in the secretomes. The assays do not contain NaCl, and the crude secretomes were dialysed to remove the salt that was included in the growth media.

Results

Xylanase and xylosidase activities of marine-derived endophytes

A qualitative plate-based assay showed that of the 16 endophyte isolates screened, eight were positive for extracellular xylanase (Table 1 and Figure 1). As reflected in the zones of clearance in the plate assay, all eight

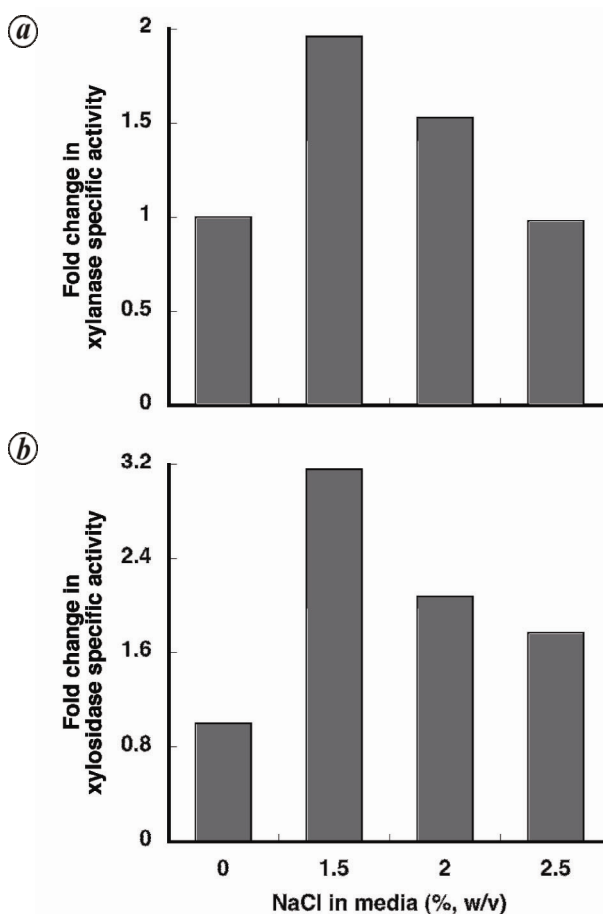


Figure 4. Effect of including sodium chloride in the media on xylanase (a) and xylosidase (b) activity secreted by *T. harzianum*. Initial velocities were determined using filtrates obtained from five day-old cultures grown in the presence of 1% (w/v) xylan and the indicated amounts of NaCl. All specific activities were normalized against that observed with *T. harzianum* grown without any salt in the medium (see Figure 3 for the actual specific activity). The initial velocities at 37°C were determined in three independent trials, and the standard deviation of the mean did not exceed 20% for both xylanase and xylosidase activities.

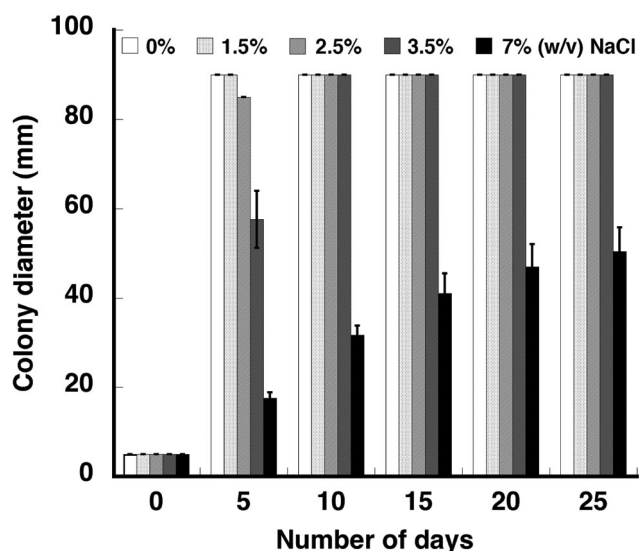


Figure 5. Effect of including NaCl in the agar media on growth of *T. harzianum*. The mean and standard deviation values were calculated from three independent measurements.

isolates produced xylanase at pH 4.5, 5.5 and 6.5; the same outcome in these three independent tests provides a measure of reproducibility for the method. We have classified the activities as only positive or negative (Table 1), since a numerical comparison is precluded by factors including differences in the rate of diffusion of the enzyme through the agar and amount of protein in a defined volume of the culture supernatant.

For a more quantitative assessment, seven fungal secretomes that scored positive for activity in the plate assay were further examined using the DNS-based spectrophotometric assay for xylanase specific activity. This analysis showed that a *Trichoderma* sp. endophytic in the brown alga *Sargassum wightii* and an *Aspergillus* sp. endophytic in the seagrass *Cymodocea serrulata* have the maximum xylanase specific activity (2.2 and 0.7 $\mu\text{mol}/\text{min}/\text{mg}$ protein respectively; Figure 3 a). Based on their ITS sequences, we identified these species as *Trichoderma harzianum* and *Aspergillus terreus* (Table 1).

Since the complete degradation of xylan to xylose entails the action of xylanases and xylosidases, we next screened the seven secretomes for xylosidase activity by evaluating their ability to cleave pNPX. *T. harzianum* emerged as the top producer of xylosidase (0.0024 $\mu\text{mol}/\text{min}/\text{mg}$ protein; Figure 3 b). Three other fungi showed ~10–20% of *T. harzianum* activity: *A. terreus* (644), *Curvularia tuberculata* (516) and *Gonatosphragmium mori* (622). Of the seven fungi examined, *T. harzianum* showed the highest activity of both xylanase and xylosidase; since the rapid removal of xylooligosaccharides by a xylosidase is likely to aid any xylanase present in the same secretome, perhaps this result is not unexpected. Given these findings, we decided to undertake further studies using *T. harzianum*.

Effect of culture age and media pH on xylanase activity secreted by T. harzianum

After empirically determining that 1% (w/v) birch wood xylan induced the maximum activity (data not shown), we investigated the effect of culture age. We assayed the *T. harzianum* culture filtrate at periodic intervals and found that the fungus produced xylanase even on day 1, with significant increases by day 4 and peaking at day 5 (Figure 2 a). The activity increased by nearly five-fold between days 1 and 5, and then decreased by ten-fold between days 5 and 8 likely due to altered culture conditions and decreased substrate (xylan) availability⁴⁰.

We then examined if the pH of the growth medium influenced the xylanase activity on day 5 with 1% (w/v) xylan as the carbon source (Figure 2 b). While the difference in xylanase activity between pH 4.5 and 5.5 was not significant (<10%), there was a near four-fold decrease when the pH of the media was increased to 6.5. This observation is consistent with the finding that other fungal species^{41–43} also exhibit optimal xylanase activity between pH 4 and 5.

Effect of NaCl on the growth of T. harzianum

Since sea water has a salinity of ~3.5% (w/v) NaCl, we examined the effect of NaCl on the growth of *T. harzianum* on an agar plate. We tested four different NaCl concentrations (1.5%, 2.5%, 3.5% and 7% (w/v), corresponding to ~0.26, 0.43, 0.6 and 1.2 M respectively), and examined the colony diameter on days 5, 10, 15, 20 and 25 (Figure 5). Seven per cent (w/v) NaCl was the highest concentration that supported growth. There was no growth difference between control (no salt) and the 1.5% (w/v) condition. In contrast, growth was slower on media containing 2.5% and 3.5% (w/v) NaCl, but the final colony diameter was no different than the control. However, with 7% (w/v) NaCl, the growth was slower and colony diameter even after 25 days was only half that observed with the control.

Inclusion of NaCl in growth media increases the xylanase and xylosidase activities of T. harzianum

We studied the effect of NaCl in the growth medium (0%, 1.5%, 2% or 2.5% (w/v)) on xylanase activity in the secretome of *T. harzianum*. The culture filtrate was dialysed before lyophilization to ensure that there was no carryover of the salt from the media in the crude secretomes. This step was necessary since the presence of salt in the assay could potentially influence the enzyme activity. Compared to the absence of NaCl (control), the specific activity of xylanase increased nearly two-fold at 1.5% and 2% (w/v) NaCl, and then decreased at 2.5% (w/v) to near-control levels (Figure 4 a). The xylosidase

activity increased by 3.2-, 2.1- and 1.8-fold respectively, when the media contained 1.5%, 2% and 2.5% (w/v) NaCl (Figure 4b). Overall, the xylanase and xylosidase activity trends parallel each other (Figure 4a and b).

Discussion

It is reasonable to expect endophytes to express xylanases in order to establish infections in angiosperms and marine algae, whose cell walls contain xylan or fuco-glucuronoxylans (in brown algae)⁴⁴. Following previous reports that many endophytic fungal species isolated from terrestrial plants produce xylanases⁴⁵, we now show that MDF endophytes express both xylanases and xylosidases (Figures 1 and 2). Further studies are required to determine how these fungi modulate xylanolytic (including esterase) activities depending on different culture conditions⁴⁶. To transition the broader bioprospecting exercise to a few lead candidates that might be useful to industry, biochemical characterization (pH/temperature optima, influence of salt/ionic liquids in the assay, nature of xylooligosaccharides generated, etc.) of these secreted enzymes is necessary. Since the structural make-up of the xylans in brown algae is different from that found in angiosperms (i.e. fuco-glucuronoxylans in brown algae versus xylans in red/green algae and higher plants)^{44,47,48}, the diversity of xylanases in MDF endophytes resident in these seaweeds is likely to be high, thus increasing the chances of identifying novel enzymes. The idea that the substrate diversity will be mirrored in the catalytic arsenal of the degrading organisms has precedents, including the recent finding of a large number of genes coding for enzymes that degrade unique algal polysaccharides in a marine alga-associated flavobacterium⁴⁹.

Our findings lend support to the use of seagrass and seaweed detritus as a solid-state fermentation (SSF) medium for growing MDF endophytes, and thereby reducing the cost of producing xylanases and xylosidases. Although SSF media such as corn cob, rice bran, sunflower head, wheat bran and tomato pomace have been used for xylanase production⁵⁰, marine plants are an attractive substitute as this would not involve the use of land, freshwater or fertilizer. However, careful attention needs to be paid to the ecological consequences from the diversion of such detritus, which also provides a habitat and food for some invertebrates and migrating birds.

Our finding of salt-inducible increases in xylanase and xylosidase activities expressed by *T. harzianum* (Figure 4) warrants further investigation given the renewed interest in salt-tolerant saccharification enzymes stimulated by the successful application of ionic liquids to deconstruct biomass^{51,52}. The increased enzyme activity that we observed could stem from either an increase in gene expression or secretion of a different enzyme altogether (thematic variants of the latter idea include post-translational modifications). There are precedents for

marine fungi tailoring their lignocellulolytic secretome as part of their salt-adaptation response^{53,54}. For example, *Phlebia* sp., a marine white-rot fungus, when grown in the presence of 3% (w/v) salt, was reported to express two manganese peroxidases neither of which was present in the non-saline secretome⁵⁴. Similarly, in the presence of 3% (w/v) sea salt, proteomes of a *Pestalotiopsis* sp., isolated from a mangrove and grown on *Rhizophora stylosa* wood chips revealed an increase in hemicellulolytic and cellulolytic activities compared to secretomes obtained from non-saline media⁵³. Interestingly, enzymes (including xylanase) secreted by this *Pestalotiopsis* sp. grown on a non-saline medium displayed a decrease in activity when salt was included in the assay⁵³. We are testing if *T. harzianum*, the MDF endophyte obtained from the brown alga *S. wightii*, does indeed secrete salt-tolerant xylanases and xylosidases.

A few *Trichoderma* species, including *T. harzianum* are known to produce xylanases¹⁹, and we now add to this list a *T. harzianum* endophytic in a brown alga. Moreover, our observations that *T. harzianum* can tolerate up to 1.2 M salt in the growth medium and increase extracellular xylanase and xylosidase activity in the presence of NaCl (Figure 4), suggest that it occupies a special environmental niche. In fact, this inference is consistent with the unexpectedly high diversity of endophytic *Trichoderma* in terrestrial plants; exploitation of novel ecological niches by *Trichoderma* has been attributed in part to their ability to jump among inter-kingdom hosts⁵⁵. Therefore, screening of marine-derived fungal endophytes (including new *Trichoderma* species) for novel cell-wall degrading enzymes is likely to be profitable.

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