

## Cellulolytic Activities of a Novel *Fomitopsis* sp. and *Aspergillus tubingensis* isolated from Philippine Mangroves

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**The ability to deconstruct plant cell wall polysaccharides is inherent in fungal endophytes. As such, discovering organisms that secrete potent cocktails of carbohydrate-active enzymes may hold the key to deconstructing waste agricultural biomass for industrial applications. Based on CMC-Congo red plate based assay, two fungal isolates derived from mangrove trees (JB10 and JB11) showed high enzymatic indices (as high as  $5.6 \pm 0.18$  for JB10). Both isolates were then grown in potato dextrose (PD), carboxymethylcellulose(CMC), and beechwood xylan (XY), and the corresponding endoglucanase, xylanase, and  $\beta$ -glucosidase activities of the enzymes present in crude culture supernatants were determined. JB11 showed significant increase in endoglucanase activity ( $0.36 \pm 0.04$  U/mL) in PD, while JB10 endoglucanase activity was similar between the three media. Interestingly, xylanase activity of both isolates was relatively high (ranging 0.26-1.0 U/mL), with JB10 xylanase activity five-fold higher in PD. Lastly, there was 2-4 fold increase detected in  $\beta$ -glucosidase activities (0.59-0.8 U/mL) in both isolates when grown in CMC or XY media. Phylogenetic analysis of the ITS sequences show that JB11 is *Aspergillus tubingensis*, while JB10 is a novel *Fomitopsis* sp. isolate.**

Keywords:  $\beta$ -glucosidase, *Aspergillus*, Endoglucanase, *Fomitopsis*, Mangrove, Xylanase

### INTRODUCTION

Lignocellulosic material, in the form of agricultural waste, is a promising biofuel feedstock or source of precursors for fine chemicals. A major obstacle in their utilization is their inherent complexity and recalcitrance to degradation. Current pretreatment methods utilizing harsh chemicals have adverse environmental effects and may preclude use of degradation products in downstream chemical synthesis (Eggeman & Elander 2005). Methods based on mild enzymatic hydrolysis of biomass have been employed and is increasingly being viewed as a greener alternative or complement to existing industrial processes (Mosier et al. 2005). Developing optimized enzyme cocktail formulations that increase biomass conversion rates and maximize yields are also being intensely studied

(Mohanram et al. 2013).

Fungi have the ability to express and secrete a cocktail of extracellular hydrolytic and oxidative enzymes that act synergistically to deconstruct lignocellulose into its components (Hatakka and Hammel 2010). The fungal proteins secreted into the media are primarily carbohydrate-active enzymes or CAZys. The CAZys are a diverse family of enzymes responsible for assembly (e.g., glycosyltransferases) and breakdown (e.g., glycosyl hydrolases and carbohydrate esterases) of complex carbohydrates (Lombard et al. 2014). For example, the deconstruction of cellulose requires the concerted effort of enzymes belonging to 12 different glycosyl hydrolase families (Lombard et al. 2014).

Continuous efforts in discovering efficient and robust cellulolytic enzymes from novel fungi are necessary, not only to understand the different determinants of enzyme-based biomass deconstruction, but also feed

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robust enzymes into industrial pipelines. The potential to discover robust and more effective enzymes is greater when isolating fungi from plants in unique ecological niches. Mangroves, bamboo, marsh grasses, sea spinach, for example, are desirable sources for fungal endophytes due to their adaptation and tolerance to salt. Mangroves are also known to use polysaccharides and other compounds as osmolytes to maintain osmotic and water potential (Liang et al. 2008). Fungal species from mangroves have been shown to be good sources of secreted wood-degrading enzymes such as carbohydrate-active enzymes (CAZys) and lignin-degrading enzymes (Arfi et al. 2013). Hence, the aim of the study is to isolate fungi from mangroves that secrete robust and efficient CAZys and characterize their enzymatic activities.

## MATERIALS AND METHODS

### Isolation of fungal endophytes

Fungal endophytes were isolated from *Avicennia marina* (Forssk.) Viehr. (Grey mangrove), *Sonneratia caseolaris* L. (Mangrove apple), and *Ipomoea pes-caprae* L. (Beach morning glory) found in Brgy. Ilog Malino, Bolinao, Pangasinan. Briefly, small terminal stem pieces were cut from the plants and extensively rinsed with 95% v/v ethanol. Air-dried samples were then stored in Ziploc® bags for transport to the lab. Stems were then cut into smaller pieces (approximately 1 cm x 2 cm) and placed in the center of a potato dextrose agar (PDA, 39 g/L) plate. Visible fungal growth from the samples were picked and transferred to new PDA plates until single isolates were obtained. Fungal isolates were preserved in sterilized barley and kept in -80° C for long-term storage. Fungal cultures were deposited in the Microbiological Research Services Laboratory (MRSL) of the UP Natural Sciences Research Institute.

### Radial growth rate measurements

Growth measurements were taken from 6 replicate plates for PDA and 1% CMC supplemented with M9 salts. Radial growth measurements were taken every day, marking four points along the circumference of fungal mycelia, until growth reached the edge of the plate. An average diameter was determined by taking 12 points from three individual plates. Radial growth rate (Kr) was estimated using colony diameter versus time during constant growth (~24-72 h) (Reeslev and Kjoller 1995).

### Plate-based enzyme screening

The CMC-Congo Red clearing assay was used as a preliminary screen for cellulases (Meddeb-Mouelhi et al. 2014). Briefly, CMC-agar plates were prepared using 1% w/v agar with 0.2% w/v medium viscosity CMC and

inoculated with mycelial plugs. Growth was monitored until the mycelia occupied approximately 1/3 of the radius of the plate. The plates were flooded with 0.1% w/v Congo red for 15-20 min and subsequently rinsed with 1 M NaCl to reveal clearing zones. The enzymatic index was calculated using the formula of Peciulyte (2007) and listed in Table 1. Isolates with EI greater than 1 were subjected to further analysis (Peciulyte 2007).

$$\text{Enzymatic index (EI)} = \frac{\text{diameter of clearing zone (cm)}}{\text{diameter of average radial growth (cm)}}$$

**Table 1.** Calculated enzymatic indices of the fungal isolates collected from mangroves.

Isolate Name	Plant Source	Enzymatic index <sup>1</sup>
JB 10	Mangrove apple ( <i>Sonneratia caseolaris</i> L. Engler)	5.60 ± 0.18
JB 11	Grey mangrove ( <i>Avicennia marina</i> Forssk. Viehr.)	1.40 ± 0.14
JB 16	Grey mangrove ( <i>Avicennia marina</i> Forssk. Viehr.)	1.30 ± 0.18
JB 17	Grey mangrove ( <i>Avicennia marina</i> Forssk. Viehr.)	1.20 ± 0.04
JB 9	Grey mangrove ( <i>Avicennia marina</i> Forssk. Viehr.)	0.88 ± 0.04
JB 8	Beach morning glory ( <i>Ipomoea pes-caprae</i> L.)	0.74 ± 0.09
JB 14	Beach morning glory ( <i>Ipomoea pes-caprae</i> L.)	0.71 ± 0.02
JB 15	Beach morning glory ( <i>Ipomoea pes-caprae</i> L.)	0.68 ± 0.05

<sup>1</sup>Average of four replicates.

### Phylogenetic analysis

The ITS-5.8S ribosomal gene sequence was used for molecular identification of the fungal isolates. Genomic DNA was extracted using the ZymoResearch Quick-DNA™ Fungal/Bacterial kit. The ITS region was amplified using universal ITS primers (ITS1: 5'TCCGTAGGTGAACCTGCGG3' and ITS4: 5'TCCTCCGCTTATTGATATGC3') (White et al. 1990). The optimized PCR conditions used were as follows: initial denaturation at 94° C for 3 min, followed by 20 cycles of denaturation at 94° C for 30 s, annealing step at 45° C for 30 s, elongation at 72° C for 30 s, and a final extension step at 72° C for 5 min. PCR products were visualized on a 1.25% w/v agarose gel and the band corresponding to the expected amplicon (~700 bp) were extracted using the QIAQuick® PCR Gel Extraction Kit (Qiagen) and sent out for sequencing (IDT Integrated DNA Technologies). The corresponding consensus

sequence was aligned with GenBank sequences using NCBI BLASTN program (Altschul et al. 1990) and similar sequences were downloaded and a phylogenetic tree was constructed using the neighbor-joining method from MEGA 7 software (Kumar et al. 2016). Bootstrap values were obtained after 1000 iterations.

#### Quantitative enzyme activity screening

Liquid cultures were prepared with slight modifications (El-Hadi et al. 2014). For CMC and xylan media, the following reagents were prepared (g/L distilled water): 1.1 g carbon source (CMC or beechwood xylan), 0.5 g  $(\text{NH}_4)_2\text{SO}_4$ , 10 g  $\text{KH}_2\text{PO}_4$ , 0.1 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.2 g NaCl. Fungi grown in potato dextrose broth (PDB) were used to inoculate liquid cultures by transferring 2 mL of mycelia in CMC and xylan media. The cultures were grown for about seven days at room temperature with discontinuous shaking (at 100 rpm) prior to extraction of culture media for enzymatic assays.

The secreted endoglucanase and xylanase activities of the fungi were measured by performing DNS (3,5-dinitrosalicylic acid) assay on crude culture supernatants (King et al. 2009). Briefly, crude filtrate was centrifuged at 8000 rpm for 20 min to ensure that there are no fungal mycelia included in the assay. Total protein concentration of the culture supernatants was obtained via Bradford assay. DNS reagent was prepared by adding the following (in w/v): 1.4% 3,5-dinitrosalicylic acid; 1.4% NaOH; 28% sodium potassium tartrate; 0.28% phenol; and 0.07% sodium metabisulfite (Xiao et al. 2005). Phenol and sodium metabisulfite were added after DNS, NaOH, and sodium potassium tartrate were combined. All absorbance measurements at 540 nm were obtained using a Thermo Fisher MultiSkan™ GO microplate reader and enzyme activities (U/mL) were calculated.

#### Determination of endoglucanase and xylanolytic activity

Endoglucanase and xylanase activities were measured using the microplate method adapted from King and co-workers (2009). Ninety  $\mu\text{L}$  of the culture supernatants was mixed with 90  $\mu\text{L}$  of substrate (0.5% CMC in 50

mM citric acid buffer, pH 4.8). The plates were sealed with Parafilm® and incubated at 50° C for 1 h. The reactions were stopped by freezing at -20° C. Sixty  $\mu\text{L}$  of the hydrolysate reaction was added to 120  $\mu\text{L}$  of DNS reagent, mixed, and heated at 95° C for 5 min. An aliquot of the DNS reaction (36  $\mu\text{L}$ ) was added to 160  $\mu\text{L}$  of double distilled water and the absorbance was read at 540 nm. Glucose standard curves (with concentrations ranging 0.8-3 mg/mL) were prepared. One unit of enzyme activity is equal to the amount of glucose (in  $\mu\text{mol}$ ) produced per minute under the above assay conditions. The substrate for measuring xylanolytic activity is 1% beechwood xylan (King et al. 2009). A xylose standard curve was prepared using concentrations ranging 0.9-5.4 mg/mL. One unit of xylanolytic activity is equal to the amount of xylose (in  $\mu\text{mol}$ ) produced per minute under the above assay conditions.

#### Determination of $\beta$ -glucosidase activity

$\beta$ -glucosidase activity was measured by using p-nitrophenyl- $\beta$ -D-glucopyranoside (pNpG) (Liu et al. 2013). Briefly, 10  $\mu\text{L}$  culture supernatants was combined with 25  $\mu\text{L}$  of 10 mM pNpG substrate, 25  $\mu\text{L}$  of 200 mM NaOAc buffer (pH 5.0), and 40  $\mu\text{L}$  of distilled  $\text{H}_2\text{O}$  and incubated at 50° C for 10 min. The reaction was terminated by adding 100  $\mu\text{L}$  of 1 M  $\text{Na}_2\text{CO}_3$  and the absorbance at 405 nm was measured. A p-nitrophenol standard curve (with concentrations ranging 5-100  $\mu\text{g}/\text{mL}$ ) was prepared. One unit of enzyme activity is equal to the amount of enzyme required to release 1  $\mu\text{mol}/\text{min}$  of p-nitrophenol under the above assay conditions. Single factor ANOVA was used in comparing the calculated enzyme activities between carbon sources.

## RESULTS

Eight previously isolated fungi from mangroves were screened for cellulolytic activity using the Congo Red plate-based assay (Figure 1). Table 1 shows a summary of the calculated enzymatic indices of the isolates. JB10 and JB11 – with enzymatic indices of  $5.6 \pm 0.18$  cm and

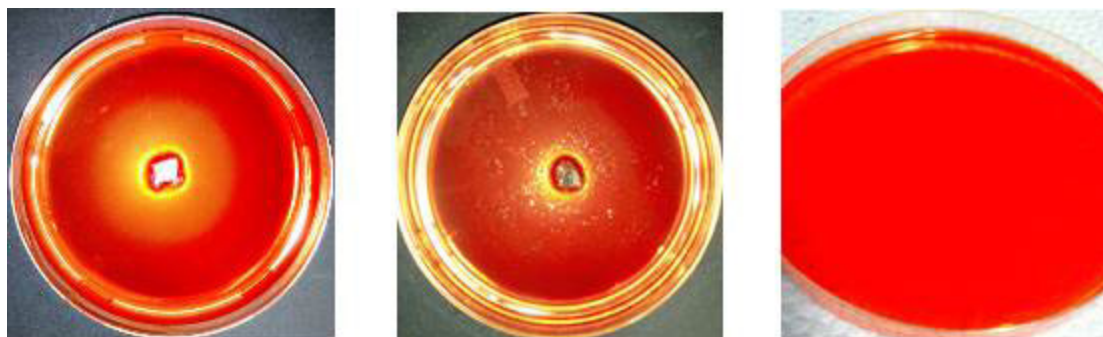


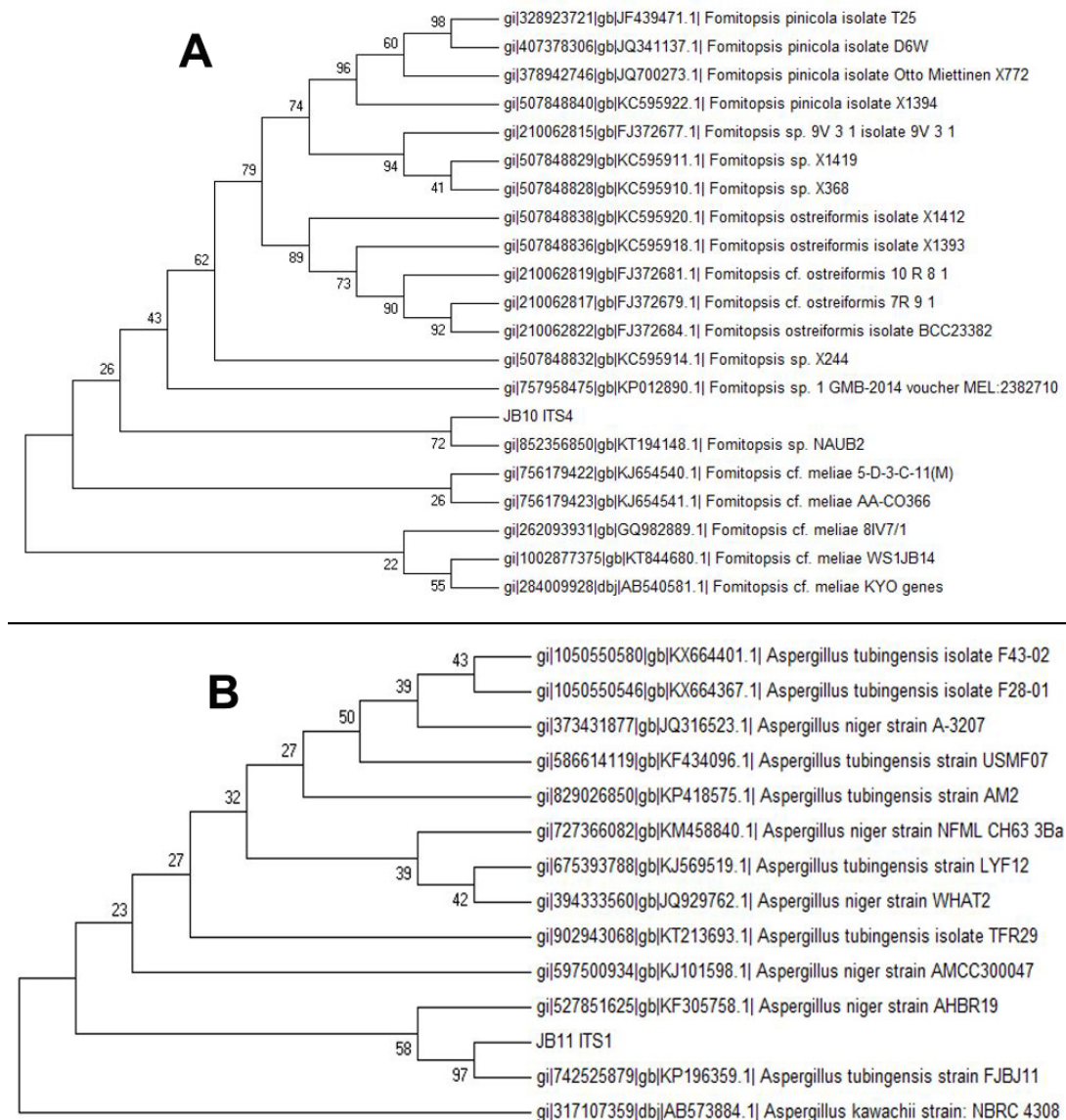
Figure 1. Congo Red plate clearing zones for JB10 (A) and JB11(B). Negative control (C) is shown.



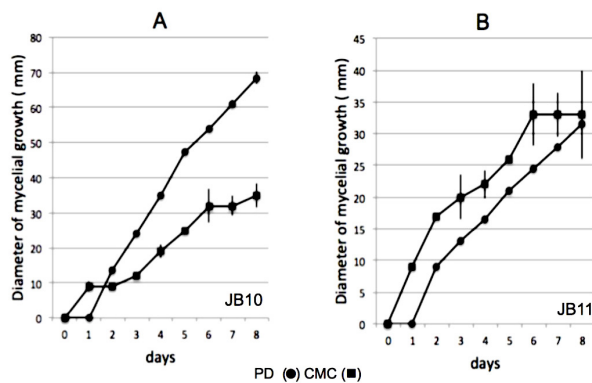
1.4 ± 0.14 cm, respectively – were chosen for further enzymatic characterization. JB10 was isolated from mangrove apple (*Sonneratia caseolaris* L.) and JB11 was isolated from grey mangrove [*Avicennia marina* (Forssk.) Viehr.]. Comparative sequence analysis of the ITS sequences show that JB10 has ~ 70% similarity with *Fomitopsis* sp. while JB11 has 97% similarity with *Aspergillus tubingensis* (Figure 2). Morphological analysis of the mycelia also supports the molecular classification of the isolates as belonging to genus *Fomitopsis* and *Aspergillus*. *A. tubingensis* and other *Aspergillus* sp. have been previously isolated from leaves of mangroves plants such as *Avicennia marina* and *Pongamiapinnata*, while *Fomitopsis* have mostly been isolated from oil palms and conifers (Debbab et al.

2013, Rungjindamai et al. 2008).

Previous studies have shown the dependence of expression and induction of various enzymatic activities as a function of feedstock or carbon source (Xie et al. 2016). The ability of the fungal isolates to utilize different carbon sources was initially demonstrated by measuring the radial growth of the fungi fed with PD or CMC as sole carbon source (Figure 3). It could be reasoned that the ability to grow in select media could be in part due to the identity and activity of the secreted enzymes that deconstruct the biomass or carbon source. It is apparent from the average growth rates that JB10 grows faster in PD (12 mm/d) than in CMC (4.5 cm/d). JB11 seems to grow similarly (~4.5 cm/d) whether fed with PD or CMC.



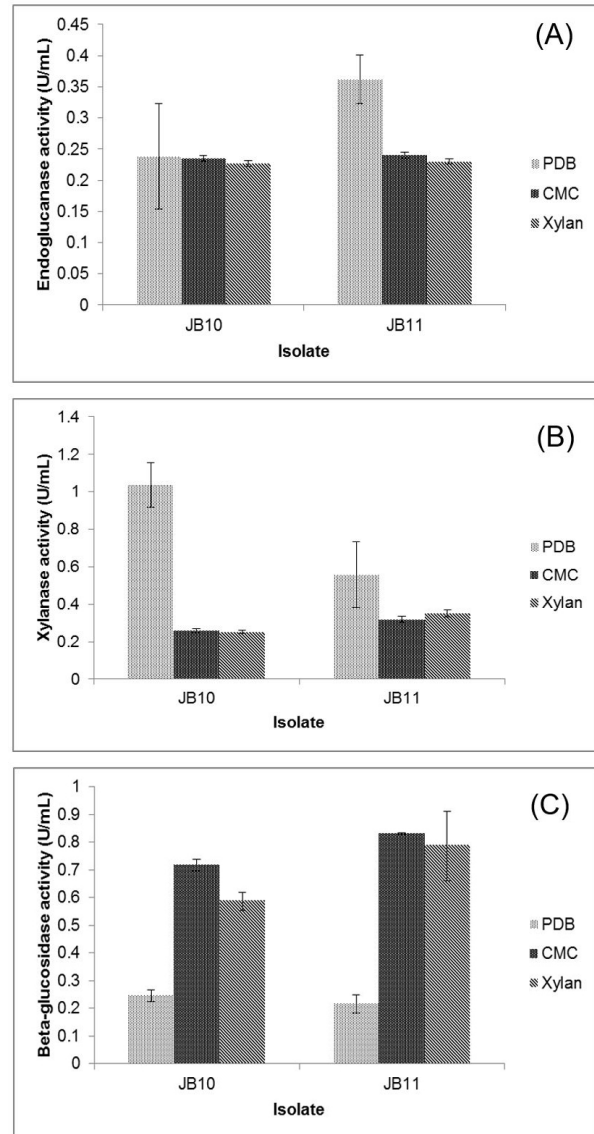
**Figure 2.** Phylogenetic trees for (A) JB10 and (B) JB11 constructed with ITS sequences. Neighbor-joining tree method was used with 1000 iterations, with bootstrap values greater than 70.



**Figure 3.** Comparison of fungal growth rates (mm/d) for JB10 (A) and JB11 (B) in PDA (closed circles) and CMC (squares). Points are calculated from the average measurements from 6 replicates.

In order to define the substrate specificity profiles of the secreted cellulolytic enzymes quantitative analysis via DNS and pNpG assay was performed. Endoglucanase, xylanase, and  $\beta$ -glucosidase activities (U/mL) of the crude supernatants grown in different carbon sources were evaluated to determine if varying the carbon source induced the production of the enzymes of interest. Daily monitoring of the enzyme activities for seven days showed no considerable difference in the total cellulolytic activity (data not shown) and therefore, the enzyme activities of these fungal cultures were quantified at day 7 for all treatments.

There was no significant difference in the endoglucanase activity of the secreted proteins (secretome) of JB10 when grown in three different media (Figure 4a, Table 2). On the other hand, JB11 endoglucanase activity was observed to be significantly greater in PD (0.36 U/mL) as compared to the more complex carbon sources, such as CMC and xylan (~0.23 U/mL). Interestingly for xylanase activities, both JB10 and JB11 had significantly greater xylanase activity in PD. Notably, xylanase activity was



**Figure 4.** Variations in glycolytic activities of secretomes obtained from growing fungi with different carbon sources. Endoglucanase (A), xylanase (B), and  $\beta$ -glucosidase (C) activities (in U/mL) of JB10 and JB11 in different types of media (PDB, CMC, Xylan) were measured.

**Table 2.** Glycolytic enzyme activities (U/mL) of JB10 and JB11 secreted proteins under different growth media.

Isolate Name	Media	Crude total protein concentration (ug/mL)	Endoglucanase activity (U/mL)*	Xylanase activity (U/mL)*	$\beta$ -glucosidase activity (U/mL)*
JB10	PDB	190 $\pm$ 8.5	0.24 $\pm$ 0.08	1.0 $\pm$ 0.12	0.25 $\pm$ 0.02
	CMC	160 $\pm$ 17	0.23 $\pm$ 0.00	0.26 $\pm$ 0.01	0.72 $\pm$ 0.02
	Xylan	140 $\pm$ 3.1	0.23 $\pm$ 0.00	0.25 $\pm$ 0.01	0.59 $\pm$ 0.03
p-values			0.97	0.0023	0.00076
JB11	PDB	150 $\pm$ 23	0.36 $\pm$ 0.04	0.56 $\pm$ 0.17	0.22 $\pm$ 0.03
	CMC	180 $\pm$ 9.6	0.24 $\pm$ 0.01	0.32 $\pm$ 0.02	0.83 $\pm$ 0.00
	Xylan	150 $\pm$ 2.3	0.23 $\pm$ 0.00	0.35 $\pm$ 0.02	0.79 $\pm$ 0.12
p-values			0.018	0.18	0.0064

\*values reported are average of 6 replicates ( $p < 0.05$ ).

least for JB10 when it was grown in xylan media (Fig. 4b, Table 2). Lastly, there was a 2- to 4-fold increase in  $\beta$ -glucosidase activities of the two isolates when grown in either CMC (0.72-0.83 U/mL) or xylan (0.59-0.79U/mL) compared to PD ( $\sim$ 0.22 U/mL) (Figure 4c, Table 2).

## DISCUSSION

Fungal endophytes from mangroves can be a potential source of robust and efficient cellulolytic enzymes. In this study, the standard CMC-Congo red plate assay was used to screen eight isolates derived from mangroves. While detectable cellulose activities were observed for almost all of the isolates, efforts were focused on the two isolates that gave the highest EI values, namely *Fomitopsis* sp. and *A. tubingensis*. Radial growth monitoring showed that CMC, as sole carbon source, could sustain the growth of the two fungi, albeit with differing growth rates. The high EI values obtained for these isolates suggest the presence of potent cell-wall degrading enzymes and thus, these isolates were further analyzed to quantitatively determine the activity of specific enzymes secreted into the culture media. The endoglucanase and xylanase activities obtained from crude culture supernatants were comparable to previously reported studies, save for the elevated  $\beta$ -glucosidase activities (Robl et al. 2013, Hmad et al. 2014). It is also likely that the high EI of *Fomitopsis* sp. may not be solely due to the glycoside hydrolases in the secretome, but also aided by carbohydrate esterases or other oxidative enzymes such as peroxidases and laccases that the fungus secretes (Hori et al. 2013). It is interesting to note how both fungi switch to different enzyme mixtures as a response to culture media. For example, JB10 expressed more enzymes with xylanase activity (almost five-fold more activity) when grown in PD. JB10 grown in more complex substrates resulted in elevated  $\beta$ -glucosidase (BGL) activity, which is consistent with previous studies demonstrating the high catalytic efficiency of purified BGL enzymes from other *Fomitopsis* isolates (Joo et al. 2009, Okamoto et al. 2011). On the other hand, JB11 endoglucanase and xylanase activity was comparable regardless of carbon source, with only  $\beta$ -glucosidase activity being induced in the presence of more complex carbohydrates. Endoglucanase activity of JB10 appears to not be affected by variations in culture media as much as that of JB11. Previous studies suggested that certain species of *Fomitopsis* are capable of generating modest exoglucanase activity under solid-state fermentation conditions or growth with the microcrystalline cellulose powder Avicel® (Yoon et al. 2007). The low endoglucanase activity of JB10 could be due to catabolite repression by glucose present in the media (Ruijter and Visser 1997). However, the fact that

endoglucanase activity did not change considerably with carbon source points to the possibility that there is basal or constitutive expression of JB10 endoglucanase genes (Ohnishi et al. 2007).

While it is recognized that *Aspergillus* sp. secrete highly active endoxyphanases (de Vries and Visser 2001), there are only a few studies on the xylanase activity of *Fomitopsis*. A highly active and thermostable xylanase was previously reported only for one *Fomitopsis* species, namely *Fomitopsis pinicola* (Shin et al. 2010). Comparison of the ITS sequences of well-studied *Fomitopsis* isolates (e.g., *F. pinicola* and *F. palustris*) with that of JB10 revealed low sequence similarity (as low as 43%) and therefore, the JB10 isolate may constitute a new species of *Fomitopsis* and source of CAZys candidate for further study.

In summary, carbohydrate-active *Fomitopsis* sp. and *A. tubingensis* fungal endophytes were isolated from mangrove species. The effects of different types of media on various glycolytic activities were evaluated for both fungi, and the results suggest that these fungi are able to shift the composition of enzymes in their secretome, depending on food source. Future work will focus on the purification, characterization, and identification of secretome CAZys from the *Fomitopsis* sp. isolate.

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