

## Species Composition and Hydrolase Enzyme (EC.3) Activity of Fungi Isolated from Thasala Mangroves, Nakhon Si Thammarat Province, Southern Thailand

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### Abstract

In the present study, we aimed to investigate diversity of manglicolous and endobiotic fungi associated with mangrove plants from Thasala mangroves, Nakhon Si Thammarat province, southern Thailand. Extracellular hydrolase enzymes (EC.3) including amylase, cellulase, lipase, protease, and pectinase from isolated fungi were evaluated. A total of 31 obligate marine fungal species was recorded from various Thasala mangroves sites. These included 24 ascomycetes (77.4 %), 1 mucoromycete (3.2 %), 3 basidiomycetes (9.7 %), 4 asexual morphs (12.9 %), and tentatively identified fungi (9.7 %). The average percentage occurrence of the fungi recorded from each site ranged from 0.4 to 16%. At all sites, the common filamentous marine fungi were *Cumulospora* sp., *Halorosellinia oceanica*, *Kallichroma tethys*, *Leptosphaeria* sp., *Rimora mangrovei*, *Lulworthia* sp., *Phoma* sp., *Halenospora varia*, *Halocyphina villosa*, *Nia vibrissa*, and *Sclerococcum haliotrephum*. Also, the most frequent marine fungi include *Eutypella naqsii*, *Sammeyersia grandispora*, *Marinosphaera mangrovei*, *Nemania maritima*, and *Verruculina enalia*. Seven mangrove plants were selected for endophytic fungal isolation including *Acanthus ebracteatus*, *Acrostichum aureum*, *Avicennia alba*, *Bruguiera cylindrica*, *Rhizophora apiculata*, *R. mucronata*, and *Sonneratia alba*. The isolation rate (IR) and colonization rate (CR) of fungal endobiota varied for all plant studied. The isolation rate ranged from 24 - 53.3 %, while colonization rate varied from 29.3 - 61.3 %. The fungal isolates were screened for the production of hydrolase enzymes (EC.3), of which 37.2 % exhibited enzyme activities. The 16 out of 28 obligate marine fungi (57.1 %) and 13 out of 50 fungal endophytes (26 %) had enzyme production efficiency toward specific substrates or an enzymatic index (EI) higher than 1.4. None of the fungal isolates depicted lipase activity. Additionally, the environmental stressors (temperature, pH, salinity) affecting enzyme production were evaluated in order to discover potential candidates for industrial application.

**Keywords:** Hydrolase enzyme, Manglicolous fungi, Species composition

### Introduction

Thailand harbors coastline lengths of 2,673 km, of which the Gulf of Thailand coasts cover 1,700 km and Andaman sea coasts cover 973 km [1]. The estimated mangrove areas in Thailand were 246,109 hectares in 2018. Nakhon Si Thammarat province is located in the south bordering part of the shoreline of the Gulf of Thailand. Especially, Thasala district harbors a large volume of mangrove area of 16,185 hectares [1].

Filamentous fungi from marine and mangrove habitats have been documented from Thailand over the past 37 years since an early study of Kohlmeyer [2]. Since then, there have been great efforts in documenting marine fungi especially manglicolous fungi with a major focus on taxonomy and their ecology [3-14]. Extensive information gathered by Jones *et al.* [11] reported the collections of marine fungi from many locations around Thailand, with 154 species documented. Dethoup and Manoch [12] later listed 152 species from the eastern region, while an extensive study from southern Thailand by Sakayaroj *et al.* [13] listed 112 marine fungal species. A paper by Suetrong *et al.* [14] reported the occurrence of 99 manglicolous fungi from eastern and southern regions. A recent paper by Devadatha *et al.* [15] documented several new marine taxa described from Thailand with total numbers listed now 303 species. Among these studies, several reported the description of new marine fungi including *Aigialus striatispora*, *Cryptosphaeria mangrovei*, *Fulvifomes siamensis*, *F. xylocarpicola*, *F. halophila*, *Helicascus*

*mangrovei*, *Morosphaeria ramunculicola*, *Pedumispora rhizophorae*, *Pseudolignicola siamensis*, *Thalespora appendiculata* [4-8,16-20].

Also, a few reports documented species composition of fungal endophytes from Thai mangrove plants and seagrasses including Chaeprasert *et al.* [21]; Sakayaroj *et al.* [22]; Buatong *et al.* [23]; Doilom *et al.* [24]; and Supaphon *et al.* [25]. The major mangrove tree family (Rhizophoraceae) and 4 major seagrass families (Cymodoceaceae, Hydrocharitaceae, Posidoniaceae, Zosteraceae) have been investigated for the presence of endophytic fungi.

Marine and endophytic fungi have been explored for the production of extracellular hydrolytic enzymes so as to obtain nutrients from their host, hydrolyze food substances and are involved in defending mechanism against pathogens [26]. These enzymes include amylase, cellulase, protease, lipase, pectinase, and laccase are of commercial interest [26,27]. In the present study, we aimed to investigate diversity of manglicolous and endobiotic fungi associated with mangrove plants from Thasala mangroves. The screening of hydrolytic enzyme from newly explored habitat may lead to the possibility to find the novel enzyme for further use. In addition, extracellular hydrolase enzymes from fungi isolated and environmental stressors affecting the enzyme production were evaluated in order to discover potential candidates for industrial application.

## Materials and methods

### Description of collecting site

Thasala mangroves are located in Thasala district, Nakhon Si Thammarat province, southern Thailand (8°36'31"N 99°57'57"E). Two major collecting sites were chosen for the present study (**Figure 1**). Site 1 is located as part of the mainland area connected to the local community. This is a healthy mangrove stand along a man-made canal, with seawater salinity varying from 20 to 30 ppt. Site 2 is connected to the mouth of the Gulf of Thailand, with seawater salinity varying from 20 to 35 ppt. The 2 sites dominated by mature and young mangrove tree species include *Avicennia marina*, *A. alba*, *Rhizophora mucronata*, *R. apiculata* and *Sonneratia alba*.

### Sampling procedure and isolation of fungi

Manglicolous marine fungal samplings were carried out from July 2019 to April 2020. Over 500 samples of decaying mangrove wood were randomly collected. Wood samples were washed using sterile seawater and observed immediately after returning to the laboratory and up to 3 weeks after incubation in damp boxes at room temperature (28±2 °C). Isolation of selected fungi was made through a single spore isolation method [28] and maintained on potato dextrose agar (PDA) prepared with natural seawater (30 ppt).

For fungal endobiotic isolation, 3 healthy leaves and branches each of 7 mangrove plants were collected: *Acanthus ebracteatus*, *Acrostichum aureum*, *Avicennia alba*, *Bruguiera cylindrical*, *Rhizophora apiculata*, *R. mucronata* and *Sonneratia alba*. All plant samples were kept in plastic bags and brought back to the laboratory for fungal isolation on the same day. Leaves and branches were cut into small segments including petiole, midrib, vein and lamina. Fungal endobiota were isolated following the procedure described by Buatong *et al.* [23]. Leaves and branches were surface-sterilized by a series of 95 % ethanol (30 s), 5 % sodium hypochlorite (5 min), 95 % ethanol (30 s), and rinsed with sterile distilled water. The sample segments were then placed on corn meal agar medium supplemented with antibiotics (penicillin G plus streptomycin sulfate 50 mg/L) to restrict bacterial growth. Plates were incubated at 25 °C for 1 week. Fungal growth was observed every day. Pure cultures were obtained by hyphal tip isolation and stored in 15 % glycerol at -80 °C.



**Figure 1** Map showing collecting sites at Thasala mangroves, Nakhon Si Thammarat province, Thailand. The map was queried from the Google Maps (<https://www.google.com/map>).

### Morphological identification

Identification of manglicolous fungi was performed based on the morphology of reproductive structures following the identification keys and recent publications by Kohlmeyer and Volkmann-Kohlmeyer [29]; Jones *et al.* [30,31], and Devadatha *et al.* [15]. Fungal endobiota obtained were identified based on their morphotypes on the selected medium.

### Enzymes screening from isolated marine and endobiotic fungi

A total of 78 marine and endophytic fungi were qualitatively screened for the presence of hydrolase enzymes (EC.3): Amylase, cellulase, lipase, protease, and pectinase. For amylase screening, all fungal isolates were inoculated with 6 mm inoculum and placed on the surface of starch agar (soluble starch 10 g, peptone 0.5 g,  $\text{KNO}_3$  1 g,  $\text{KCl}$  0.1 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g,  $(\text{NH}_4)_2\text{SO}_4$  0.1 g,  $\text{NaH}_2\text{PO}_4$  0.1 g and agar 15 g, natural seawater 1000 mL, pH 6.8), at 28 °C. After 7 days of incubation, the plates were flooded with iodine solution for measuring the enzymatic starch hydrolysis. For cellulase screening, the fungal isolates were inoculated on the CMC agar (carboxymethyl cellulose (CMC) 10 g,  $\text{KNO}_3$  1 g,  $\text{KH}_2\text{PO}_4$  1 g,  $\text{NaCl}$  15 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.01 g, peptone 0.2 g, and agar 15 g, natural seawater 1000 mL, pH 6.8), and incubated at 28 °C for 7 days. The hydrolysis of cellulose by cellulase activity was detected by flooding with 1 % congo red solution for 15 min and 1 %  $\text{NaCl}$  for 15 min. To examine the protease production, the fungal isolates were inoculated on skim milk agar (Skim milk 10.0 g, Yeast extract 1.0 g,  $\text{KNO}_3$  3.0 g,  $\text{NaCl}$  2 g,  $\text{K}_2\text{HPO}_4$  2 g,  $\text{MgSO}_4$  0.05 g,  $\text{CaCl}_2$  0.02 g, agar 20 g, natural seawater 1000 mL) and incubated at 28 °C for 7 days. The clear zones around the growth of mycelium represents protease activity after incubation. The extracellular pectinase production was examined using pectin agar (pectin 10 g,  $\text{KH}_2\text{PO}_4$  0.5 g,  $\text{NaCl}$  0.2 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.01 g,  $\text{CaCl}_2$  0.15 g, yeast extract 1.0 g, and agar 20 g, natural seawater 1000 mL), and incubated at 28 °C for 7 days. After incubation, the plates were treated with Lugol's iodine solution, and the clear zone around the growth of fungi were measured as pectinase activity. For lipase screening, the palm oil agar (palm oil 10 g, yeast extract 2.0 g,  $\text{K}_2\text{HPO}_4$  0.5 g,  $\text{KH}_2\text{PO}_4$  0.5 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.1 g,  $\text{NaCl}$  0.1 g, gum arabic 1.0 g, Rhodamine B 0.001 g, natural seawater 1000 mL, pH 6.8), at 28 °C for 7 days. The zone of orange fluorescence around mycelium were detected under UV light using a transilluminator. The appearance of a clear zone surrounding the fungal colony was measured and calculated in terms of efficiency of hydrolysis or enzymatic index (EI) by the ratio of clear zone and colony diameter of each isolate. Fungi with a ratio higher than 1.4 were selected for molecular identification by ribosomal DNA sequencing.

### Environment stressors affecting enzyme production (temperature, pH, salinity)

The effect of 3 different temperatures (room temperature, 37, 45 °C), pH (5, 7, 9) and salinity (0, 30 ppt) on starch, skim milk, CMC, and pectin degradation were studied. All conditions were incubated for 15 days. The plates were detected the enzyme activity by flooding specific reagent or a clear zone observation as described above.

### DNA extraction and molecular identification of isolates

Fungi with EI higher than 1.4 were selected for molecular identification by ribosomal DNA sequencing. Genomic DNA extraction was performed following the procedure by E.Z.N.A Soil DNA Kit (OMEGA, BIO-TEX Inc., USA). Large subunit and ITS1, ITS2, 5.8S ribosomal DNA were amplified using fungal universal primers [32,33] with One PCR™, (GeneDirex Inc., Taiwan). PCR products were purified and directly sequenced by the Macrogen (Korea). The sequences were analyzed along with other sequences obtained from the GenBank database. Sequences were aligned and refined visually in BioEdit version 7.0.5.3 [34]. Phylogenetic trees were performed in PAUP 4.0b10 [35].

### Statistical analyses

The percentage occurrence of each species was calculated as follows: Number of occurrences of particular species  $\times 100$  divided by total number samples examined. Percentage colonization = number of samples supporting fungi  $\times 100$  divided by number of samples examined. Average number of fungi per sample = total number of fungal isolates divided by total number of substrata supporting fungi [36]. The diversity of fungi at each collecting site was assessed based on the diversity indices: Species diversity index (Shannon-Wiener, Simpson's diversity), species evenness (Equitability), and species richness (Margalef) [36]. Endophytic isolation rate (IR) = total number of isolates yielded by a given sample divided by total number of leaf disc segments [37]. Endophytic colonization rates (CR) = total number of leaf disc segments in a sample yielding  $\geq 1$  isolate divided by total number of leaf disc segments [37]. The clear zone diameter of enzyme secretion and enzymatic index (EI) were expressed as mean  $\pm$  standard deviation (SD) ( $n = 3$ ). The significance level of the variables was determined using the 1-way analysis of variance (ANOVA) at the level of 95 % ( $p < 0.05$ ) and paired t-test analysis by SPSS trial version (IBM SPSS Statistics for Windows, IBM, Armonk, NY, USA).

## Results and discussion

### Frequency of occurrence and diversity of manglicolous and endophytic fungi

The percentage of occurrence of filamentous marine fungi obtained from Thasala mangroves varied from 0.4 to 16 % at both sites (**Table 1**). Thirty-one marine fungi representing 24 ascomycetes (77.4 %), 1 mucoromycete (3.2 %), 3 basidiomycetes (9.7 %), 4 asexual morphs (12.9 %), and tentatively identified taxa (9.7 %). Although a lower number of species was recorded at site 1, greater Shannon-Wiener, Simpson's diversity indices, equitability species evenness and Margalef species richness indices were observed at site 1 than at site 2. At Thasala mangroves, site 1 is a natural forest located near local community (approximately 20 years old), while site 2 is newly planted forests 8 - 10 years ago. The dominant tree species at site 1 include *A. marina*, *A. alba*, *Bruguiera cylindrical*, *R. mucronata*, *R. apiculata*, and *Sonneratia alba*. Site 2 harbors *R. mucronata* and *R. apiculata* as the most abundant tree species. The higher diversity of mangrove plants may support a wider range of fungal taxa at site 1. Moreover, the abundance and availability of decayed wood at site 1 might be favorable condition for fungal colonization [38].

In a comparison of fungal diversity in Thailand, number of marine fungal species varied from 42 to 112 in different geographical locations. For example, 81 species from 600 *Nypa* palm samples documented from Samut Songkhram [39], 76 species from 650 samples in Ranong [9], 112 species from 1932 samples in Khanom National Park, Nakhorn Si Thammarat [13], 78 species from 910 samples in Trat, 52 species from 479 samples in Prachuap Khiri Khan, 42 species from 526 samples in Phetchaburi [14]. This figure may vary owing to the variation of geographical locations, nature and age of mangroves. The present study revealed 31 species from 510 wood samples, in which the figure is lower than the other sites. The low species diversity of marine fungi at Thasala mangroves was attributed to the scarcity of dead wood arising from a young mangrove stand. Our study was similar to a study conducted at a small and young mangrove stand (established 30 years ago) at Moorea mangroves, Hawaii and they revealed only small number of fungi from this site [40]. The small number of samples collected in this study might support a lower range of fungal taxa. Devadatha *et al.* [15] opinioned that a study with a larger number of samples results in a distinct percentage of occurrence than a study with a smaller number of samples. In addition, marine fungal diversity is dependent on the nature of the host tissue; succession on the substrate,

fungi interaction and horizontal or vertical zonation of fungi, and the physicochemical features of mangrove habitats (e.g. hydrostatic pressure, light, osmotic effects, oxygen level, pH, pollutants, tidal range, salinity and temperature) [15,38].

**Table 1** Percentage occurrence of manglicolous marine fungi.

Fungi	Percentage occurrence (%)		
	Site 1	Site 2	Site 3
<b>Ascomycota</b>			
<i>Aniptodera chesapeakensis</i>	-	1.2	0.6
<i>Cumulospora</i> sp.*	1.2	1.6	1.4
<i>Eutypella naqsii</i> **	0.8	16.0	8.2
<i>Halorosellinia oceanica</i> *	2.3	-	1.2
<i>Halosphaeriopsis mediosetigera</i>	0.8	0.4	0.6
<i>Kallichroma glabrum</i>	0.4	-	0.2
<i>Kallichroma tethys</i> *	1.5	2.4	2.0
<i>Leptosphaeria</i> sp.*	1.9	0.4	1.2
<i>Rimora mangrovei</i> *	3.8	0.8	2.4
<i>Sammeyersia grandispora</i> **	0.8	10.8	5.7
<i>Lulworthia</i> sp. 1 *	3.5	1.6	2.5
<i>Marinosphaera mangrovei</i> **	1.9	10.0	5.9
<i>Morosphaeria velataspora</i>	-	0.4	0.2
<i>Nemania maritima</i> **	7.7	3.2	5.5
<i>Pedumispora rhizophorae</i>	0.8	-	0.4
<i>Periconia prolifica</i>	1.2	0.4	0.8
<i>Phoma</i> sp.*	1.9	0.4	1.2
<i>Saagaromyces ratnagiriensis</i>	-	0.4	0.2
<i>Dyfrolomyces rhizophorae</i>	-	0.4	0.2
<i>Savoryella lignicola</i>	0.4	-	0.2
<i>Sclerococcum haliotrephum</i>	1.5	0.4	1.0
<i>Swampomyces triseptatus</i>	-	0.4	0.2
<i>Verruculina enalia</i> **	7.7	10.0	8.8
<i>Halenospora varia</i>	1.5	0.4	1.0
<b>Mucoromycota</b>			
<i>Mucor</i> sp.	-	0.4	0.2
<b>Basidiomycota</b>			
<i>Calathella mangrovei</i>	0.8	0.8	0.8
<i>Halocyphina villosa</i> *	4.6	1.6	3.1
<i>Nia vibrissa</i> *	2.7	-	1.4
Tentatively identified fungi	-	3.6	1.8
Total number of collections	129	170	299
Total number of taxa	22	26	31
Number of samples examined	260	250	510
Number of samples colonized	125	91	216
Percentage colonization (%)	49.6	68.0	58.8
Average number of fungi per sample	1.03	1.86	1.45
Species diversity index (Shannon-Weiner)	3.196	2.522	
Species diversity index (Simpson's)	0.944	0.878	
Species evenness (Equitability)	0.624	0.487	
Species richness (Margalef)	6.640	5.405	

\*Common fungi (1 - 5 %), \*\*Frequent fungi (> 5 - 10 %)

Devadatha *et al.* [15] and Jones *et al.* [41] recently listed a total of 850 manglicolous fungi from locations around the world. Numbers of mangrove fungi listed for Thailand are now 303 species. India accommodates the highest number (339), followed by Malaysia (171), China (150), Brunei (134), and Hawaii (107) [15,41]. The most frequently recorded marine fungi (> 10 % occurrence) collected in the present study include *Eutypella naqsii* (16 %) and *Sammeyersia grandispora* (10.8 %). *Sammeyersia grandispora* as well *Antennospora quadricornuta*, *Lignincola laevis*, *Sclerococcum haliotrephum*, and *Verruculina enalia* were listed as the most frequently encountered mangrove fungi in the Indian Ocean mangroves [15]. In central, eastern and southern regions of Thailand, *Astrosphaeriella striatissima*, *Corollospora maritima*, *Lindra thallasiae*, *Linocarpon appendiculatum*, *Linocarpon nypae*, *Oxydothis nypae*, *Rimora mangrovei*, *Salsuginea ramicola*, *Sarvoryella lignicola*, and *Trichocladium nypae* were found as the highly frequent fungi (> 10 %). While the frequent species (5 - 10 %) have been documented including *Aigialus grandis*, *Aniptodera* sp., *Anthostomella* sp., *Dictyosporium elegans*, *Fasciatispora lignicola*, *Halocryptosphaeria bathurstensis*, *Halorosellinia oceanica*, *Helicascus kanaloanus*, *Leptosphaeria australiensis*, *Lignincola laevis*, *Morosphaeria velatospora*, *Quintaria lignatilis*, *Saagaromyces abonnis*, *Sammeyersia grandispora*, *Sclerococcum haliotrephum*, and *Verruculina enalia* (**Table 2**).

**Table 2** Core filamentous marine fungi found from central, eastern, and southern locations in Thailand. [9,11-14,39].

Frequent (5 - 10 % occurrence)	Very frequent (> 10 % occurrence)
<i>Aigialus grandis</i>	<i>Astrosphaeriella striatissima</i>
<i>Aniptodera</i> sp.	<i>Corollospora maritima</i>
<i>Anthostomella</i> sp.	<i>Lindra thallasiae</i>
<i>Dictyosporium elegans</i>	<i>Linocarpon appendiculatum</i>
<i>Fasciatispora lignicola</i>	<i>Linocarpon nypae</i>
<i>Halocryptosphaeria bathurstensis</i>	<i>Oxydothis nypae</i>
<i>Halorosellinia oceanica</i>	<i>Rimora mangrovei</i>
<i>Helicascus kanaloanus</i>	<i>Salsuginea ramicola</i>
<i>Leptosphaeria australiensis</i>	<i>Sarvoryella lignicola</i>
<i>Lignincola laevis</i>	<i>Trichocladium nypae</i>
<i>Morosphaeria velatospora</i>	
<i>Quintaria lignatilis</i>	
<i>Saagaromyces abonnis</i>	
<i>Sammeyersia grandispora</i>	
<i>Sammeyersia</i> sp.	
<i>Sclerococcum haliotrephum</i>	
<i>Verruculina enalia</i>	

The isolation rate (IR) and colonization rate (CR) of fungal endobiota for all plants studied varied from 24 - 53.3 and 29.3 - 61.3 %, respectively (**Table 3**). Our study supports data by Xing *et al.* [42]; Liu *et al.* [43]; Sun *et al.* [44]; and Li *et al.* [45]. For example, Xing *et al.* [42] studied 5 mangrove plant species and revealed that the colonization rate of endophytic fungi ranges from 8 - 54 %. Sun *et al.* [44] identified fungal endophytes from desert halophytes and found the colonization rates ranging from 35 to 100 % in stems and leaves. Generally, haloplants, mangrove plants, and marine seaweeds show higher densities of endophyte colonization, typically greater than 80 - 100 % [46-49]. However, fungal endophytes colonizing the healthy seagrasses were relatively low, only in the range of 0.2 - 3.3 % [22].

In the present study, diversity indices indicated the difference in fungal diversity among different plant species and tissue segments. High diversity indices were noted for branch and petiole, whereas low diversity indices were reported in vein and lamina. The endophyte assemblages dominated by different tissue types could be reflected in tissue preferences of the individual taxa [50]. The greater number of endophytes obtained from the branch and petiole in this study could be because the physical properties of the branch and petiole tissues might affect spore retention and spore deposition [51]. Factors affecting the frequency of occurrence, colonization, and diversity of endophytic fungi might be attributed to biotic and abiotic factors, such as characteristics of forest stands that differ markedly in plant host diversity and climatic conditions. Seasonal and spatial variation in the infection frequencies is largely dependent on the host density, surrounding vegetation, type and phase disposition of the plant organ, as well as the isolation procedure and the number and size of samples [51-53].

**Table 3** Colonization and isolation rates of endophytic fungi obtained from mangrove plants.

Plant Species	Tissue segments	Isolation rate (% IR)	Colonization rate (% CR)	Species diversity indices			
				Shannon-Weiner	Simpson's diversity	Species evenness (Equitability)	Species richness (Margalef)
<i>Acanthus ebracteatus</i>	Branch	14.7	13.3	0.365	0.843	0.111	10.697
	Petiole	10.7	6.7	0.360	0.920	0.109	7.697
	Midrib	4	2.7	0.244	0.991	0.074	2.697
	Vein	6.7	6.7	0.312	0.972	0.095	4.697
	Lamina	0	0	-	-	-	-
	<b>Total</b>	<b>37.3</b>	<b>29.3</b>				
<i>Acrostichum aureum</i>	Branch	26.7	26.7	0.346	0.763	0.115	9.666
	Petiole	10.7	12	0.366	0.853	0.122	7.666
	Midrib	2.7	8	0.230	0.995	0.077	1.666
	Vein	0	0	-	-	-	-
	Lamina	0	0	-	-	-	-
	<b>Total</b>	<b>26.7</b>	<b>33.3</b>				
<i>Avicennia alba</i>	Branch	14.7	13.3	0.362	0.913	0.101	10.721
	Petiole	13.3	13.3	0.355	0.929	0.099	9.721
	Midrib	4	4	0.207	0.995	0.058	2.721
	Vein	6.67	10.7	0.274	0.984	0.076	4.721
	Lamina	9.3	9.3	0.318	0.967	0.089	6.721
	<b>Total</b>	<b>48</b>	<b>50.7</b>				
<i>Bruguiera cylindrical</i>	Branch	13.3	25.3	0.326	0.706	0.113	9.654
	Petiole	5.33	6.7	0.334	0.961	0.116	3.654
	Midrib	2.7	6.7	0.244	0.993	0.084	1.654
	Vein	2.7	2.7	0.244	0.993	0.084	1.654
	Lamina	0	0	-	-	-	-
	<b>Total</b>	<b>24</b>	<b>41.3</b>				
<i>Rhizophora apiculata</i>	Branch	16	13.3	0.361	0.915	0.098	11.729
	Petiole	10.7	13.3	0.321	0.964	0.087	7.729
	Midrib	12	12	0.335	0.954	0.091	8.729
	Vein	6.7	10.7	0.259	0.987	0.070	4.729
	Lamina	8	12	0.284	0.981	0.077	5.729
	<b>Total</b>	<b>53.3</b>	<b>61.3</b>				
<i>Rhizophora mucronata</i>	Branch	10.7	13.3	0.364	0.836	0.124	7.660
	Petiole	8	13.3	0.364	0.912	0.124	5.660

Plant Species	Tissue segments	Isolation rate (% IR)	Colonization rate (% CR)	Species diversity indices			
				Shannon-Weiner	Simpson's diversity	Species evenness (Equitability)	Species richness (Margalef)
	Midrib	4	10.7	0.291	0.982	0.099	2.660
	Vein	2.7	2.7	0.236	0.994	0.080	1.660
	Lamina	0	0	–	–	–	–
	<b>Total</b>	<b>25.3</b>	<b>40</b>				
	<i>Sonneratia alba</i>	Branch	8	13.3	0.321	0.966	0.094
	Petiole	9.3	13.3	0.339	0.952	0.100	6.706
	Midrib	4	10.7	0.361	0.917	0.106	8.706
	Vein	1.3	4	0.113	0	0.033	0.706
	Lamina	9.3	9.3	0.339	0.952	0.100	6.706
	<b>Total</b>	<b>40</b>	<b>52</b>				

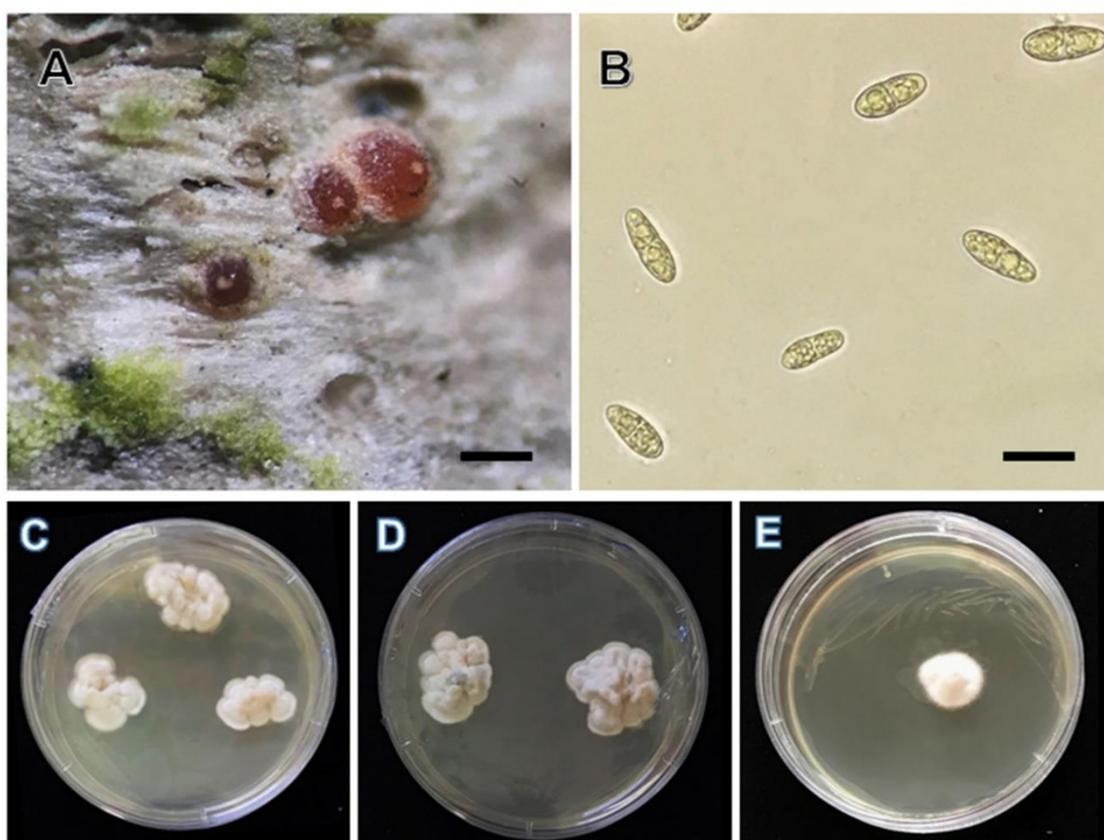
### Screening of extracellular hydrolase enzyme production

The 78 strains screened, 16 out of 28 obligate marine fungi (57.1 %) and 13 out of 50 fungal endophytes (26 %) had enzyme production efficiency toward specific substrates or an enzymatic index (EI) higher than 1.4. For obligate marine fungi, 10.7, 35.7, 28.6, and 32.1 % of fungi screened were shown to produce amylase, cellulase, protease, and pectinase, respectively. Ten isolates of obligate marine fungi and 7 isolates of fungal endophytes producing only 1 enzyme exhibiting EI values ranging from 1.45 to 4.56 (Table 4). The obligate marine fungi JS22 (*Saagaromyces abonnis*) and JS29 (*Kallichroma tethys*) showed the highest amylase (EI = 2.79) and cellulase activity (EI = 3.16), respectively, while isolate JS21.1 (*K. tethys*) also exhibited the highest protease and pectinase activities (EI = 4.56 and 4.47). For marine fungal endophytes, 4, 8, 8, and 24 % of fungi screened produced amylase, cellulase, protease, and pectinase activities, respectively. Isolate AA1 (xylariaceous fungus) had the highest amylase (EI = 3.18), cellulase (EI = 2.50), protease (EI = 2.52) and pectinase (EI = 3.13) activities on agar. The illustration of the 3 isolates exhibiting highest enzyme activity are shown in Figure 2.

**Table 4** Enzyme index (EI) of 29 active fungal isolates. Different letters mean that the data are statistically different from the least significant difference ( $p < 0.05$ ).

No.	Isolate	Source	Enzymatic Index				
			Amylase	Cellulase	Protease	Lipase	Pectinase
1	JS2	Mangrove wood	-	1.52 <sup>g</sup>	1.21	-	-
2	JS3		-	1.69 <sup>f</sup>	2.17 <sup>f</sup>	-	1.77 <sup>ef</sup>
3	JS9		-	-	2.93 <sup>d</sup>	-	-
4	JS15		1.06	1.74 <sup>ef</sup>	1.93 <sup>g</sup>	-	1.47 <sup>gh</sup>
5	JS17		-	2.68 <sup>b</sup>	1.23	-	-
6	JS17.1		-	1.96 <sup>de</sup>	1.24	-	-
7	JS20		-	-	-	-	3.33 <sup>d</sup>
8	JS24		-	1.38	1.04	-	4.33 <sup>ab</sup>
9	JS25		-	1.29	-	-	4.17 <sup>bc</sup>
10	JS12		-	-	3.78 <sup>c</sup>	-	-
11	JS21.1		2.46 <sup>c</sup>	2.03 <sup>d</sup>	4.56 <sup>a</sup>	-	4.47 <sup>a</sup>
12	JS22		2.79 <sup>b</sup>	2.05 <sup>d</sup>	4.19 <sup>b</sup>	-	3.92 <sup>c</sup>
13	JS27.1		-	1.87 <sup>e</sup>	1.89 <sup>gh</sup>	-	-
14	JS27.3		-	1.45 <sup>h</sup>	1.07	-	1.20
15	JS29		1.95 <sup>e</sup>	3.16 <sup>a</sup>	2.51 <sup>e</sup>	-	3.27 <sup>d</sup>
16	JS35		-	1.09	-	-	1.72 <sup>ef</sup>

No.	Isolate	Source	Enzymatic Index				
			Amylase	Cellulase	Protease	Lipase	Pectinase
17	RM4	<i>Rhizophora mucronata</i>	1.27	1.62 <sup>fg</sup>	1.65 <sup>hi</sup>	-	1.50 <sup>efh</sup>
18	RM9		1.06	1.27	1.62 <sup>i</sup>	-	1.46 <sup>gh</sup>
19	SA3	<i>Sonneratia alba</i>	-	-	-	-	1.56 <sup>ef</sup>
20	AA1	<i>Avicennia alba</i>	3.18 <sup>a</sup>	2.50 <sup>c</sup>	2.52 <sup>e</sup>	-	3.13 <sup>d</sup>
21	AA3		1.08	1.66 <sup>fg</sup>	1.31	-	1.48 <sup>gh</sup>
22	AA5		1.07	-	1.59 <sup>i</sup>	-	1.45 <sup>gh</sup>
23	AAU4		1.45 <sup>f</sup>	1.24	1.34	-	-
24	AAU8	<i>Acrostichum aureum</i>	1.35	-	-	-	1.52 <sup>efh</sup>
25	AAU9		-	-	-	-	1.81 <sup>e</sup>
26	AAU11		-	1.09	-	-	1.47 <sup>gh</sup>
27	AE8		<i>Acanthus ebracteatus</i>	-	-	-	-
28	BC6	<i>Bruguiera cylindrica</i>	-	1.49 <sup>h</sup>	1.19	-	2.08 <sup>e</sup>
29	2AA2	<i>Avicennia alba</i>	-	1.28	-	-	1.75 <sup>ef</sup>



**Figure 2** The 3 isolates exhibited highest enzyme production. A) Ascomata of JS29 (*Kallichroma tethys*), B) Ascospores of JS21.1 (*K. tethys*), C) Colony morphology of JS21.1 on PDA prepared in natural seawater after 10 days cultivation, D) Colony morphology of JS29 on PDA prepared in natural seawater after 10 days cultivation, E) Colony morphology of AA1 (xylariaceous fungus) on PDA prepared in natural seawater after 5 days cultivation. Scale bars A = 0.25 cm, B = 20  $\mu$ m.

Fungi isolated from various substrata in the marine environments have been found to produce extracellular enzymes of commercial interest, and they play an important role as the primary decomposers in marine ecosystems [54-56]. Endophytic fungi possess extracellular hydrolytic enzymes necessary for lignocellulosic degradation mainly xylanases, cellulases, protease, phenol-oxidase [56]. Moreover, marine fungi efficiently produce pectinase because this enzyme is important for penetrating carbohydrate containing cell walls and leads to cell lysis [57]. *Halorosellinia oceanica* produced the greatest activity of cellulase compared to other obligate marine fungi tested [58]. Other genera inhabiting mangrove wood proved to produce amylase and cellulase include species of ascomycetes and basidiomycetes e.g. *Acrocordiopsis*, *Aigialus*, *Ascocratera*, *Corollospora*, *Cryptovalsa*, *Halocyphina*, *Kallichroma*, *Linocarpon*, *Massarina*, *Nia*, *Periconia*, *Rimora*, and *Sclerococcum* [59-61]. Our study supports the earlier reports on the ability of *K. tethys* to degrade mangrove wood [54,62]. None of the fungi tested in this study showed lipase activity, although lipase activity was seen in the marine basidiomycetes *Halocyphina vilosa* and *Nia vibrissa* and mangrove endophytes, as lipase activity suggests their ability to use fats as energy source [63,64].

Fungal endophytes obtained from mangroves, salt marsh plants, and marine algae were shown to produce extracellular enzymes especially amylase and cellulase, and get involved in litter decomposition after senescence [65,66]. These fungi include species of *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Hypoxylon*, *Penicillium*, *Pestalotiosis*, and Pleosporales sp. [65,66]. The xylariaceous endophytic fungus AA1 isolated from the leaves of the mangrove tree *Avicennia alba* exhibited the highest amylase production. Members of xylariaceous fungi found growing as endophytes in plant tissues are also reported as potential producers of amylase [56,64-66]. Protease and pectinase activities were seen in some of the fungi screened. *Kallichroma tethys* exhibited the highest protease and pectinase activities, while *Corollospora maritima* performed detectable pectinase amount [67].

#### Effect of environmental stressors on enzyme production

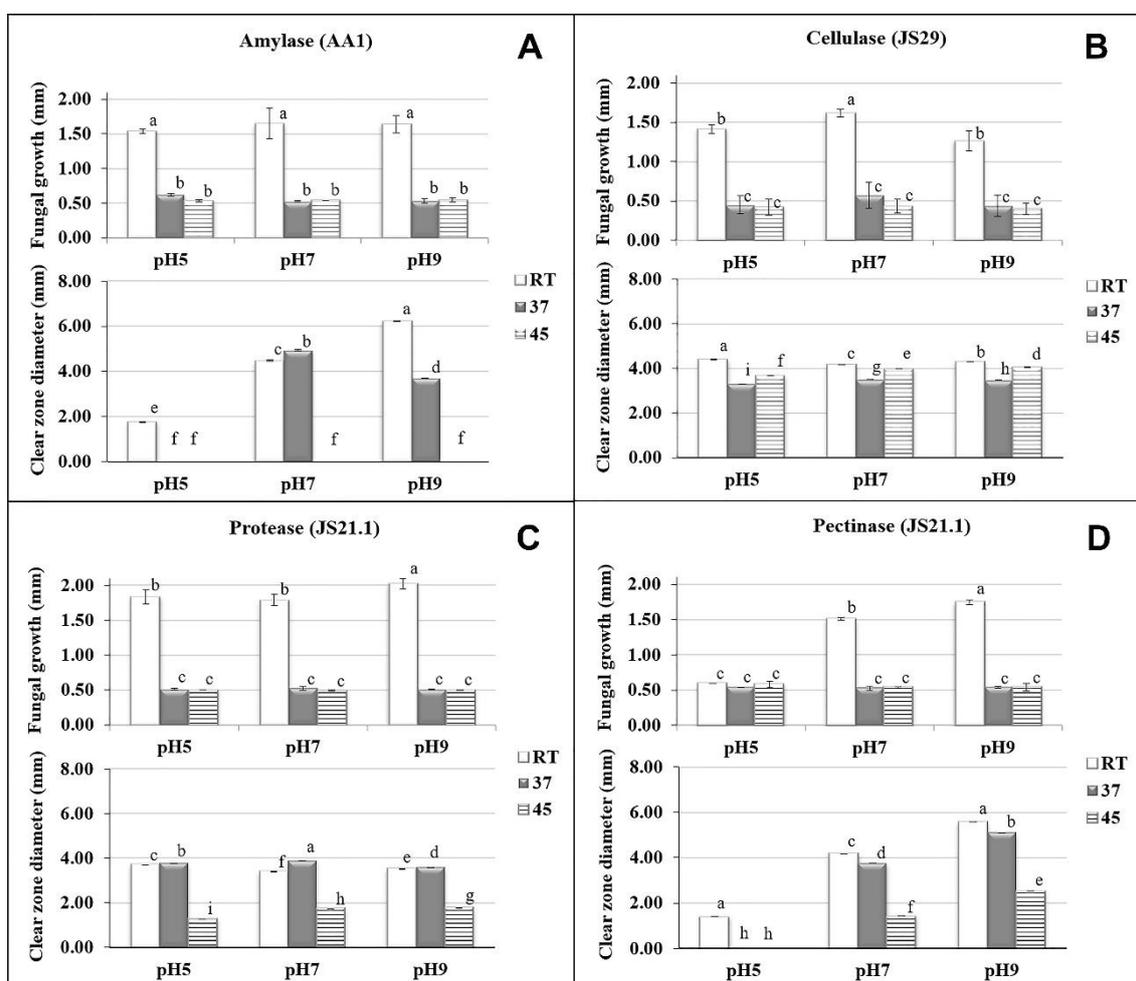
Marine fungi inhabiting mangrove habitats have been challenged with the fluctuation of environmental stressors, which might influence the growth and enzyme production [68,69]. The environmental factors including temperature, pH, and salinity were tested with isolates AA1 (xylariaceous fungus), JS29 (*K. tethys*), and JS21.1 (*K. tethys*), which produced the highest amylase, cellulase, pectinase, and protease activities. The fungus AA1 showed a wide tolerance of temperature and pH for growth and amylase production, and it preferred enzyme production at pH 7 and 9 (**Figure 3A**, **Table 5**). The fungal growth ranged from 0.53 to 1.65 cm after 15 days incubation, and the maximum growth was shown at room temperature ( $28 \pm 2$  °C) and decreased with increasing temperature. This fungus had the highest amylase production at room temperature and in the neutral (pH 7) and alkaline pH range (pH 9). The clear zone surrounding the colony after iodine solution treatment was 6.21 cm at pH 9 followed by 4.47 cm at pH 7, and 1.75 cm at pH 5, while the enzyme secretion at 37 °C was high at 4.94 cm at pH 7 and could not detect extracellular amylase at 45 °C under all pH conditions. Many studies on amylase production by *Aspergillus oryzae*, *A. ficuum*, *Botryodiplodia theobromae*, *Rhizopus oryzae*, and *Penicillium fellutanum* were reported the optimum temperature of 30 °C [70]. Maria *et al.* [64] also discovered that neutral pH at 7 and alkaline pH at 9 stimulated the production of amylase for some marine endophytes.

The isolate JS29 (*K. tethys*) had optimum growth at room temperature and pH 7, while the cellulase production was highest at pH 5. The enzyme production was reduced at all pH values with increasing temperature (**Figure 3B**). The EI tendency at all conditions increased depending on the incubation time. The highest EI value indicating the efficiency of enzyme production was pH 9 and 45 °C after 15 days (EI = 10.36) (**Table 5**). The results demonstrated that temperature strongly affected fungal growth, whereas varying pH slightly affected both growth and enzyme production. Although its growth was pressured at 37 and 45 °C, it might be stimulated to produce enzymes for substrate hydrolysis in order to survive. The trend of pH tended to be acidic at pH 5 and room temperature, with a significant difference in the specific pH condition of each enzyme may be related to enzyme-substrate binding, catalytic activity and substrate ionization as well as protein structure. It was also supported that the high cellulase production from *Aspergillus niger* and *Trichoderma* sp. exhibited at pH range between 5 - 8 and temperature between 40 - 50 °C [71].

For protease production, JS21.1 (*K. tethys*) grew well at room temperature at pH 9 with a fungal diameter of 2.03 cm (**Figure 3C**). Moreover, growth was also hardly observed at 37 and 45 °C of incubation. After 15 days of incubation, the diameter of the clear zone was measured ranging from 1.26 - 3.87 cm. The largest clear zone formation was 3.87 cm at pH 7 and 37 °C, followed by 3.76 cm at pH 5 and 3.7 cm at pH 5, and room temperature. Temperature had a greater effect on growth and protease

production of JS21.1. This fungus produced protease in a wide range of pH values, implying that it could tolerate a wide range of pH values in some habitats with changes over time. Protease production from microorganisms has been widely reported to be acidic, neutral or alkaline depending on the organisms and location of the sample. For example, the maximum protease activity of *P. fellutanum* isolated from mangrove rhizosphere was at 30 °C and pH 8.5 [72]. Enzyme protease of *Pestalotiopsis* sp. isolated from mangrove angiosperms showed highest activity in both neutral and alkaline pH [73], and *Aspergillus* species exhibited maximal protease production at pH 4 and 5 [74].

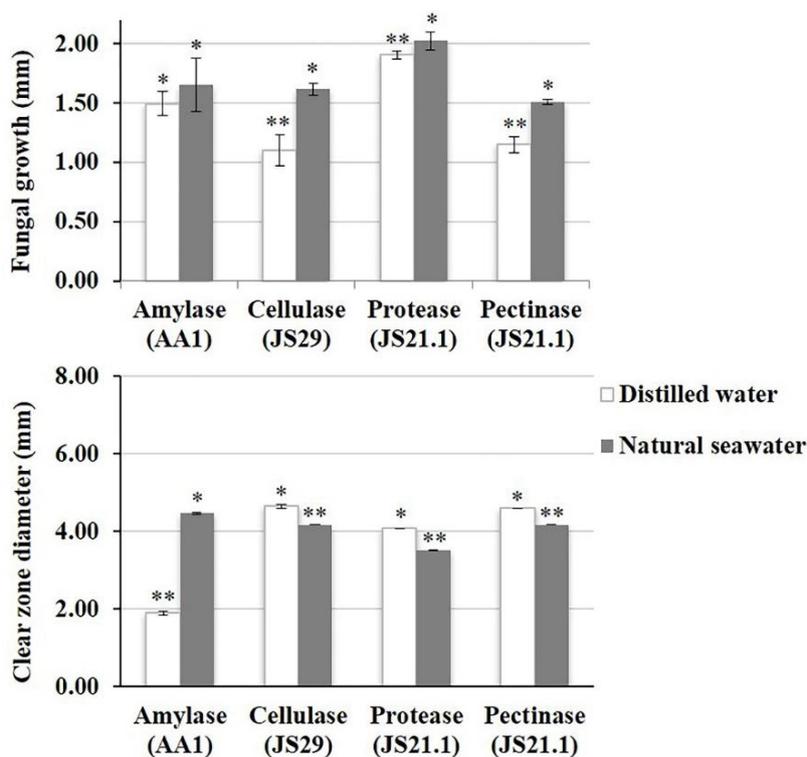
Moreover, isolate JS21.1 had a high efficiency for pectinase production. It had the highest growth of 1.74 cm at pH 9 and 1.51 cm at pH 7, and room temperature, with no significant difference. Pectinase production was the highest at pH 7 and 9, and the lowest production at pH 5. The maximum zone of clearance was found to be 5.58 cm at pH 9, and room temperature, followed by 5.10 cm at pH 9, and 37 °C with a significant difference (**Figure 3D**). The maximum EI value of protease production was 7.36 at pH 7 and 37 °C, while pectinase production exhibited 9.39 at pH 9 and 37 °C. The optimum for growth was at pH 9 and room temperature, although the effectiveness of enzyme production was the same pH and at 37 °C. This finding was in agreement with the reports of Pedrolli and Carmona [75] and Ketipally and Ram [76] who studied *Aspergillus giganteus* and *A. oryzae* isolated from mangrove soil, which had the highest production at pH 6 - 6.5 and 35 °C and above 40 °C. *Pestalotiopsis* sp. isolated from *Acanthus ilicifolius* had the highest pectinase production at both pH 7 and 9 after 6 - 9 days of fermentation [73], therefore, neutral and alkaline pH values did not affect pectinase production.



**Figure 3** Effect of temperature (RT = room temperature (28±2 °C), 37, 45 °C) and pH (5, 7, 9) on enzyme production. A) amylase production by isolate AA1, B) cellulase production by isolate JS29, C) protease production by isolate JS21.1, D) pectinase production by isolate JS21.1. Statistically significant differences were assumed at  $p < 0.05$ . All values are presented as mean ± standard error (n = 3).

**Table 5** Enzymatic index (EI) of enzyme production by selected fungi. All values are presented as mean  $\pm$  standard error (n = 3).

Sample	Time (Days)	EI (RT)			EI (37 °C)			EI (45 °)		
		pH 5	pH 7	pH 9	pH 5	pH 7	pH 9	pH 5	pH 7	pH 9
AA1	0	–	–	–	–	–	–	–	–	–
Amylase	5	3.39 $\pm$ 0.21	2.83 $\pm$ 0.08	2.89 $\pm$ 0.12	–	5.51 $\pm$ 0.12	4.54 $\pm$ 0.30	–	–	–
	10	2.12 $\pm$ 0.25	2.75 $\pm$ 0.07	3.24 $\pm$ 0.14	–	6.65 $\pm$ 0.14	5.30 $\pm$ 0.35	–	–	–
	15	1.14 $\pm$ 0.01	2.73 $\pm$ 0.36	3.80 $\pm$ 0.28	–	9.38 $\pm$ 0.17	6.99 $\pm$ 0.42	–	–	–
JS29	0	–	–	–	–	–	–	–	–	–
Cellulase	5	1.37 $\pm$ 0.20	2.33 $\pm$ 0.31	2.19 $\pm$ 0.32	4.61 $\pm$ 1.52	3.27 $\pm$ 1.23	3.62 $\pm$ 1.37	6.62 $\pm$ 1.98	5.80 $\pm$ 1.56	1.26 $\pm$ 0.35
	10	2.56 $\pm$ 0.15	2.39 $\pm$ 0.11	3.35 $\pm$ 0.56	6.10 $\pm$ 2.16	6.16 $\pm$ 2.36	3.86 $\pm$ 1.61	9.35 $\pm$ 3.05	8.56 $\pm$ 2.70	2.32 $\pm$ 1.13
	15	3.10 $\pm$ 0.12	2.58 $\pm$ 0.08	3.43 $\pm$ 0.36	7.74 $\pm$ 2.33	6.54 $\pm$ 2.24	8.55 $\pm$ 3.19	9.22 $\pm$ 2.60	9.44 $\pm$ 2.15	10.36 $\pm$ 2.06
JS21.1	0	–	–	–	–	–	–	–	–	–
Protease	5	2.36 $\pm$ 0.19	2.25 $\pm$ 0.09	2.62 $\pm$ 0.10	4.60 $\pm$ 0.10	4.53 $\pm$ 0.29	4.44 $\pm$ 0.05	2.28 $\pm$ 0.00	3.04 $\pm$ 0.07	2.82 $\pm$ 0.03
	10	2.14 $\pm$ 0.13	1.61 $\pm$ 0.11	1.90 $\pm$ 0.07	6.47 $\pm$ 0.15	6.26 $\pm$ 0.42	6.22 $\pm$ 0.06	2.48 $\pm$ 0.00	3.32 $\pm$ 0.08	3.26 $\pm$ 0.04
	15	2.02 $\pm$ 0.11	1.90 $\pm$ 0.09	1.74 $\pm$ 0.07	7.33 $\pm$ 0.17	7.36 $\pm$ 0.46	6.96 $\pm$ 0.07	2.52 $\pm$ 0.00	3.50 $\pm$ 0.08	3.52 $\pm$ 0.04
JS21.1	0	–	–	–	–	–	–	–	–	–
Pectinase	5	2.71 $\pm$ 0.06	2.98 $\pm$ 0.10	3.48 $\pm$ 0.04	–	5.36 $\pm$ 0.32	5.46 $\pm$ 0.25	–	2.48 $\pm$ 0.10	4.92 $\pm$ 0.62
	10	2.55 $\pm$ 0.00	2.68 $\pm$ 0.02	3.04 $\pm$ 0.11	–	7.00 $\pm$ 0.40	7.55 $\pm$ 0.21	–	2.42 $\pm$ 0.03	5.03 $\pm$ 0.52
	15	2.33 $\pm$ 0.00	2.76 $\pm$ 0.03	3.19 $\pm$ 0.07	–	7.12 $\pm$ 0.40	9.39 $\pm$ 0.26	–	2.67 $\pm$ 0.03	4.72 $\pm$ 0.49

**Figure 4** Bar graphs showing paired t-test analysis on the effect of salinity on fungal growth and enzyme production. Statistically significant differences were assumed at  $p < 0.05$ . All values are presented as mean  $\pm$  standard error (n = 3).

All 3 fungi tested preferred growth in natural seawater (30 ppt). They could produce enzymes in a medium containing distilled water and had higher cellulase, protease and pectinase production (4.08 - 4.64 cm), except for amylase production (1.89 cm) after 15 days incubation (**Figure 4**). The 2 obligate marine fungi (JS29 and JS21.1) had greater enzyme production in a medium without salt, while an endophytic fungus AA1 showed higher amount of amylase in a medium containing salt. The effect of salinity strongly impacted fungal growth and enzyme production. The result from this study was similar with the study by Pointing *et al.* [58] who reported that increasing salinity reduced enzyme production in some of the obligate marine fungi tested. A majority of fungi from mangroves were shown to produce enzymes such as cellulase, xylanase, and laccase in the media prepared with seawater [58,77,78]. This might probably be the result of the fluctuation of salinity in the marine environments.

## Conclusions

We present here the diversity of marine and endophytic fungi from Thasala mangroves in Nakhon Si Thammarat province and their extracellular hydrolase enzymes of commercial interest. This study reported the occurrence of manglicolous fungi collected at Thasala mangrove, not previously studied before. Low species diversity of manglicolous fungi at Thasala in comparison with other locations in Thailand. This probably was attributed to the scarcity of dead wood arising from a young mangrove stand, the small number of samples collected, the nature of host tissue, and the physicochemical features of mangrove habitats. Our data support earlier observations that marine fungi and endophytic fungi are potential sources of hydrolase enzymes. The 57 % of obligate marine fungi and 26 % of fungal endophytes had hydrolase enzyme (EC.3) production efficiency toward specific substrates. Three isolates AA1 (xylariaceous fungus), JS29 (*Kallichroma tethys*), and JS21.1 (*K. tethys*) demonstrating the highest amylase, cellulase, pectinase, and protease activities. They were further challenged for the environmental factors affecting growth and enzyme production, including pH, temperature, and salinity. Neutral and alkaline conditions stimulated higher production of amylase and pectinase. Temperature affected the growth and protease production of an obligate marine fungus JS21.1. Salinity had impacted fungal growth and enzyme production, although responses varied from species to species. Further studies should include optimization of culture conditions, enzyme purification, and kinetic studies of hydrolase enzymes from marine fungi for conspicuous application with environmentally friendly technological development.

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