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Bioactivities of Mangrove-Associated Filamentous Fungi from Jepara, Indonesia: Anti-Bacterial Metabolites and Wood-Degrading Enzymes

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Abstract

Background: Mangrove-associated fungi are widely known due to their bioactivities, especially their antibacterial and lignocellulose-degrading enzymes activity. However, research on their bioactivity in Indonesia, particularly in Jepara Regency remain limited. This research aims to explore the antibacterial and wood-degrading enzymatic properties from mangroves-associated fungi of Jepara, Indonesia.

Methods: A total of 43 isolates were successfully collected from leaf and barks of *Excoecaria agallocha*, *Acanthus ilicifolius*, as well as *Lumnitzera racemosa*. Antibacterial screening against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) was then conducted to all isolates, where 1 isolate was active against *E. coli*, 12 against *S. aureus*, and 5 isolates active against both pathogens. All active isolates were identified through microscopic observation.

Results: Isolates with the most consistent inhibition zone, namely AB1, E2D2, E2B4, and LB10 continued to have metabolite extraction and antibacterial assay through disc-diffusion methods against the same 2 pathogens. Isolate E2B4 was able to produce the largest halo zone diameter measuring 22.28±1.59 *mm* at concentration of 50 *mg/mL* against *S. aureus*. Based on preliminary phytochemical screening, crude extract of E2B4 contains flavonoid and alkaloid as secondary metabolite. To search for further bioactivity, wood-degrading enzymatic assay was conducted, where the result showed that 6 isolates were actively produced cellulase, 1 laccase, 7 tannase, and 5 lignin peroxidase. DNA barcoding revealed that AB1, E2D2, and E2B4 isolates were identified as *Phaeophleospora eucalypticola*, *Curvularia lunata*, and *Paraconiothyrium* sp., respectively.

Conclusion: Therefore, these findings highlight mangrove-associated fungi bioactivities potential through screening assay and provide critical information regarding the rarely studied Indonesian fungi.

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Introduction

Mangroves are highly specialized plants found in intertidal zones of tropical or subtropical areas, where they inhabit 25% of total coastline area and present in 112 different countries [1,2]. Due to their adaptation to saline environment, mangroves can easily thrive in coastline area where terrestrial vegetation are not able to grow [3]. Mangrove ecosystems provide impactful service in relation to ecological function and particularly for human societies [4,5]. Mangrove contribute to maintaining water quality, produce fuel and charcoal, resource for multiple medicines by producing beneficial natural compounds, as well as maintaining human nutrition by preserving fisheries production and food web stability [5,6]. In addition, mangrove is home to coastal biodiversity, particularly fungal biodiversity [7].

Mangrove-associated fungi represent an important component of marine microbial communities, where these fungi are known to live in extreme and fluctuating environments [7]. Mangrove-associated fungi live in high-salinity condition, tidal inundation, and lignocellulosic substrates [8]. This pressure stimulates the fungal bioactivity, therefore driving the secondary

metabolite production and enzymatic activity [9,10]. Antibacterial activity is a good example for this phenomenon, where endophytes can produce several metabolites, including steroids, phenols, terpenoids, and many different phytochemical compounds which have been proven to be useful for mangrove to survive bacterial infection [11]. With the Antimicrobial Resistance (AMR) occurrence of human pathogens [mainly *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*)], it is important to search for new sources of antibiotic and mangrove-associated fungi with its capability to produce antibacterial metabolites can be a new solution to counteract this medical issue [12].

Mangrove-associated fungi are also known to produce wood-degrading enzymes which includes cellulase, tannase, and lignocellulolytic enzyme (particularly laccase and lignin peroxidase) [13,14]. These properties are valuable for nutrient cycling by degrading mangrove tree litters, while also beneficial to neutralize toxic substrate such as industrial dye and pesticides [15,16]. Furthermore, the production of lignocellulolytic enzyme is known to affect the production of secondary metabolite such as nitroaryl which contains antibacterial properties [17]. Thus, investigating the relationship between antibacterial activity and wood-degrading enzyme production in mangrove-associated fungi holds significant value in medical and ecological study.

Indonesia, home to many mangrove species, is the country with the largest mangrove forests in the world, where all 38 provinces harbor mangrove ecosystems, comprising 77 species of mangroves [18]. As such, previous studies have reported how Indonesian mangroves host multiple species of mangrove-associated fungi (*e.g. Aspergillus, Penicillium, Neopestalotiopsis, Trichoderma*) and prospecting the secondary metabolite as well as wood-degrading enzymes [19-21]. However, some regions, such as Jepara Regency, remain unexplored. Given the importance of the antibacterial activity and wood-degrading enzyme, the objective of this study was to: (1) Highlight the biodiversity of mangrove-associated fungi from Jepara mangroves, (2) Explore the antibacterial metabolites and wood-degrading enzymes of mangrove-associated fungi from Jepara Regency, (3) Unraveling the understudied fungal species through ITS identification.

Materials and Methods

Sample collection and fungi isolation

Mangrove-associated fungi samples were collected from mangrove forest of Science and Techno Park of Diponegoro University, Awur Bay, Jepara Regency, Indonesia (6.62248154S; 110.6386947E). 2 body parts of mangrove were collected, including leaves and bark of *Excoecaria agallocha* (*E. agallocha*), *Acanthus ilicifolius* (*A. ilicifolius*), and *Lumnitzera racemosa* (*L. racemosa*). Each collected samples were moved to sterilized ziplock bag and stored in a coolbox [22]. The sample surface sterilization was done by soaked them in sterilized sea water for 1 *min*, aquadest 1 *min*, 2% sodium hypochlorite for 30 *s*, and finally rinsed with sterile sea water for 1 *min* [23]. Fungi isolation was conducted through direct planting method, where leaf and bark samples were tucked in sterile Potato Dextrose Agar (PDA) medium containing 70% sea water and 50 *mg/L* chloramphenicol to prevent bacterial growth. Each isolated samples were incubated at room temperature (28±2°*C*) for 7 days [24]. An environmental control was prepared in order to differentiate the undesired isolate outside of targeted fungi [25].

Fungi purification and macroscopic identification

Each isolated fungi which successfully grew on isolation media will be inoculated in new media. To prevent isolated the same species of fungi from different host, morphological identification was conducted by observing visible traits, such as color, growth rate, media pigmentation, and colony structure. Purified fungi were incubated for another 7 days, and macroscopic identification were conducted by observing the form, color, margin, elevation, and texture of fungi colonies [26,27].

Antibacterial screening

Antibacterial screening was carried out with agar plug method against 2 different pathogens, using *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. Pathogens were inoculated from Nutrient Agar (NA) to Nutrient Broth (NB) and cultured for 1×24 *hr* on a shaker at 100 *rpm*. After the culture, pathogens were suspended in a 0.86% physiological saline solution while matching its turbidity to 0.5 McFarland Standard. Pathogens were inoculated onto Mueller Hinton Agar (MHA) using a sterile cotton swab (M size) until each surface of the media was fully covered. Fungi isolates (5-7 days old) were inoculated using the base of blue tip (7.5 *mm* in diameter) by placed them on the surface of MHA containing pathogen test [28]. All antibacterial screening were incubated for 24, 48, and 72 *hr* at room temperature (28±2°*C*) [24,25]. Positive result was indicated by the formation of halo zone around the plug while measured with caliper every 24 *hr* to clarify the consistency of formation. Blank PDA served as negative control. The screening was conducted in duplicate.

Microscopic morphological traits observation

Isolates with antibacterial potential will have their microscopic morphological traits observed by applying slide culture method. To prevent undesired fungi growth from environment, each essential equipment (glass slide, petri dish, and cover glass) were sterilized using autoclave (121°*C* for 20 *min*) and burned by Bunsen burner before used. A sterile glass slide was placed in a petri dish, and a square piece of sterile PDA medium (approximately 1×1 *cm*) was placed on top of the slide [29]. Spores of isolate were inoculated on each side of the piece and the media is placed with a sterile cover glass [30]. The culture was sealed and incubated at room

temperature for 3-5 days, depending on the growth rate of the isolate. After incubation period is done, the cover glass moved into a new glass slide with Lactophenol Cotton Blue on it. The morphological traits using binocular microscope with magnification of 100× was observed [31]. Each slide cultures were done in duplicate.

Mass culture and crude extraction

Isolates with the most promising antibacterial activity were prepared for mass culture and metabolite extraction. Fungi were cultured in solid media for 7 days, and 6-8 pieces of the fungi were inoculated into 1000 *ml* erlenmeyer containing 300 *ml* of Potato Dextrose Broth (PDB) [32,33]. Isolates were cultured on 90 *rpm* shaker for 14 days while regular observation was performed to make sure that each culture were not contaminated. After that, metabolite extraction was done by separate the broth media and mycelium through filtrate paper [34].

The broth was moved into new erlenmeyer and 1:1 amount of ethyl acetate was poured to the broth. The broth was macerated with the solvent for 72 *hr* on shaker at 90 *rpm*. After maceration completed, organic phase and solvent phase was separated using separatory funnel and evaporated at 40°*C* until crude extract can be collected [33]. The crude extract was re-dissolved in dimethylsulphoxide (DMSO) to obtain 4 different concentrations, which were 50, 25, 12, 5, and 6,25 *mg/ml*, respectively. All extract were incubated at 4°*C* before used [35].

Antibacterial assay

Antibacterial assay was performed using disc-diffusion method and the concentrated metabolites were tested against *E. coli* and *S. aureus*. The pathogens were cultured and inoculated with the same procedure as antibacterial screening. After the MHA was covered in pathogen test, sterile filter paper discs with the size of 6 *mm* were soaked in 20 *µl* of fungi extracts (concentration of 50, 25, 12,5, and 6,25 *mg/ml*) and let it be absorbed for about 30 *s*. Paper discs were then placed on top of media [36]. For further comparation, 30 *µg*/disc of amoxicillin was used as positive control due to their broad-spectrum compatibility against gram-positive and negative pathogens [37]. On the other hand, DMSO 10% was used as negative control [38]. The petri dishes were incubated at room temperature (28±2°*C*) for 24, 48, and 72 *hr*, while any inhibition zone was measured in *mm* scale using caliper. Each extract assay was done in duplicate.

Preliminary phytochemical screening

Phytochemical screening was done to qualitatively detect any potential phytochemical properties in fungi extract. The phytochemical target includes flavonoid, tannin, alkaloid, steroid, and saponin screening [39]. Flavonoid screening was conducted by mixing 1 *ml* of concentrated extract with 4 droplets of HCl 37% and 25 *mg* of magnesium powder, where positive reaction was the presence of red or pink color [40]. Tannin screening was done by dissolving 5 *ml* of extract with 2 *ml* of 5% $FeCl_3$ and positive reaction can be observed when the reagent turned into dark blue or dark-greenish brown [39]. For alkaloid screening, extract was dissolved in 10 *ml* of EtOAc solvent and mixed with a few droplets of HCl 37%. Filtrate were separated to 3 reaction tube with each tube containing 3 *ml* of filtrate. The tubes were mixed with a few drops of Mayer, Wagner, and Dragendorff, respectively, where the positive reactions were white, brown, and orange precipitation respectively [40]. For steroid, 3 *ml* of extract were dissolved with 1 *ml* Liebermann-Burchard, where blue and green formation shows that the extract contains steroid [40]. Lastly, procedure for saponin was done by dissolving 0.5 *ml* of extract to 5 *ml* of distilled water and shook for 1 *min* straight. Consistent formation of foam means the extract contain saponin [41].

Enzymatic assay

Each isolate with antibacterial activity from antibacterial screening was continued into enzymatic assay. This was a semi-qualitative assay, where 7-14 days old fungi were inoculated into the test medium containing required enrichment and the enzymatic activity was determined using the following enzymatic index formula [42]:

$$Enzymatic\ Index\ (EI) = \frac{Clear\ zone\ diameter - colony\ diameter}{Colony\ diameter}$$

The first enzymatic assay was cellulase, where PDA media containing 1% Carboxymethylcellulose (CMC). Fungi isolate (7-14 days) were inoculated into media test and incubated for 72 *hr*. To observe the visible degradation zone, 0.3% Congo red was poured into the media test and let it absorb for a few minutes. Positive result was indicated by the presence of clear zone around colonies [7].

Second enzymatic assay was laccase and tannase, where both activities can be observed using PDA with 0.2% tannic acid was used as an enrichment. Due to slow production of these enzymes, the plates were incubated and observed for 2, 7, and 14 days. Laccase production was revealed by the presence of brown zone around the colony, while tannase was demonstrated by presence of clear zone [43,44].

The last enzymatic assay was lignin peroxidase, where the assay used PDA containing 100 *mg/L* of methylene blue. The plates incubated and observed for 2, 7, and 14 days. The presence of clear zone around colonies indicated the presence of lignin peroxidase activities [45]. All enzymatic assay was conducted in duplicate.

DNA extraction, amplification, and electrophoresis

DNA extraction was carried out by re-culturing the isolate into sterile PDA for 7 days. The DNA extraction was done by following Quick-DNA™ Fungal/ Bacterial Miniprep Kit (D6005, Zymo Research)

protocol. The extracted DNA were then amplified through Polymerase Chain Reaction (PCR) by adding 12.5 *µl* if PCR master mix (Promega), 1 *µl* of ITS1 primer, 1 *µl* of ITS4 primer, 1 *µl* of DNA template, and 9.5 *µl* of ddH_2O to obtained 25 *µl* PCR mix. The PCR reaction were carried out with initial denaturation at 95°*C* for 3 *min*, 35 cycles of denaturation at 95°*C* for 10 *s*, annealing at 52-53°*C* for 30 *s*, and extension at 72°*C* for 45 *s* while the cycle ended with post-extension at 72°*C* for 10 *min* [25,27]. The DNA visualization was performed through 0.8% of agarose gel and 50 *ml* of TBE buffer with 1× concentration. 2.5 *µl* of PCR product were loaded into the agarose, while the electrophoresis was running at 100 *V* for 30 *min*. The electrophoresis result was visualized through gel documentation system [46].

DNA barcoding and phylogenetic construction

PCR product with desired electrophoresis result were sequenced to 1st BASE (Malaysia) by Sanger Sequencing method. The sequence and chromatogram result were aligned and analyzed using MEGA 11.0 version. The aligned sequences were identified using Basic Local Alignment Search Tool (BLAST) to obtain the species of isolates. The phylogenetic tree was made using Maximum-Likelihood (ML) algorithm, Kimura 2-parameter model with the help of 1000 replication of bootstrap [27].

Results

Isolation and macroscopic observation

This research managed to isolate a total of 43 mangrove associated fungi from leaf and bark of *E. agallocha*, *A. ilicifolius*, and *L. racemosa*, where 27 isolates were collected from leaves while 16 others collected from bark. Each sample were isolated from one individual, except for *E. agallocha* which was isolated from 2 different individual. Each isolated fungi were characterized by multiple morphology factor, where the most distinguishable characteristic were colony form, coloration, and margin. The other observable morphological traits such as form, margin, elevation, and texture of each isolate were distinct to one another, which helped in differentiate each isolate. Isolation plate can be seen in figure 1 and number of isolates can be observed in figure 2.

Antibacterial screening

The antibacterial screening with agar plug method shows that 1 isolate actively inhibited *E. coli*, 11

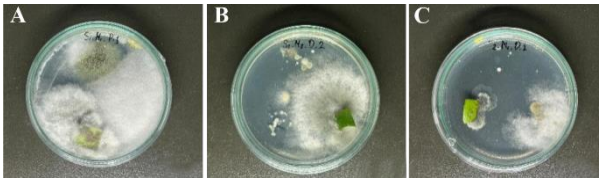

Figure 1. Isolation of mangrove-associated fungi from (A) *E. agallocha*, (B) *A. ilicifolius*, (C) *L. racemose.*

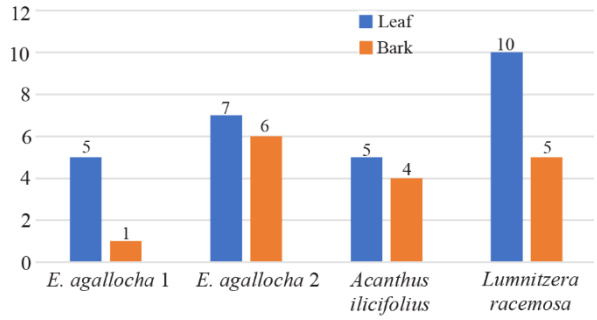

Figure 2. Number of isolates based on species and plant parts.

isolates were active against *S. aureus*, and 6 isolates against both pathogens. However, isolate AB1, E2D2, E2B4, as well as LB10 inhibited both pathogens consistently and continued into metabolite extraction and antibacterial assay. 41.86% of total isolate were proven to produce antibacterial activity. Overall, the result of the antibacterial screening shows that *S. aureus* was more susceptible compared to *E. coli*. The results are provided in table 1.

Microscopic morphological observation

The microscopic observation included 5 morphological trait identifications, including conidia, metulae, conidiophore, vesicle, phialides, and hyphae. Observed structures such as vesicles and conidiophores in several isolates (*e.g.*, AD3, E2D2, LD10, LB10) strongly suggests their affiliation with *Trichoderma*, *Curvularia*, and *Penicillium* like-taxa. The microscopic observation result can be seen in figure 3.

Mass culture and extraction

Crude extract dry weight of mangrove-associated fungi extracts can be seen in figure 4. Among all 4 obtained extracts, isolate AB1 produced the least amount of extract (36.2 *mg*/300 *ml* PDB) while isolate E2D2 generated the crudest extract (73.6 *mg*/300 *ml* PDB).

Antibacterial assay

Fungi extract from ethyl acetate solvent were diluted into 4 different concentrations, which were 50, 25, 12.5, and 6.25 mg/ml^{-1}. Antibacterial assay shows that there were 3 extracts with antibacterial activity, including extracts of AB1 produced inhibition zone against both pathogens, E2D2 inhibited *S. aureus* only, while E2B4 produced the highest value of inhibition zone against both pathogens. Extract of E2B4, later identified as *Paracniothyrium* sp., managed to produce a promising inhibition zone, up to 22.28±1.59 *mm* against *S. aureus* and 6.10±1.55 *mm* against *E. coli* with concentration of 50 mg/ml^{-1} at 24 *hr* of incubation. Other extract, especially produced by isolate AB1 and E2D2 (identified as *Phaeophleospora eucalypticola* and *Curvularia lunata*, respectively) produced less inhibition zone than E2D2. AB1 extract manage to inhibit *E. coli* at concentration of 100 and

Table 1. Antibacterial Screening Result from Mangrove-Associated Fungi Isolates

Mangrove host	Pathogen	*Escherichia coli*			*Staphylococcus aureus*		
	Isolate code	Observation time (*hr*)					
		24	48	72	24	48	72
E. agallocha							
	E1B1	-	-	-	+	+	+
	E1D3	-	-	-	+	+	+
	E2B1	+	-	-	+	+	-
	E2B4	+	+	+	+	+	+
	E2D2	+	+	+	+	+	+
	E2D3	-	-	-	+	-	-
	E2D4	-	-	-	+	+	+
A. ilicifolius							
	AB1	+	+	+	+	+	+
	AD2	-	-	-	+	+	-
	AD3	-	-	-	+	+	-
	AD4	-	-	-	+	+	+
	AD5	-	-	-	+	+	-
L. racemosa							
	LB1	+	+	-	-	-	-
	LB5	-	-	-	+	+	+
	LD2	+	+	-	+	+	-
	LD3	-	-	-	+	+	+
	LD5	-	-	-	+	+	+
	LB10	+	+	+	+	+	+
Negative Control		0±0	0±0	0±0	0±0	0±0	0±0

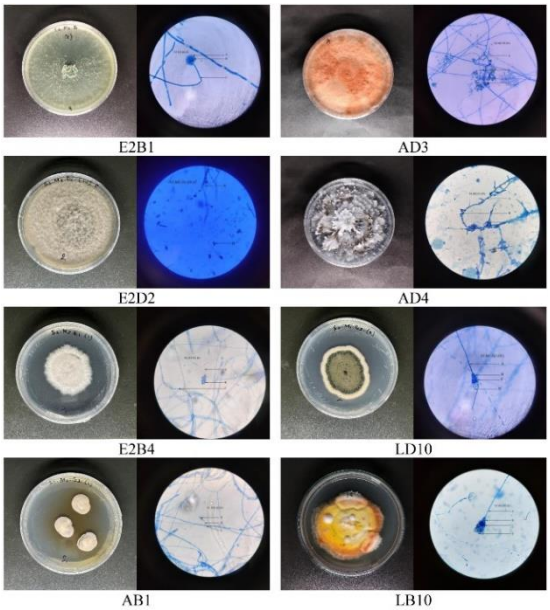

Figure 3. Microscopic view of isolates with antibacterial activity.

50 *mg/ml*$^{-1}$ (2.78±2.38 and 2.9±3.39 *mm*, respectively at 24 *hr*) and a minimal inhibition activity towards *S. aureus*. On the other hand, isolate E2D2 is limited to inhibiting *S. aureus* at each concentration up to 48 *hr*. Lastly, extract from isolate LB10 did not produce any inhibition zone towards both pathogen (data not shown). The antibacterial inhibition data is presented in table 2, while the *in vitro* imagery assay can be seen in figure 5.

Preliminary phytochemical screening

Phytochemical screening consisted of 5 metabolite detections, which included flavonoid, tannin, alkaloid, steroid, and saponin. Result shows that metabolite extract of AB1 contained alkaloid, E2D2 contained tannin and alkaloid, E2B4 contained flavonoid and alkaloid, while L10 only contained alkaloid. The screening result can be observed in table 3.

Wood degrading enzyme properties

Wood degrading enzyme assay was performed through semi-quantitative approach for enzymatic activity calculation. Cellulase result consists of 6 active isolates (Table 4), where isolate with codename LB5 produces enzymatic index as much as 1.40±0.49, making it the most productive cellulase producer. Within 6 active isolates, 3 were isolated from *A. ilicifolius* while the other 3 came from *E. agallocha*. The second and third enzyme are laccase and tannase, where there was only 1 isolate can actively produce laccase and 6 manage to produce tannase. Isolate with the codename of E2D2 is the only laccase producing isolate and manage to produce the highest productivity reaching 1.21±0.25 index value at 14 days of incubition. This isolate was obtained from *E. agallocha* and its extract manage to inhibit *S. aureus* growth. On the

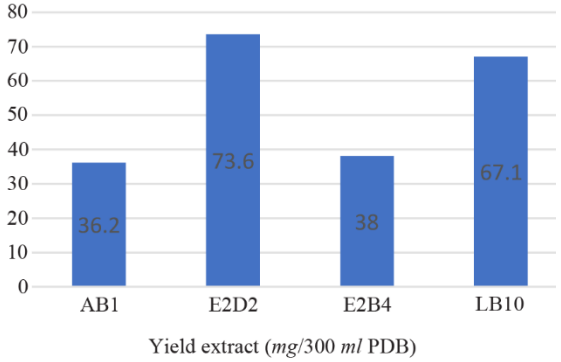

Figure 4. Crude extract dry weight of mangrove-associated fungi from mass culture.

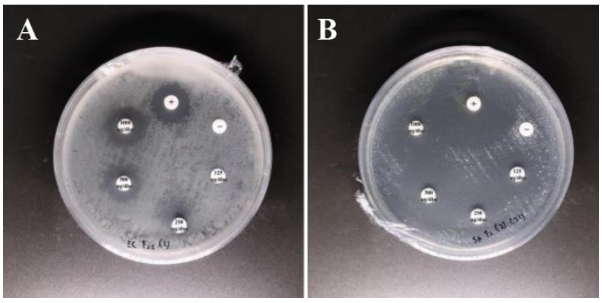

Figure 5. Antibacterial assay of mangrove-associated fungi extract of E2B4 against *E. coli* (A) and *S. aureus* (B).

other hand, isolate with the highest tannase productivity is AB1 with 1.26±0.15 at 7 days as the highest index value. This isolate comes from *A. ilicifolius*, which its extract apparently contains antibacterial activity against *E. coli*. For tannase, there were 3 isolates coming from *A. ilicifolius* and 3 others were isolated from *E. agallocha* proven to be able to produce tannase. The laccase and tannase activity can be seen in figures 6 and 7 respectively, while the index was provided in table 5.

The last enzymatic assay in this study is lignin peroxidase, where there were 5 isolates which managed to produce lignin peroxidase. The highest index productivity comes from isolate with codename of AB1, where it was capable to produce as much as 0.30±0.02 at 14 days. However, isolate LB10 managed to clear out all methylene blue contained in media. Due to punctiform colonies dispersal into whole media, the degradation of methylene blue size was just as much as the colony size, causing index value to stay at 0±0. Therefore, LB10 can be considered as the most productive lignin peroxidase producer in this research, which were isolated from the leaf of *L. racemosa*. Interestingly, lignin peroxidase result is the only enzymatic test where most of the active isolates were isolated from *L. racemosa*. The lignin peroxidase activity can be seen in figure 8 while the enzymatic index is served in table 6.

Isolate identification

DNA barcoding or species identification were conducted using ITS 1 (Internal Transcibe Spacer) gene marker. The *ITS* gene was successfully amplified and visualized in gel agarose (Figure 9). DNA barcoding and phylogenetic result shows 3 different identified isolates with the most promising potential, which are AB1 as *Phaeophleospora eucalypticola* (99.82%), E2D2 as *Curvularia lunata* (100%), and E2B4 as *Paraconiothyrium* sp. (99.30%). BLAST result details were presented in table 7. This result further supported by the phylogenetic tree (Figure 10), where sequence of isolate AB1, E2D2, and E2B4 were closely related to *P. eucalypticola*, *C. lunata*, and *Paraconiothyrium* sp., respectively.

Table 2. Antibacterial Assay of Crude Extract against *E. coli* and *S. aureus*

Pathogen test / Isolate code	Concentration (mg/ml^{-1})	*Escherichia coli*			*Staphylococcus aureus*		
		Inhibition Zone Diameter (*mm*)			Inhibition Zone Diameter (mm)		
		24 hr	*48 hr*	*72 hr*	*24 hr*	*48 hr*	*72 hr*
AB1							
	50	2.78±2.38	0.75±2.07	0.61±0.11	1.5±0.32	0.53±0	0.35±0.49
	25	2.9±3.39	2.18±0.02	2.1±2.54	0.73±1.03	0±0	0±0
	12.5	0±0	0±2.66	0±0	0±0	0±0	0±0
	6.25	0±0	0±0	0±0	0±0	0±0	0±0
E2D2							
	50	0±0	0±0	0±0	2.61±0.44	1.46±0.14	0±0
	25	0±0	0±0	0±0	2.15±0.54	1.31±0.02	0±0
	12.5	0±0	0±0	0±0	1.73±0.18	0.91±0.11	0±0
	6.25	0±0	0±0	0±0	1.66±0.14	0.35±0.49	0±0
E2B4							
	50	**6.1±1.55**	4.33±2.30	2.76±1.97	**22.28±1.59**	20.4±0.32	19.11±0.82
	25	4.43±2.40	2.43±1.60	1.5±2.12	20.65±1.10	18.38±1.24	18.05±1.39
	12.5	2.3±0.75	0±0	0±0	17±2.45	14.91±0.11	14.86±1.31
	6.25	0±0	0±0	0±0	10.83±2.45	5.33±7.54	0±0
C (+)	30	8.36±0.23	7.5±0.04	7.31±0.02	28.96±0.47	27.66±1.36	27.25±0.77
C (-)	0	0±0	0±0	0±0	0±0	0±0	0±0

Note: C(+): Positive control, Amoxicillin, C(-): Negative Control, blank DMSO.

Table 3. Phytochemical Screening Result

Isolate extract	Flavonoid	Tannin	Alkaloid	Steroid	Saponin
AB1	-	-	+	-	-
E2D2	-	+	+	-	-
E2B4	+	-	+	-	-
LB10	-	-	+	-	-

Table 4. Cellulase Enzymatic Index

Isolate code	Cellulase enzymatic index
AD3	0.42±0.45
E2B4	1.04±0.06
AD5	1.23±0.12
E1D3	1.21±1.39
LB5	1.40±0.49
LB10	0.31±0.44

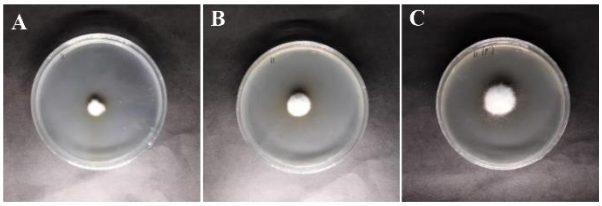

Figure 6. Laccase activity of isolate E2D2 with incubation time of (A) 2 days, (B) 7 days, and (C) 14 days.

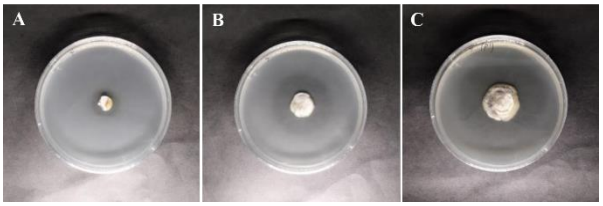

Figure 7. Tannase activity of isolate AB1 with incubation time of (A) 2 days, (B) 7 days, and (C) 14 days.

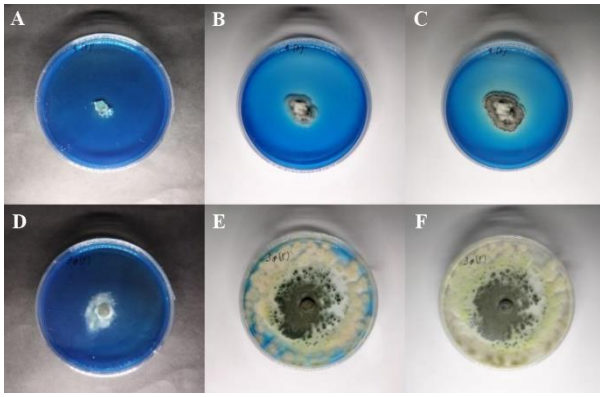

Figure 8. Lignin peroxidase assay with incubation period of 2, 7, 14 days of isolate (A-C) AB1 and (D-F) E2B4.

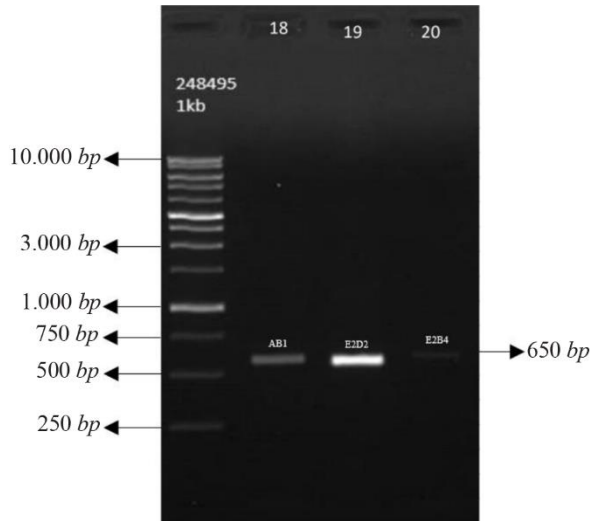

Figure 9. Its marker band in 0.8% agarose gel.

Discussion

This study evaluates the bioactivities of mangrove-associated fungi specifically evaluating the biodiversity, antibacterial activity, as well as wood-degrading enzyme activity through semi-quantitative approach. Insight associated with biodiversity of mangrove-associated fungi has been extensively studied, but it is still somewhat scarce for specific host mangroves. *A. ilicifolius*, *E. agallocha*, and *L. racemosa* are good example for the previous statement, where *A. ilicifolius* has been known to host a diverse number of species [47] while information regarding *E. agallocha* and *L. racemosa* has been quite limited [48-50]. Therefore, the result regarding total isolated fungi is critical to pinpoint the fungi diversity from the 3 mangrove species. A total of 43 different fungal isolates were isolated from the 3 mangroves, where leaf hosts more fungi than bark. The dominant number of isolates itself can be highly varied, which might be influenced by multiple factors such as the tree age, variation in sampling sites, or even dispersion difference in mangrove microbiome itself [51]. Moreover, *E. agallocha* was isolated 2 times to see how the same species but different individual can accommodate different number of fungi. There were multiple factors that can influence this phenomenon, such as age, heterogenicity, spatial differences of the trees, and the microbial dispersion [51]. Nevertheless, this research still lacks in that information, hence why it is important to highlight the possibilities for further research.

Antibacterial screening result proves the previous information regarding Gram-positive bacteria sensitivity towards antibacterial activity in comparison with

Table 5. Laccase and Tannase Enzymatic Index

Isolate code	Laccase assay			Tannase assay		
	2 Days	7 Days	14 Days	2 Days	7 Days	14 Days
AB1	0±0	0±0	0±0	1.01±0.07	1.26±0.15	1.20±0.16
E2D2	0.75±0.38	0.85±0.05	1.21±0.25	0±0	0±0	0±0
AD3	0±0	0±0	0±0	0.87±0.14	0.09±0.02	0.41±0.02
AD4	0±0	0±0	0±0	0±0	0.63±0.08	0.01±0.01
E1B1	0±0	0±0	0±0	0±0	0.09±0.04	0±0
E2D3	0±0	0±0	0±0	0.08±0.01	0.04±0.03	0.07±0.01
E2B4	0±0	0±0	0±0	0.69±0.01	0.44±0.05	0.35±0.18

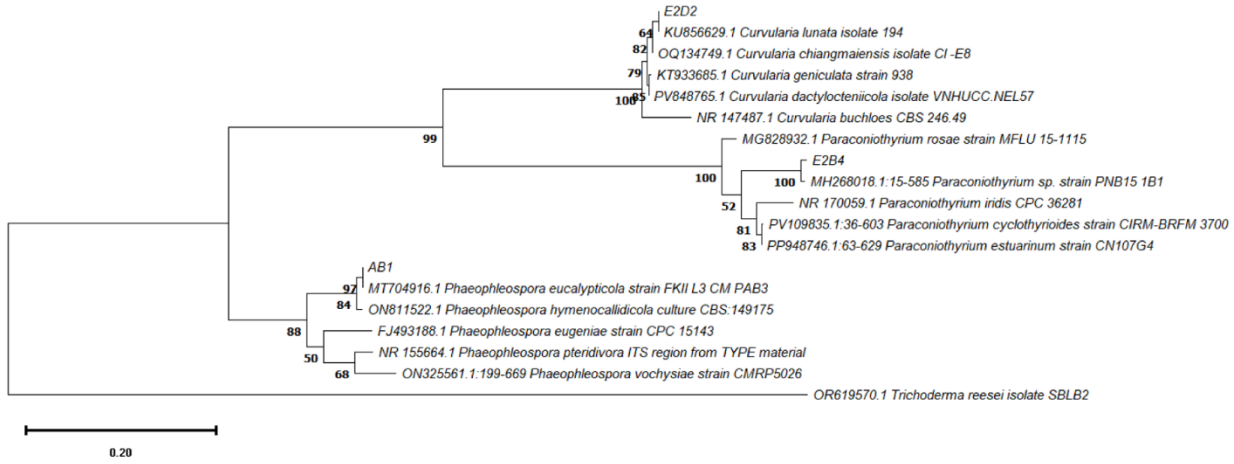

Figure 10. Phylogenetic tree of mangrove-associated fungi (MJ method, 1000 bootstrap, kimura 2-parameter model).

Table 6. Lignin peroxidase enzymatic index

Isolate code	2 days	7 days	14 days
LB1	0±0	0±0#	0±0#
AB1	0.0±0.00	0.24±0.08	0.30±0.02
LD2	0.24±0.08	0.18±0.05	0.05±0.00
E2B4	0.15±0.04	0.26±0.12	0.06±0.01
LB10	0±0	0±0#	0±0#

Note: *: Colony size exceeds the clear zone

Gram-negative bacteria. Gram-positive bacteria susceptibility is caused by the lack of Lipopolysaccharides (LPSs) in their outer membrane and instead possess thicker peptidoglycan, whereas LPSs act as a primary barrier in Gram-negative bacteria. Peptidoglycan does not confer the same restrictiveness to antibiotic penetration compared to LPSs and thus making Gram-positive bacteria more sensitive [52]. The result of antibacterial screening itself shows that some isolates manage to maintain the inhibition zone up to 72 *hr*, proving that some mangrove-associated fungi produced effective metabolites to inhibit the growth of pathogen within the time interval. This can be explained through its original ecosystem itself, where mangrove fungi live in such an extreme environment [53]. Mangrove ecosystem with multiple stress factors (such as high salinity, nutrient poor condition, and tide exposure) drives fungi that lived alongside mangrove to produce antibacterial compound that were capable to maintain the inhibition zone [54].

The screening result shows that 41.86% of total isolate were able to produce antibacterial activity. However, the remaining isolate with no inhibition zone cannot simply be considered as isolate with no antibacterial activity. The inactivity of fungi isolate can be affected by the antibacterial metabolite that were not able to diffuse into the media and making it unable to affect the growth of pathogen [55]. Furthermore, this research only highlighted the antibacterial activity towards *E. coli* and *S. aureus*. Some antibacterial products or metabolite can only inhibit a certain species of pathogens and it is still possible for other isolate to inhibit specific pathogen that were not tested in this study [56].

The microscopic morphological observation reveals the information regarding possible genus of isolates with positive antibacterial activity from previous screening. A total of 18 isolates were observed microscopically and characterized structure such as vesicles and conidiophores in several isolates (*e.g.*, AD3, E2D2, LB10, LB10) strongly suggests their affiliation with *Trichoderma*, *Curvularia*, and *Penicillium* like-taxa, which are among the most frequently encountered genera in mangrove habitats [57]. This result

Table 7. Sequence Species Identification Result through BLAST, NCBI

Host	Isolate code	Identified species	Query cover	Homology	Accession No.
A. ilicifolius	AB1	*Phaeophleospora eucalypticola*	98%	99.82%	PX758444
E. agallocha	E2D2	*Curvularia lunata*	99%	100%	PX758445
E. agallocha	E2B4	*Paraconiothyrium* sp.	100%	99.30%	PX758446

can be proven by species identification through DNA barcoding, where isolate E2D2 has the conidia structure of *Curvularia* sp. and later confirmed as *Curvularia lunata* through ITS-based identification. Other isolate such as LB10 and LD10 have monoverticillate branches, and visible traits such as phialide, conidium, branch, and stipe that strengthen the suggestion of both isolates being *Penicicillium* sp [58].

Metabolite extract by ethyl acetate solvent indicates the bioactivity profile of mangrove-associated fungi, especially AB1, E2D2, and E2B4, which later were proven by the phytochemical result. Among all the recorded result, metabolite extract of E2B4, later identified as *Paraconiothyrium* sp., managed to produce the highest inhibition zone against both pathogens, while other isolates were not able to produce the high result of inhibition zone with the same concentration (namely AB1 and E2D2). Antibacterial activity profile can be classified into weak (<5 *mm*), moderate (5-10 *mm*), strong (11-20 *mm*), and very strong (>20 *mm*) [55,59]. Based on the classification, extract of E2B4 can be considered as moderate towards *E. coli* and very strong against *S. aureus*. In addition, at concentration of 25, 12.5, and 6.25 mg/ml^{-1} managed to consistently preserve the inhibition zone up to 48-72 *hr*. The result itself generally had close inhibition zone size with positive control. However, due to significant difference in concentration, it is still quite incomparable and need further study such as MIC/MBC to subsequently confirm the capability of the extract [60]. Fractionation and compound purification can also be done to further enhance the purity and antibacterial capability.

Other extract, such as AB1 (*Phaeophleospora eucalypticola*) managed to produce inhibition zone with a weak inhibition zone against both pathogens, while E2D2 only managed to produce inhibition zone against *S. aureus*. Lastly, isolate LB10 did not manage to produce any form of inhibition in the assay. There were multiple causes that can influence this result, which includes incompatible nutrient, incubation time, and the need to optimize the environmental and medium condition such as temperature, salinity, as well as pH [61,62]. Previous study has already pinpoint how medium composition and pH can naturally affect the optimum antibacterial activity and should be considered for further study [61,63]. Solvents can also directly affect the possible extracted metabolite, since solvent works by binding the metabolite with the same polarity and ultimately affect the antibacterial capability [64].

Phytochemical screening result supports the previous antibacterial assay result, where isolates with the most promising activity, such as AB1, E2D2, and E2B4, show the main secondary metabolite presence including alkaloid, flavonoid, and tannin. These components are known to be essential in antibacterial activity because it disintegrate cell membrane, precipitate protein, and inhibit many essential enzymes produced by bacteria [65,66]. The occurrence of alkaloid in each active isolate indicates how these nitrogen-based compound might be the main contributor towards bacteriostatic and bacteriocidic activity of *S. aureus* and *E. coli* assay.

The detected flavonoid in E2B4 also tightly correlates with the promising inhibition activity in antibacterial assay [67]. This phytochemical is mainly functioning as antioxidant agent and capable to destroy bacterial lipid membrane through the complex creation of protein and phospholipid [67,68]. Moreover, the tannin found in extract of E2D2 can potentially increase the antibacterial synergistic effect through metal ion exhalation and deactivate bacterial adhesin [69,70]. This discovery is aligned with how mangrove-associated fungi tend to produce a wide variety class of secondary metabolite, including phenolic and aromatic where this compound plays crucial role in extreme environment and microbes competition [10,11].

The absence of saponins and steroids in the tested extract indicates that the biosynthesis of potential compounds such as triterpenoid and glycosidic compounds were not dominant under the culture condition. This finding opens new opportunities to reoptimize the fermentation medium and culture parameters to optimize the expression of latent biosynthetic pathway (mainly silent gene cluster), which are often found in marine endophytic fungi [71]. Thus, the combination of phytochemical profiling and antibacterial assay confirms many possibilities for mangrove-associated fungi of Jepara Regency as a natural source for bioactive compounds for pharmaceutical and marine biotechnology applications [72]. To further confirm the availability of specific metabolite, deeper analysis such as Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS) is needed [73].

The variety enzymatic of mangrove-associated fungi shows that the fungi have different physiological strategy to degrade lignocellulose component of wood, reflecting the functional heterogeneity within the fungal community itself. This finding is corresponded with previous research [74], which reported how man-

grove endophytic fungi serves an important role in the process of biomass restructuration through hydrolytic and oxidative enzymes, thereby supporting nutrient cycle in coastline ecosystem. However, although several isolates (*e.g*., AB1 and E2B4) exhibiting strong antibacterial activity also showed significant result in cellulase, tannase, or even lignin peroxidase, the present data demonstrate correlation rather than a direct casual relationship between enzymatic capability and antibacterial metabolite production [75]. The biosynthetic pathways of oxidative enzymes such as laccase and peroxidases are known to mediate the production of complex aromatic compounds such as quinones and oxidized phenols, which normally possess antibacterial bioactivity [76,77]. In addition, tannase enzymes were found in several isolates with capability to cleave the ester bonds of plant tannins into gallic acid and ellagic acid, where these two compounds do have high antibacterial as well as antioxidant activities [2,78]. Nevertheless, alternative mechanisms cannot be excluded, especially the independent regulation of secondary metabolite biosynthesis and non-phenolic antibacterial metabolite which is unrelated to lignocellulase enzyme [79]. Therefore, it is important to suggest a deeper analysis regarding the functional linkage, mainly metabolomic profiling and gene expression analysis to confirm how wood-degrading enzymes plays an important role in antibacterial formation [80]. Therefore, these findings highlight the ecological connection of enzymatic diversity in mangrove-associated fungi and noteworthy in terms of biotechnological relevance, while also underscoring the need to validate possible mechanism [81].

Among all the isolated fungi, 3 isolates have been significantly potential in antibacterial and enzymatic activity. The first isolate is AB1 as *Phaeophleospora eucalypticola* (99.82%), E2D2 as *Curvularia lunata* (*C. lunata*) (100%), and E2B4 as *Paraconiothyrium* sp (99.30%). Each of these isolates were widely known due to their biological properties. For instance, *C. lunata* is notably recognized to produce antibacterial metabolite due to gene cluster Nonribosomal Peptide Synthetase (NRPS) and Polyketide Synthase (PKS), which directly affect high production of antimicrobial [82]. Moreover, *C. lunata* is widely known to produce laccase enzyme, hence why *C. lunata* of this research completely corresponds with many previous researches [83]. *Paraconiothyrium* genus is also widely recognized to synthesize antibacterial metabolite that were effective against *E. coli* and *S. aureus*, while produce a wide variety of enzyme such as cellulase and lignin-degrading enzyme [84,85]. On the other hand, *Phaeophleospora eucalypticola* bioactivity still remains understudied, especially with the minimum record of antibacterial and enzymatic properties. Therefore, this research provides a new information regarding its capability to produce metabolite against *E. coli* and *S. aureus*, while at the same time produce some interesting enzyme such as tannase and lignin peroxidase.

All the isolates were identified based on ITS1 gene marker. ITS region, especially ITS1 and ITS2 are present between 18S and 28S rRNA, where this location is more diverse and suitable to identify a species of fungus [86,87]. Hence, ITS region is considered to be a perfect gene marker with taxonomic resolution up to species and genus level, making it a more proper choice for fungus identification [87]. However, ITS region still holds its own flaw, where there was closely related fungus share the same ITS sequence [87]. For example, *Aspergillus* and *Penicillium*, as well as *Cortinarius* and *Fusarium* need additional DNA markers to differentiate among closely related taxa [87,88]. Therefore, it is recommended to consider other gene marker or nomenclature such as IGS, TUB2, RPB1 and 2, TEF1, LNS2, PGK, SSU, TOP1, as well as COX1 and COX2 for species identification *via* DNA barcoding approach [88,89].

ITS-based identification provides further insight towards the taxonomic diversity and biogeographic profile of mangrove-associated fungi of Indonesia. Specifically, the results reveal the fungal species whose host associations and geographic distributions have been poorly investigated in previous studies. Several species show discrepancies with existing records, especially in terms of host association and regional occurrence. For instance, *C. lunata* (E2D2) has been isolated from multiple mangrove and plants in Indonesia [90,91]. However, to our knowledge, its association with *E. agallocha* has been quite limited [83,92,93]. Therefore, the results regarding bioactivity of *C. lunata* associated with *E. agallocha* provides valuable information for further research about *C. lunata* potential itself. The second species is *P. eucalypticola*, where this species has been detected to be an associative of *A. ilicifolius* in Taiwanese mangrove ecosystem through metabarcoding study [47]. Yet, to the best of our knowledge, information or published isolation records about *P. eucalypticola* from Indonesia is not yet well documented [47,94,95]. Finally, *Paraconiothyrium sp*. have been recognized as mangrove-associated fungal community [95]. However, the information regarding its isolation in Indonesia has not been reported, despite its possible availability in Indonesia [96]. Together, these findings suggest that mangrove-associated fungal diversity in Indonesia shows how much room of improvement that can be achieved in the Indonesian research of mangrove-associated fungi diversity, specifically characterization and uncovering possible bioprospection of these fungi.

Conclusion

To conclude, there were 43 isolated fungi from 3 species of mangrove from Awur Bay, Jepara Regency, Indonesia, where 27 fungi isolated from leaf and 16 others came from bark. This research proves that

mangrove-associated fungi of Jepara were indeed capable to actively produce antibacterial metabolites based on antibacterial screening, antibacterial assay, and phytochemical screening. Not only that, the isolates also positively produce wood-degrading enzymes including cellulase, laccase, tannase, as well as lignin peroxidase. Among every isolated fungus, isolates with the codename of AB1 (*Phaeophleospora eucalypticola*), E2D2 (*Curvularia lunata*), as well as E2B4 (*Paraconiothyrium* sp.) were proven to produce antibacterial as well as some wood-degrading enzyme. E2B4 (*Paraconiothyrium* sp.) produced the most effective antibacterial extract against test pathogens, especially towards *S. aureus* with the inhibition zone as much as 22.28±1.59 *mm*.

This research managed to point out a new information about the biological activity of *P. eucalypticola*, where the isolates can produce antibacterial activity against *E. coli* and *S. aureus* based on antibacterial assay. To the best of our knowledge, this is the first report of *P. eucalypticola* in Indonesia, *C. lunata* as *E. agallocha*-associated fungi, and *Paraconiothyrium* sp. as mangrove-associated fungi in Indonesia.

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Conflict of Interest

The authors declare no conflict of interest. This research did not receive any funding from commercials, public agencies, or non-profit organizations.

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