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Potential of fungi isolated from a mangrove ecosystem in Northern Sulawesi, Indonesia: Protease, cellulase and anti-microbial capabilities

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Abstract. ***Trianto A,*** *Radjasa OK, Subagiyo, Purnaweni H, Bahry MS, Djamaludin R, Tjoa A, Singleton I, Diele K, Evan D. 2021. Potential of fungi isolated from a mangrove ecosystem in Northern Sulawesi, Indonesia: Protease, cellulase and anti-microbial capabilities. Biodiversitas 22: 1717- 1724.* The high and relatively unexplored diversity of fungi present in the mangrove ecosystem represents a source of novel biotechnological importance. This study explored the potential of fungi isolated from the mangrove ecosystems to produce proteases and cellulases (commercially important enzymes) and their ability to inhibit pathogenic *Vibrio* species. Random samples of root, branch, leaf, sediments and litters were collected from 5 different mangrove sites in Manado, North Sulawesi, as a source of fungal isolates. The fungi were isolated on malt extract agar (MEA) and potato dextrose agar (PDA). The isolates were identified mainly based on the molecular methods (18S gene sequence) and examined for their ability to produce proteases, cellulases, and activity against several *Vibrio* species. Altogether 288 species of fungi were isolated from all samples. The fungi, isolated from leaves showed the highest diversity. A fungal isolate 19 Mba-C2-1 *Fusarium equiseti* from *Avicennia* sp. leaf showed the highest protease activity. While, the isolate 19 MT-05-3 *Hypocrea* sp. from sediment had the highest cellulase activity. From the root of *Rhizophora* sp., the isolate 19 MT-04-3 identified as *Trichoderma viride* had the strongest activity against a range of *Vibrio* species. This work indicates the high potential of fungi isolated from mangrove ecosystems as a source of commercially important enzymes and novel antimicrobial compounds.

Keywords: Aquaculture diseases, bioprospecting, Eurotiomycetes, fungal enzyme, internal transcribed spacer, Sordariomycetes

INTRODUCTION

Mangrove is an essential component of our ecosystems and has huge but relatively unexplored biodiversity–particularly fungal biodiversity. These fungi could have huge biotechnology potential for production of enzyme and antibacterial compounds. Cellulase and protease enzyme has promising biological prospects for discovering potential biocatalysts for use in hydrolysis of lignocellulosic materials and proteic residues. These enzymes can increase and ensure viable production of second-generation ethanol from different and alternative sources (Immaculatejeyasanta et al. 2011; Ramesh et al. 2014). *Vibrio* is a pathogenic-bacteria found in the environment and community with a high-risk infection (Igbinosa and Omoruyi 2016).

Manado is the largest coastal population in North Sulawesi, Indonesia, having 4.6% of 161 km^2 area under forest and mangrove. It is an interesting area to harboring the richness of microorganisms. Mangrove forests are uniquely valuable coastal wetlands in the transition zone between land and sea, which moderates freshwater flows from inland while coping with tidal inundation. They sustain millions of people globally, contributing to their survival and welfare through protection against coastal erosion, provision of food and material for construction and firewood, and through filtering of water-borne pollutants, which improves the water quality (Brown and Djamaluddin 2017; Djamaluddin 2018; Hadika and Karuniasa 2020). Mangrove forests are also globally important carbon sinks with carbon densities exceeding 8 times those typical for terrestrial tropical forests (Hossain 2016). They are considered as high priority habitats in climate change mitigation and adaptation strategies (Nehren et al. 2017; Indarsih and Masruri 2019). Mangrove fungi are known to be rich sources of enzymes and secondary metabolites with

various applications such as proteinase, cellulose, and antibacterial compounds (Sari et al. 2017; Maitig et al. 2018; Sibero et al. 2018). Many studies have shown the importance of using mangrove-derived fungi. The enzymes derived from mangrove-associated microorganisms have economic value for industrial and medical purposes. Previous study has shown that *Aspergillus niger*, *Halocyphina villosa*, and *Lignicola longirostris are* known to produce protease and cellulase (Immaculatejeyasanta et al. 2011).

Protease is an enzyme that performs proteolysis (protein catabolism by hydrolysis of peptide bonds). Proteases are used in industry, medicine, and daily life, e.g., in drug production, controlling blood clotting, as a substituent of detergents (Kamath et al. 2010). Proteases have been applied in environmental bioremediation of protein-polluted areas (through excess feeds) near fish or shrimp ponds to improve water quality (de Souza et al. 2015). Thus, proteases can also act as biocontrol of pathogens, such as *Vibrio*. Cellulase is an enzyme that can break the cellulose bonds into oligo, di, or mono-saccharides. It breaks cellulose through hydrolysis into simple saccharides called cellodextrin (Kelecom 2002). Unlike other compounds, cellulose is an abundant natural biopolymer on earth. Microorganisms, such as fungi, produce cellulase to degrade cellulose by hydrolyzing the glycoside linkages of cellulose. Previous studies have reported that six fungi such as *Acremonium* sp. *Alternaria chlamydospora*, *Alternaria* sp., *Aspergillus* sp., *Fusarium* sp., and *Pestalotiopsis* sp., isolated from mangrove root of *Avicennia marina*, to produce cellulase (Maria and Sridhar 2002).

Vibrio is one of the notorious pathogens, and a leading cause of shrimp and fish aquaculture disease. Bacteria from the genus *Vibrio* have caused enormous losses in the shrimp and fish industry due to mass death and slowing of the growth rate of the fingerlings. The *Vibrio* also infects people consuming raw or undercooked seafood as a case in Japan where a man has reported to be infected by *V. vulnificus* through fish (Li et al. 2018). The objective of this study was to explore the mangrove-associated fungi as sources of protease and cellulase enzymes and/or anti-*Vibrio* compounds.

MATERIAL AND METHODS

Location of sampling sites

The natural mangroves are naturally formed mangrove ecosystems, while the restoration mangroves are the mangrove forests that humans have replanted. Samples were collected from 9 to 11 April 2019, from two natural and three restoration sites viz. Likupang Restoration (MSr), 1°40'33.82"N/125° 3'17.24"E; Likupang Natural (MSn), 1°40'41.76"N/125° 3'14.20"E; Tiwoho Natural (MT), 1°35'57.01"N/124°51'32.25"E; Bawoho Restoration (MBa), 1°34'51.69"N/124°49'3.26"E; Buyat Restoration (MB), 0°50'57.01"N/124°42'27.56"E (Figure 1). The collected samples (leaf, branch, root, and sediments) were put into sterile plastic bags to avoid contamination and brought to the laboratory in a cool-box (4 °C) for further treatment.

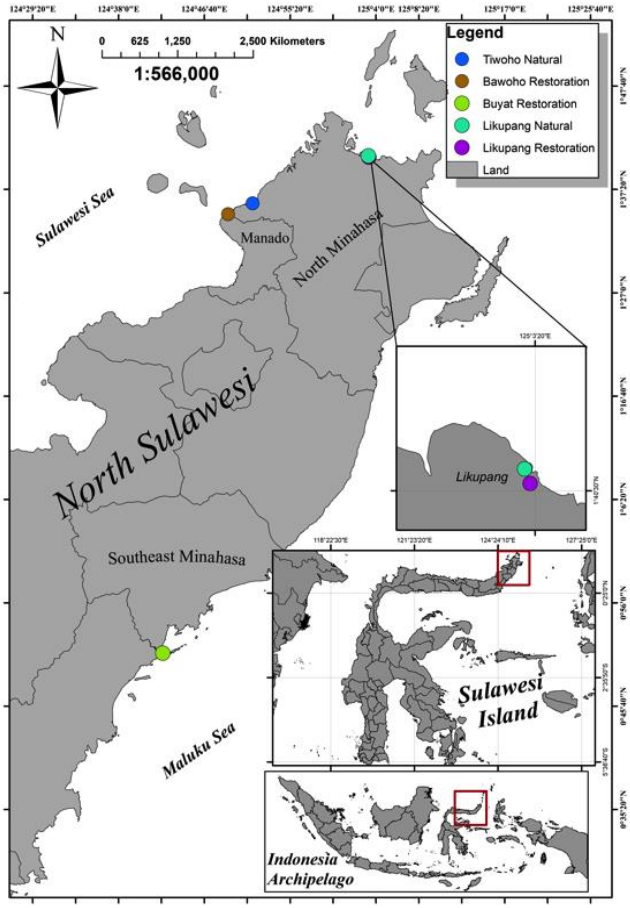

Figure 1. Map of the study areas in the Manado, North Sulawesi, Indonesia. Mangroves showing five sampling sites; Likupang Restoration, MSr (1); Likupang Natural, MSn (2); Tiwoho Natural, MT (3); Bawoho Restoration, MBa (4); and Buyat Restoration, MB (5)

Screening for cellulolytic and proteolytic of fungi

A total of 288 fungal isolates were tested for their ability to produce cellulolytic and proteolytic enzymes. For Proteolytic screening, the isolates were inoculated on PDA media supplemented with 1 % skimmed milk powder and incubated for 3-5 days at 30 °C. A clear zone around the colony indicated the presence of protease activity (Bonugli-Santos et al. 2015). Screening of cellulolytic activity was done by inoculating the fungi on the media (containing 1% peptone, 0.05% yeast extract, 1% Carboxymethylcellulose (CMC), and 3% bacteriological agar) and then incubated for 3–5 days at 30 °C, and transferred into a refrigerator (4 °C) for overnight. Cellulase activity was detected by the presence of clear zone around the fungal colony after the addition of Congo Red. The clear zone diameter was classified into weak (< 2 mm), medium (3-4 mm), and strong (>5mm) based on the proteolytic and cellulolytic activities of fungi.

Antibacterial assay

The *Vibrio* strains were chosen for antibacterial assay, which was conducted using the overlay method as used in the previous study (Trianto et al. 2019). The fungal isolates were inoculated on MEA media in triplicate. After the

growth of isolates which usually takes 1-7 days depending upon the growth rate, the *Vibrio* containing soft agar was poured onto the plates. The soft agar was composed of (0.3% (w/v), nutrient broth, 1% (w/v) NaCl and 0.7% (w/v) agar), containing one of the indicator strains with concentration of 0.5 McFarland. The following strains were used for antibacterial testing: *Vibrio harveyi, V. vulnificus*, and *V. parahaemolyticus.* The plates were incubated at the optimum temperature for bacterial growth (37 ± 2 °C) for 24h. The anti-*Vibrio* activity was defined by the presence of clear zones around the bacterial isolates. The clear zone diameter formed was also used to classify the antibacterial activity of fungi into weak (< 2 mm), medium (3-4 mm), and strong (>5mm).

Molecular identification of the active fungi

The DNA of active isolates was extracted using the Zymo DNA kit. The universal primer, internal transcribed spacer (ITS) was used for the fingerprint region for fungal barcoding using polymerase chain reaction (PCR) thermal cycler. The PCR mix contained GoTaq® Green Master Mix 12.5µl, ITS 1 primer 0.25–2.5µl, ITS 4 primer 0.25–2.5µl, DNA template 1-5 µl, Nuclease-Free Water to total 25µl. The thermal cycler setting used was denaturation at 95°C for 1 min; 34 cycles of denaturation at 95°C for 3 min, annealing at 56.1°C for 1 min, extension at 72°C for 1 min; last extension at 72°C for 7 min and cooling at 4°C until recovery of the samples. The PCR products were visualized by electrophoresis process, and sequencing was undertaken at Genetika Science, Jakarta Indonesia, and continued to 1st Base, Malaysia. The results were compared with other sequences in the NCBI database using BLAST. The phylogenetic tree of sequence results was constructed by MEGA 7.0 (Kumar et al. 2016).

RESULTS AND DISCUSSION

Fungal isolates

A total of 288 fungal isolates were collected from 5 different locations (Figure 1) having vegetation of four genera of mangrove, viz. *Sonneratia* sp., *Rhizophora* sp., *Avicennia* sp., and *Lumnitzera* sp. Figure 2 showed the mangrove vegetation in North Sulawesi.

Isolation of mangrove-associated fungi

The total number of samples collected was 84 from 5 sites (Figure 1), which is shown in Figure 3. The highest number of samples was collected from Tiwoho Natural with 29 samples, followed by Likupang Restoration and Buyat Restoration locations, from which 17 and 16 samples were collected, respectively. Tiwoho Natural area was the most interesting location with respect to the diversity of mangrove samples obtained. This area is part of Bunaken National Park that is a protected mangrove area. The total number of fungi that were successfully isolated was 288 isolates. The highest amount of fungi was 96 isolates from Tiwoho Natural. The ratio from 5 locations (MSr, MSn, MT, MBa, MB) showed that the ratio of fungal association to the number of the sample was to be around 2.6 to 3.7. Interestingly, the highest ratio of 3.7 was observed in Buyat Restoration.

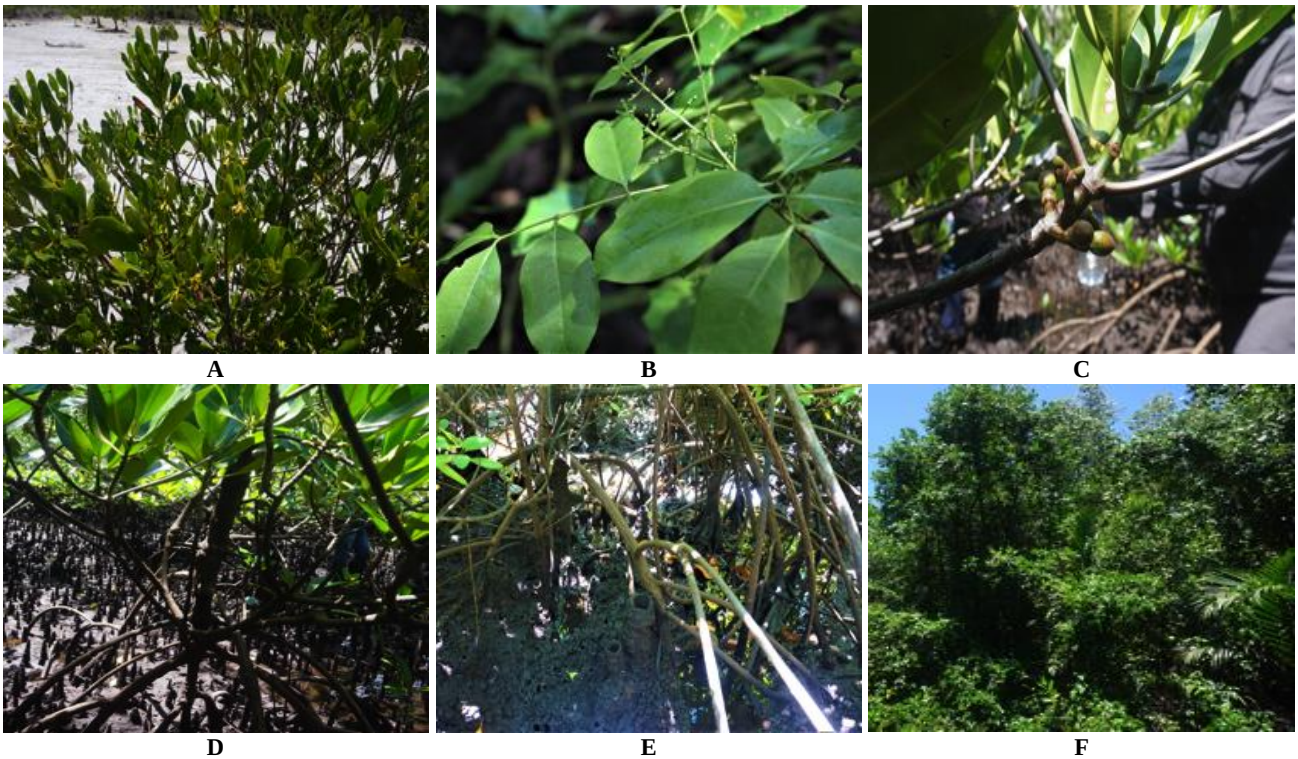

Figure 2. Samples of the study areas in Manado, North Sulawesi, Indonesia. A. *Sonneratia* sp., B. *Rhizophora* sp., C. *Lumnitzera* sp., D. Root and sediment of *Avicennia* sp., E. Litters, F. Mangrove forest

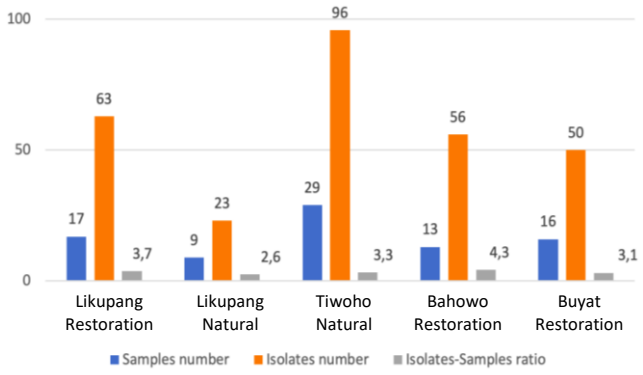

Figure 3. The number of samples and corresponding isolates collected from several locations in North-Sulawesi and their isolate-sample ratio

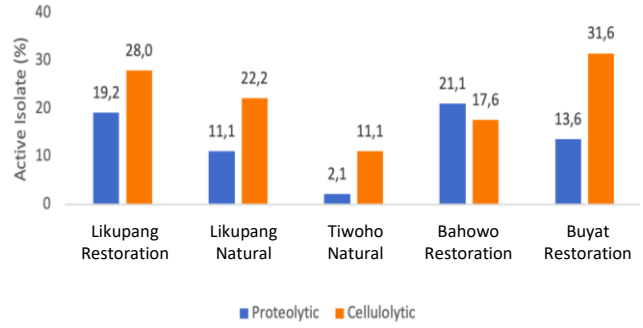

Figure 4. The fungal isolate (%) having cellulolytic and proteolytic activities

The cellulolytic and proteolytic activities

It was observed that the fungal isolates had cellulolytic and proteolytic activities, as shown in Figure 4. The percentage of the proteolytic enzyme was 2.1% to 21.1%. Moreover, the range of cellulolytic enzyme was identified at 11.1% to 31.6%. Each location had distinct potential activity in terms of enzyme production. The location in Bahowo restored and Buyat Restoration had higher production of the enzyme with proteolytic and cellulolytic activities respectively, than other places. Tiwoho Natural ranked lowest in terms of enzyme production. Two other locations, Likupang Natural and Bahowo Restored had a similar result of proteolytic enzyme in range 11.1 to 21.1% and cellulolytic enzyme in range 17.6 to 22.2%.

The fungi isolated from leaves were found to be the most potent sources of the enzymes, where 33.3% of them produced protease and cellulase (Figure 5). Followed by roots, whose isolates produced 25% cellulases and 20% of proteolytic enzyme. Branches produced 5.6% of cellulases and 11.1% of proteolytic enzyme, moreover, sediment isolates produced 26.7% of cellulases and 6.7% of proteolytic enzyme, while, isolates from litters did not have any proteolytic activity.

Antibacterial assay

The result of antimicrobial activity of mangrove-fungi associated with *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* was shown in Figure 6 and Figure 7. Based on the collection sites, most of them had antimicrobial activity with varying potential. The highest anti-*Vibrio* activity (47.6%) was represented by fungal isolate from Bawoho Restoration which had antimicrobial activity against *V. harveyi*, While, fungal isolates from Likupang Natural had the highest antimicrobial activity (20%) against *V. vulnificus*. A total 45.1% of fungal isolate from Tiwoho Natural had highest antimicrobial activity against *V. parahaemolyticus*. Then, based on the fungal association of the part of mangrove (Figure 7), it was known that most of them had strong antimicrobial activity against *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus*. There is no antimicrobial activity against *V. vulnificus*, and *V. parahaemolyticus* as observed on the fungal isolates from Litters. The strongest activity was investigated on fungi from sediment against *V. harveyi* at 45.5%.

Table 1. Isolate source, BLAST identified potential species and biological activity of fungi associated mangrove as proteolytic, cellulolytic and anti-*Vibrio*

Isolate code	Source of mangrove part	Biological activity			Identified species	ACC number
		Proteolytic	Cellulolytic	Anti-*Vibrio*		
19 Mba-C2-4	Leaf of *Avicennia* sp.	+			*Pestalotiopsis theae*	AY924274.1
19 Mba-C2-1	Leaf of *Avicennia* sp.	+			*Fusarium equiseti*	KT459349.1
19 Mba-C1-1	Branch of *Avicennia* sp.			+	*Penicillium citrinum*	KT844552.1
19 MSr-B3-4	Root of *Sonneratia* sp.	+	+		*Fusarium equiseti*	MF471699.1
19 MSr-B3-5	Root of *Sonneratia* sp.	+			*Pestalotiopsis microspora*	KT459349.1
19 MB-B7-4	Litters *Rhizophora* sp.		+		*Nigrospora sphaerica*	KC505176.1
19 MSr-B2-3	Leaf of *Sonneratia* sp.		+		*Hypocrea jecorina*	MN310399.1
19 MT-10-2	Leaf of *Rhizophora* sp.		+		*Aspergillus aculeatus*	MH892845.1
19 MT-04-3	Root of *Rhizophora* sp.			+	*Trichoderma viride*	MK841023.1
19 MSr-C4-3	Sediment		+		*Diaporthe stewartii*	KU204517.1
19 MT-05-3	Sediment		+		*Hypocrea* sp.	MG711900.1

Noted: Likupang Restoration (MSr); Likupang Natural (MSn); Tiwoho Natural (MT); Bawoho Restoration (MBa); Buyat Restoration (MB)

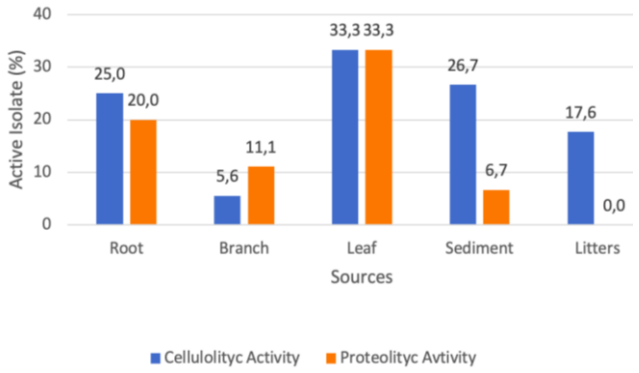

Figure 5. The fungal isolate (%) from various samples having cellulolytic and proteolytic activities

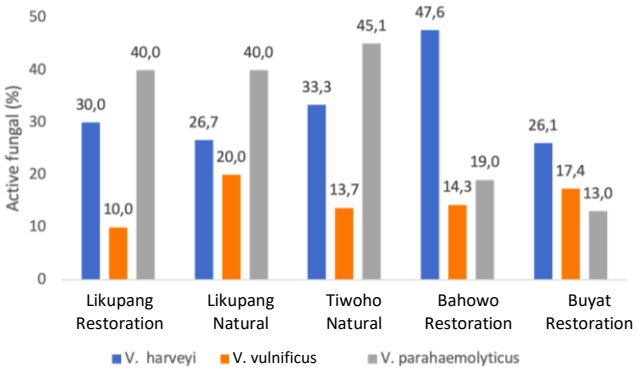

Figure 6. Site wise fungal isolate (%) active against *Vibrio harveyi*, *V. vulnificus*, and *V. parahaemolyticus* (%)

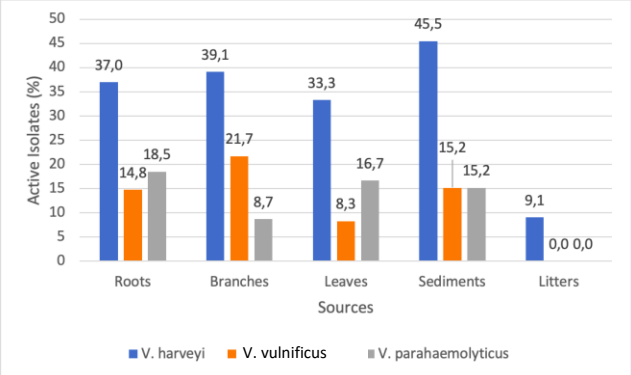

Figure 7. Source-wise fungal isolate (%) active against *Vibrio harveyi*, *V. vulnificus*, and *V. parahaemolyticus*

Molecular identification of fungal isolates

Phylogenetic trees were build using maximum likelihood method with bootstrap replicates of 1000 in MEGA 7.0.26 bioinformatics software. The tree represents phylogenetic diversity between restoration area and natural area. The accession number of each species was written in bold and italic style right after the species name. the scale bar at the bottom of the figure represents the distance of evolutionary sequence.

Biological activity of isolated fungi

The proteolytic and cellulolytic activity as well as anti-*Vibrio* activity of the fungal isolates, their BLAST identified potential species, source of mangrove parts are shown in Table 1. *Pestalotiopsis theae* and *Fusarium equiseti* isolated from leaf of *Avicennia* sp., *Fusarium equiseti*, *Pestalotiopsis microspora PKT2*, and *Fusarium equiseti* isolated from root of *Sonneratia* sp. were the five fungi which had proteolytic activities. The genus *Pestalotiopsis* causes leaf spots, petiole/rachis blights, and sometimes a bud rot of palms. In other words, unlike the other leaf spot and petiole blight pathogens, which attack either the leaf blade or the leaf petiole, *Pestalotiopsis* attacks all parts of the leaf from base to tip (Elliott 2018). *Fusarium equiseti* is commonly found in tropical and subtropical areas, and is considered to be a weak pathogen on cereals and is occasionally to be associated with fusarium head blight infected kernels. Isolation of *Fusarium* species in greater number and frequency may be due to the high nutrient level in the mangrove ecosystem (Selvi and Sivakumar 2013).

Discussion

Mangrove ecosystems are a relatively unexplored source of fungal diversity and these fungi represent a potential important commercial reservoir of novel enzymes and compounds with novel activities e.g. new antibiotics, larvacides, etc. (Thatoi et al. 2013; Pringgenies et al. 2018; Sibero et al. 2018). Bonugli-Santos et al. 2015 reported that mangrove associated fungi are endowed with rich sources of enzymes with biotechnological application, such as hydrolytic and/or oxidative enzymes, with alginate lyase, amylase, cellulase, chitinase, glucosidase, inulinase, keratinase, ligninase, lipase, nuclease, phytase, protease, and xylanase.

This work focused on isolating fungi from natural and restored mangrove systems in Northern Sulawesi and it was thought that the different sites could harbor different species due to variation in mangrove species, tidal systems, and restoration methods used. A total of 288 species with the highest diversity found in Tiwoho Natural. Fungi in classesSordariomycetes and Eurotiomycetes were among the most prevalent in mangrove environments which is in agreement with previous studies (Lee et al. 2019). Previous study has reported the diversity of fungi associated with different parts such as fruits, leaves, pneumatophores, and sediments. They reported that fruit and leaf is the highest amount. On average, our study showed that fungi isolate produced protease and cellulose enzymes. The fungal association from leaf showed the highest production of proteolytic and cellulolytic enzymes (Figure 5). Mangrove fungal cellulolytic activity is reported to be affected by environmental conditions (pH, temperature, substrates), fungal community, and culture conditions (Hossain 2016). Fungi that have proteolytic activity have the ability to produce protease enzymes that are secreted into their environment. The proteolytic enzyme works to hydrolyze protein compounds into oligopeptides, short-chain peptides and amino acids. This extracellular protease enzyme is very important for bacterial life because it provides nitrogen

compounds that can be transported into cells. The types of fungi that can secrete protease have great potential to be used as a source of aquaculture probiotics, especially in shrimp farming (Setyati et al. 2016).

Our study showed Likupang has complex aquatic dynamics. Likupang-restored mangrove area had abandoned shrimp pond with water rich in protein and cellulose from the waste of shrimp and unconsumed feed. The abundance of pollutant sources from the ponds provides a source of nutrients needed by microbes, therefore Likupang-restored had a high number of isolates and isolate-sample ratio (Saiya and Katoppo 2015; Ruete et al. 2016). Moreover to the contrary, it was surprising that in Likupang-Natural has the lowest ratio of isolates to samples. Sridhar and Seetharam (2001) reported loss of over 300 species of aquatic hyphomycete species due to negative effect of polluted water on fungal diversity. In laboratory experiments, water pollutants that contain low concentrations of Cd, Cu, and Zn have been shown to inhibit growth and reproduction of aquatic hyphomycetes and fungi respond by synthesizing specific stress peptides (Krauss et al. 2011).

Mangrove leaves were the best source of fungal isolates producing protease and cellulase enzymes (Figure 5). The study of Chi et al. (2019) revealed that fungi associated with leaves of mangrove had a highly diverse fungal community, where a total of 110 taxa were recovered from isolation and metabarcoding methods; among them, Ascomycota was dominant, which includes *Corynespora cassiicola* (6.90%), *F. oxysporum* (6.40%) and *Guignardia* sp. (6.40%). Only specifically fungal isolates sourced from sediments reported anti-*Vibrio* activity. The fungi that live in seawater sediment are reported to grow well than in freshwater. Sediment nutrient content is affected by physiology and environment among the mangrove vegetation. Previous studies have reported that fungi from different locations, bottom sediment and depth, identified Phycomycetes, Ascomycetes, and Deuteromycetes (Sivakumar 2013).

Fungi use mechanisms like extracellular precipitation, valence transformation and active uptake (e.g. bio-sorption to cell wall and pigments, intracellular compartmentation, complexation and crystallization, and sequestration) and therefore could be used to degrade, accumulate or remove metal pollutants. Thus, screening of metal tolerant fungi has the potential of providing strains with improved metal accumulation. However, the search for microorganisms capable of metal bio- sorption and sequestration has mainly focused on contaminated sites (Ojuederie and Babalola 2017; Igiri et al. 2018).

An interesting fungi *Hypocrea jecorina* have been found in litters of *Rhizophora* sp. with cellulolytic enzymatic activity. *Hypocrea jecorina* (anamorphic *Trichoderma reesei*) is a saprophyte noted for its ability to abundantly secrete native hydrolytic enzymes. These enzymes are used in various industrial applications, such as pulp and paper production, and the food and feed industries, and the textile industry (Steiger et al. 2011). *Penicillium citrinum* and *Trichoderma viride* have been successfully isolated from branch of *Avicennia* sp. and root of *Rhizophora* sp., respectively.

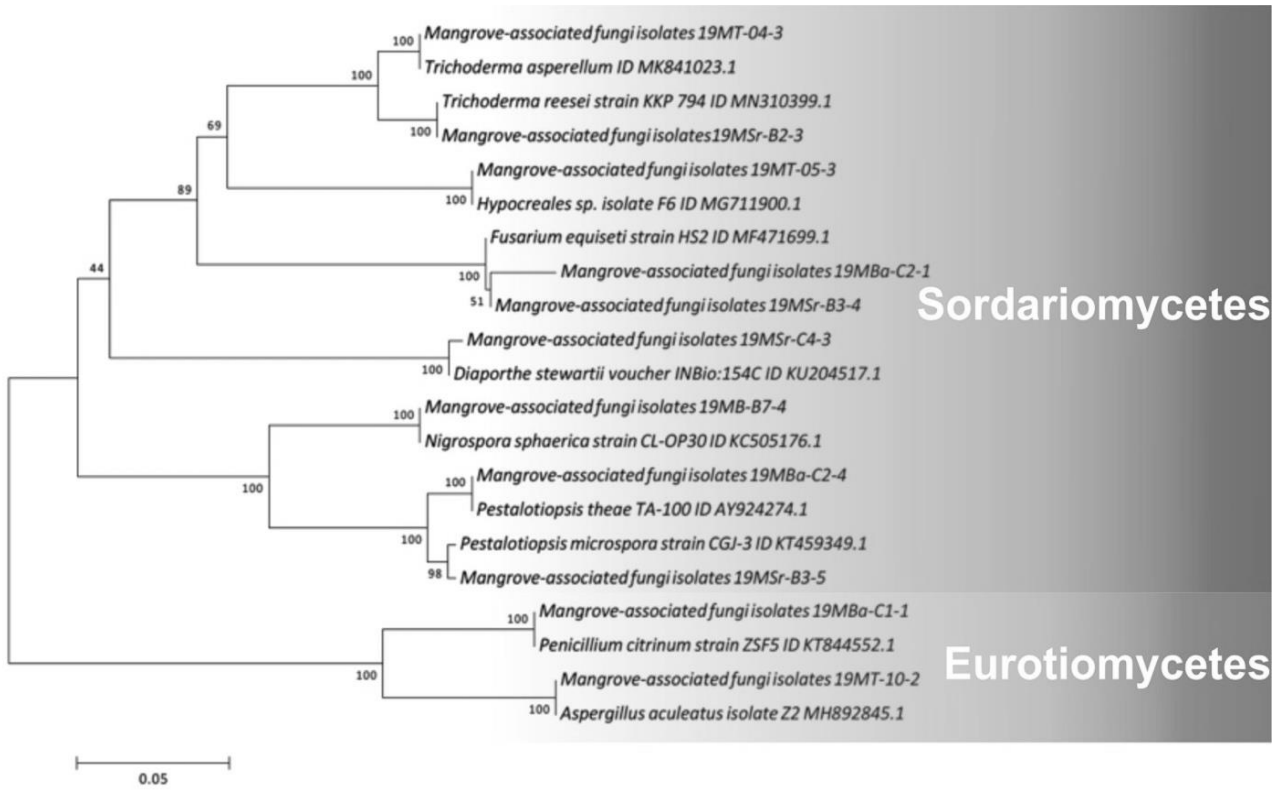

Figure 8. Phylogenetic trees constructed with sequence of active isolates along with BLAST derived sequences

We showed anti-*Vibrio* activity of *Penicillium citrinum* and *Trichoderma viride*. Previous study has shown four new compounds, penicitrinone E, penicitrinol J, penicitrinol K, and citrinolactone D, that were isolated together with six known compounds from the marine-derived *Penicillium* sp. ML226 (Wang et al. 2013). While, penicitrinone E, penicitrinol J and penicitrinol K showed modest selective cytotoxicity against HepG-2 cell line, citrinolactone D showed weak cytotoxicity against HepG-2 and HeLa cell lines. penicitrinol J and penicitrinol K also showed mild antimicrobial activity against *Staphylococcus aureus* (Wang et al. 2013).

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