

## KORESPONDENSI PAPER

**JUDUL** : POTENTIAL OF FUNGI ISOLATED FROM A MANGROVE ECOSYSTEM IN NORTHERN SULAWESI, INDONESIA: PROTEASE, CELLULASE AND ANTI-MICROBIAL CAPABILITIES

**JURNAL** : Biodiversitas

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1	Manuscript submission	17 Januari 2021	Messages: Comments for the Editor <ul style="list-style-type: none"><li>Cover letter</li><li>Submit manuscript</li></ul>	2 3 4-15
2	Manuscript sent for review	19 Februari 2021	Notifications: Editor Decision <ul style="list-style-type: none"><li>Response from Editor</li></ul>	16
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Note	From
<p>Dear Editor of Journal Biodiversitas,</p> <p>We are submitting a manuscript entitled: Biotechnological potential of fungi isolated from a mangrove ecosystem in Northern Sulawesi: protease, cellulase, and anti-microbial capabilities. The manuscript is not under consideration of any journal. Hopefully, it can be accepted.</p> <p>Best regards,</p> <p>Agus</p>	<p>atrianto 2021-01-17 11:48 PM</p>

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## COVERING LETTER

Dear **Editor-in-Chief**,

I herewith enclosed a research article,

**Title:**

Biotechnological potential of fungi isolated from a mangrove ecosystem in Northern Sulawesi : protease, cellulase and anti-microbial capabilities

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Semarang, 12 May 2020

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(fill in your name, no need scanned autograph)

Agus Trianto

1                   **Biotechnological potential of fungi isolated from a mangrove**  
2                   **ecosystem in Northern Sulawesi : protease, cellulase and anti-**  
3                   **microbial capabilities**

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19                   Manuscript received: DD MM 2016 (Date of abstract/manuscript submission). Revision accepted: ..... 2016. (8 pt)

20                   **Abstract.** The high and relatively unexplored diversity of fungi present in the mangrove ecosystem represents a source of novel biotechnological  
21                   importance. This study explored the potential of fungi isolated from the mangrove ecosystems to produce proteases and celluloses (commercially  
22                   important enzymes) and their ability to inhibit pathogenic *Vibrio* species. Random samples of root, branch, leaf, sediments, and litters were collected  
23                   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  
24                   potato dextrose agar (PDA). The isolates were identified mainly based on the molecular methods (18S gene sequence) and examined for their ability  
25                   to produce proteases, cellulases, and activity against several *Vibrio* species. Altogether 288 species of fungi were isolated from all samples. The fungi  
26                   originally from leaves showed the highest diversity. A fungal isolate 19 Mba-C2-1 *Fusarium equiseti* was isolated from *Avicennia* sp. leaf showed the  
27                   highest protease activity. While, the isolate 19 MT-05-3 *Hypocreales* sp. isolated from sediment has the highest cellulase activity. From the root of  
28                   *Rhizophora* sp., the isolate 19 MT-04-3 identified as *Trichoderma viride* having the strongest activity against a range of *Vibrio* species. This  
29                   preliminary work indicates the high potential of fungi isolated from mangrove ecosystems as a source of commercially important enzymes and novel  
30                   antimicrobial compounds.

31                   **Keywords:** Fungi, mangrove, protease, cellulose, anti-microbial activity  
32

## INTRODUCTION

Mangrove is an essential component of our ecosystems have a huge but relatively unexplored diversity – particularly relevant for fungal biodiversity. These fungi could have huge biotechnology potential as producing enzyme and as antibacterial compound. Cellulose and protease enzyme has bioprospecting as a promise strategy for the discovery of potential biocatalysts for use in hydrolysis of lignocellulosic materials as well as proteic residues. These enzymes can increment and turn viable the production of second generation ethanol from different and alternative sources (Immaculatejeyasanta et al. 2011, Ramesh et al. 2014). *Vibrio* is pathogenic bacteria that found in environment and community that have a high risk infection (Igbiosa and Omoruyi, 2016).

Manado is the largest coastal population in North Sulawesi, Indonesia, with covered 4.6% of 161 km<sup>2</sup> of the total area is included the forest and mangrove. It is an interesting area to expose the richness of microorganisms. Mangrove forests are uniquely valuable coastal wetlands in the transition zone between land and sea, moderating 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 Djameluddin 2017, Djameluddin 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 (Lusi et al. 2017, Maitig et al. 2018, Sibero et al. 2018). Many researchers have shown the importance of using mangrove-derived fungi. The enzymes derived from mangrove-associated microorganisms have economic value in industrial and medical. Previous research has shown that *Aspergillus niger*, *Halocyphina vilosa*, and *Lignicola longirostis* is known to produce protease and cellulose (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 drug 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 able to break the cellulose bonds become oligo, di, or mono-saccharides. It breaks cellulose through a hydrolysis reaction into simple saccharides called cello dextrin (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 reported that six fungi such as *Acremonium* sp., *Alternaria chlamydospora*, *Alternaria* sp., *Aspergillus* sp., *Fusarium* sp., and *Pestalotiopsis* sp., isolated from mangrove root of *Avicenia marina*, produce cellulases (Maria and Sridhar 2002).

*Vibrio* is one of the notorious pathogens since its leading cause of shrimp and fish aquacultures disease. Bacteria from the *Vibrio* genus have caused enormous losses in the shrimp and fish industry due to mass death and slower the growth rate of the cultivan. 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* originally from fish (Li et al. 2018). The objective of this study is to explore the mangrove-associated fungi as sources of protease and cellulases enzymes and/or anti-*Vibrio* compounds.

## MATERIAL AND METHODS

### Sampling sites location

The natural mangroves are naturally formed mangrove ecosystems, while the restoration mangrove is mangrove forests that have been replanted by humans. Samples were taken on 9 to 11 April 2019, we selected two natural and three restoration sites: Likupang Restoration (**MSr**), 1°40'33.82"N/125° 3'17.24"E; Likupang Natural (**MSn**),

82 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),  
83 1°34'51.69"N/124°49'3.26"E; Buyat Restoration (MB), 0°50'57.01"N/124°42'27.56"E (Fig. 1). The collected  
84 samples (leaf, branch, root, and sediments) were put into sterile plastic bags avoid the contamination and brought to  
85 the laboratory in a cool-box (4°C) for further treatment.

86

#### 87 **Screening for cellulolytic and proteolytic of fungi**

88 A total of 288 fungal isolates was tested for their ability to produce cellulolytic and proteolytic enzymes.  
89 Proteolytic screening, the isolates were inoculated on PDA media supplemented with 1 % skimmed milk powder  
90 and incubated for 3-5 days at 30 °C. A clear zone around the colony indicates the presence of protease activity  
91 (Bonugli-Santos et al. 2015). Screening of cellulolytic was done by inoculating the fungi on the media (contained  
92 1% peptone, 0.05% yeast extract, 1% Carboxymethylcellulose (CMC) and 3% agar bacteriological) and then  
93 incubated for 3–5 days at 30 °C, and transferred into a refrigerator (4 °C) for overnight. Cellulose activity was  
94 detected by the presence of clear zone around the fungal colony after the addition of Congo Red. Base on the clear  
95 zone diameter formed the proteolytic and cellulolytic of fungal activities were classified into weak (< 2 mm),  
96 medium (3-4 mm), and strong (>5mm).

97

#### 98 **Antibacterial assay**

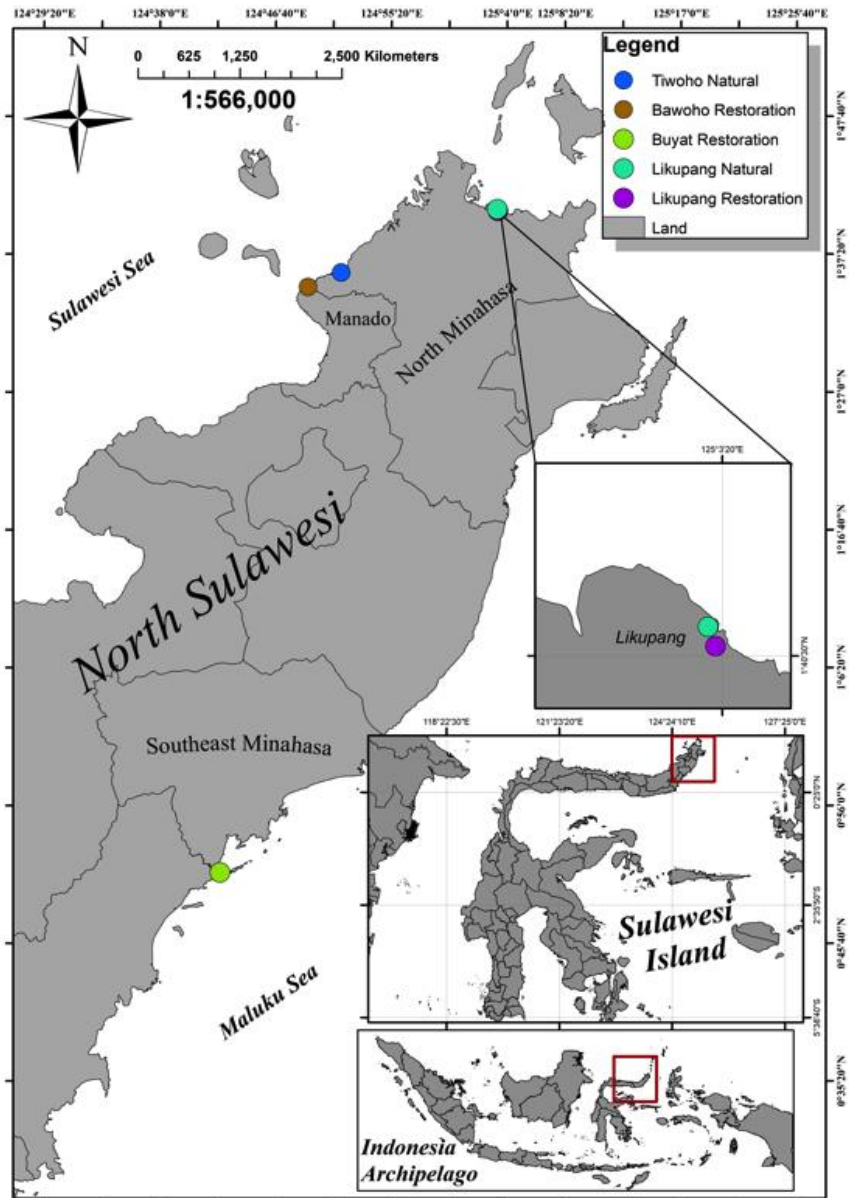
99 The *Vibrio* strains were chosen for antibacterial assay, which conducted using the overlay method as used in the  
100 previous method (Trianto et al. 2019). The fungal isolates were inoculated on MEA media in triplicate. After the  
101 isolates grew (depend on the growth rate, usually 1-7 days), the *Vibrio* containing soft agar were poured onto the  
102 plates. The soft agar consists of (0.3% (w/v), nutrient broth, 1% (w/v) NaCl and 0.7% (w/v) agar), containing one of  
103 the indicator strains with concentration was 0.5 McFarland. The following strains were used for antibacterial testing:  
104 *Vibrio harveyi*, *V. alginolyticus*, and *V. anguillarum*. The plates were incubated at the optimum temperature for  
105 bacterial growth ( $37 \pm 2^\circ\text{C}$ ) for 24h. The anti-*Vibrio* activity was defined by the presence of clear zones around the  
106 bacteria isolates. The clear zone diameter formed was also used to classify the antibacterial of fungal activity into  
107 weak (< 2 mm), medium (3-4 mm), and strong (>5mm).

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#### 109 **Molecular identification of the active fungi**

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111 The DNA of active isolates was extracted using the Zymo kit DNA. The universal primer, internal transcribed  
112 spacer (ITS) was used for the fingerprint region for fungal barcoding using polymerase chain reaction (PCR)  
113 thermal cycler. The mix PCR contained GoTaq® Green Master Mix 12.5µl, ITS 1 primer 0.25–2.5µl, ITS 4 primer  
114 0.25–2.5µl, DNA template 1-5 µl, Nuclease-Free Water to total 25µl, The thermal cycler setting was denaturation at  
115 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  
116 min; last extension at 72°C for 7 min and cooling at 4°C until recovery of the samples. The PCR products were  
117 visualized by electrophoresis process, and sequencing was undertaken at Genetika Science, Jakarta Indonesia, and  
118 continued to 1st Base, Malaysia. The results were compared with other sequences in the NCBI database using  
119 BLAST. The phylogenetic tree of sequence results was constructed by MEGA 7.0 (Kumar et al. 2016).

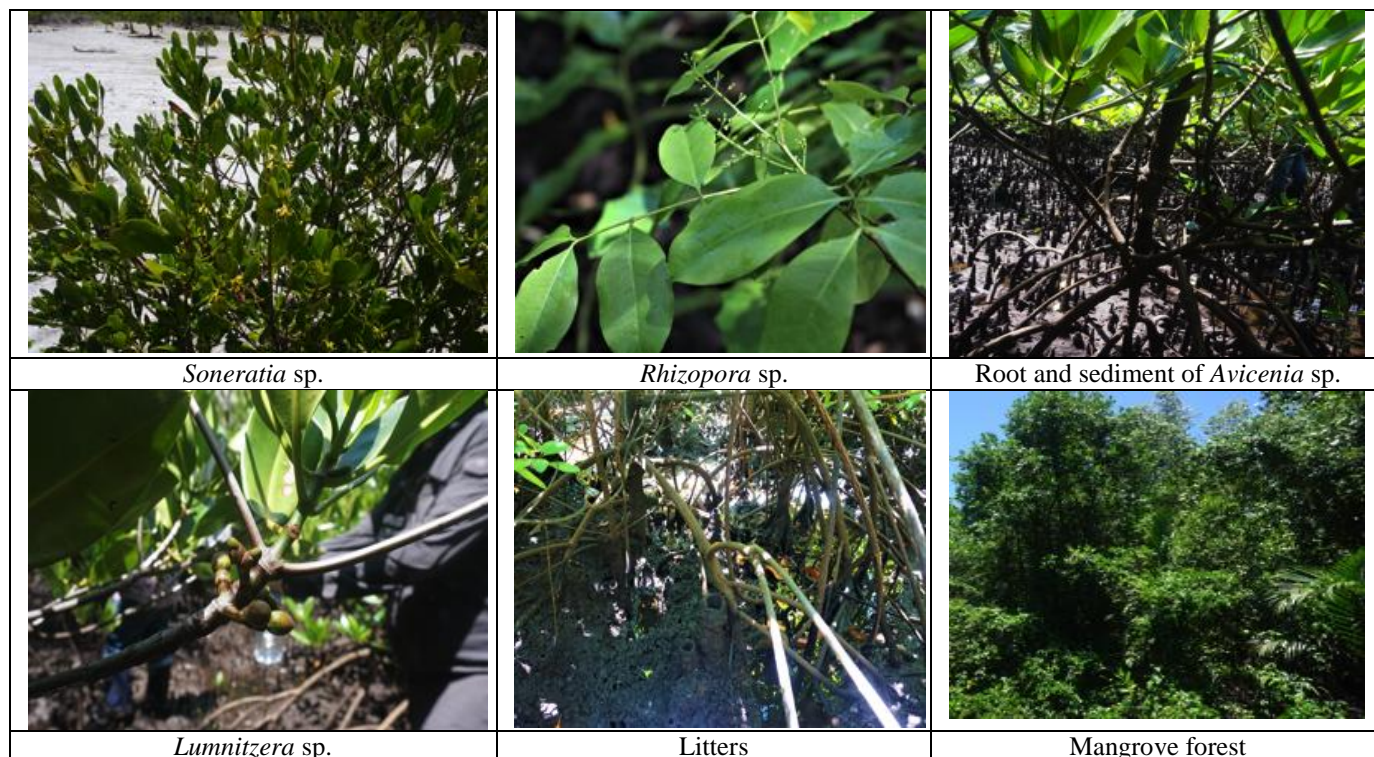


**Fig 1.** Map of the study areas in the Manado, North Sulawesi. Mangroves were collected at five sampling sites; Likupang Restoration, **MSr** (1); Likupang Natural, **MSn** (2); Tiwoho Natural, **MT** (3); Bawoho Restoration, **MBa** (4); and Buyat Restoration, **MB** (5)

## RESULTS AND DISCUSSION

### Fungal isolates

A total of 288 fungal isolates were collected from 5 different locations that contained from four genus of mangrove, viz *Soneratia* sp., *Rhizophora* sp., *Avicenia* sp., and *Lumnitzera* sp. Figure 2 showed the diversity source of samples in North Sulawesi. It showed the condition in the mangrove area.



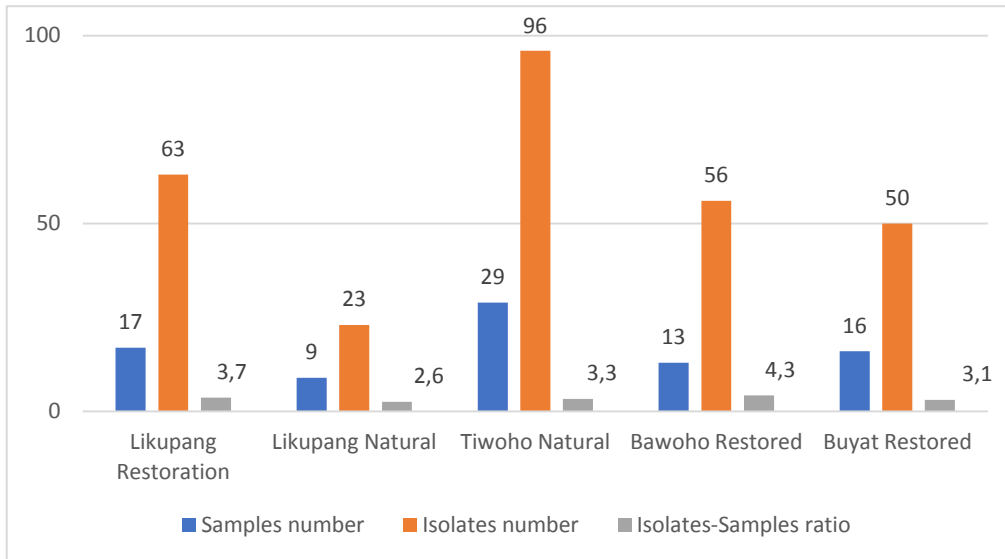
**Fig 2.** Samples of the study areas in the Manado, North Sulawesi.

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### Isolation of mangrove-associated fungi

Mangrove samples were collected from 5 different locations in North Sulawesi province, as shown in Figure 1. The types of mangrove that have been collected are *Rhizophora sp.*, *Avicenia sp.*, *Soneratia sp.*, and *Lumnitzera sp.* The total number of samples collected was 84 from the whole area, as shown in Figure 3. The highest number of samples was collected from Tiwoho Natural with 29 samples followed by Likupang Restoration and Buyat Restored location, which are 17 and 16 samples, respectively. Tiwoho Natural area is the most interesting location with the diversity mangrove samples obtained. This area is part of Bunaken National Park that a protected mangrove and ecological area. A 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 4 location (**MSr**, **MSn**, **MT**, **MBa**, **MB**) showed that the ratio of fungal association to the number of the sample was around 2.6 to 3.7. Interestingly, the highest ratio was observed in Buyat Restored, which is 4.3.

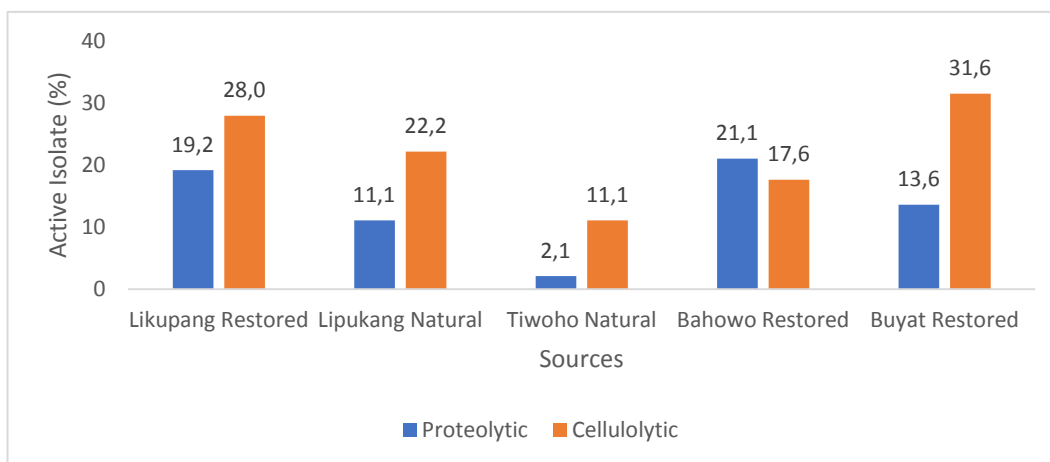




**Fig 3.** The number of samples collected from several locations in North-Sulawesi and their fungal and fungal - samples ratio.

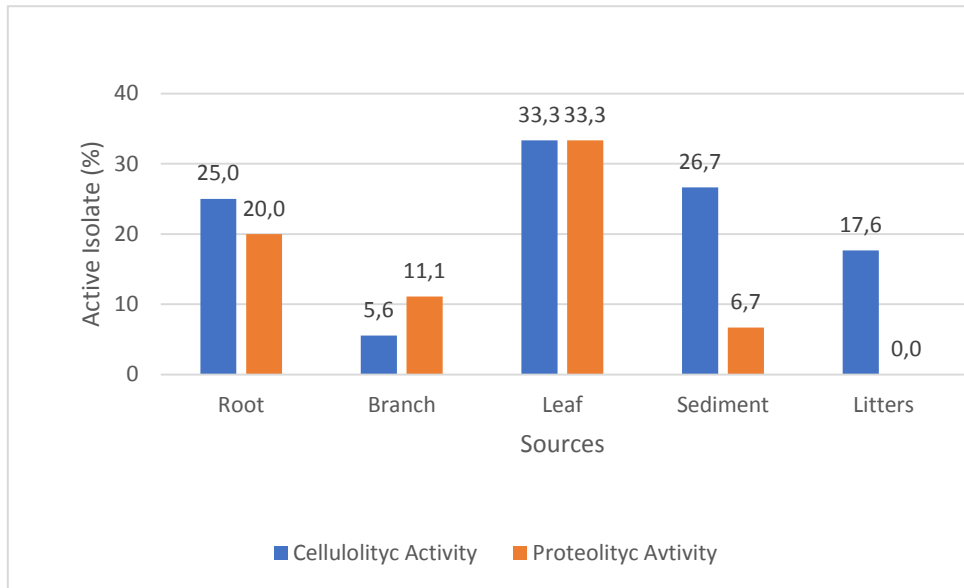
### The celluloses and proteolytic activities

We observed that the fungal had celluloses and proteolytic activities, as shown in Figure 4. Every single location has a different characterization to produce the enzyme. The percentage of the cellulolytic enzyme is 2.1% to 21.1%. Moreover, the percentage of the proteolytic enzyme was identified at 11.1% to 31.6%. The location in Bahowo restored, and Buyat Restored had a higher production of the enzyme proteolytic and celluloses than other places. Tiwoho Natural that is close to the Bunaken National Park, had the lowest enzyme production. Two others locatios, Likupang Natural and Bahowo Restored had a similar result of proteolytic enzyme in range 2.1 to 21.1% and cellulolytic enzyme in range 17.6 to 22.2%.



**Fig 4.** The fungal having celluloses and proteolytic activities (%).

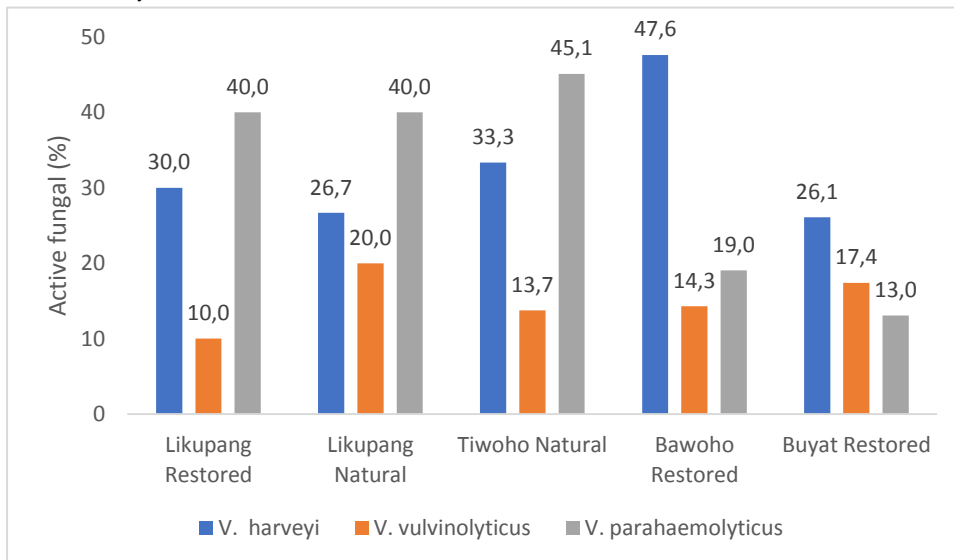
The fungi isolated from leaves indicated the most potent sources of the enzymes, where 33.3% of them produce the protease and cellulose, as shown in Figure 5. Followed by the root is 25% of celluloses and 20% of proteolytic enzyme. The branch produced 5.6% of celluloses and 11.1% of proteolytic enzyme, moreover sediment produced 26.7% of celluloses and 6.7% of proteolytic enzyme. In the part of litters had no proteolytic activity.



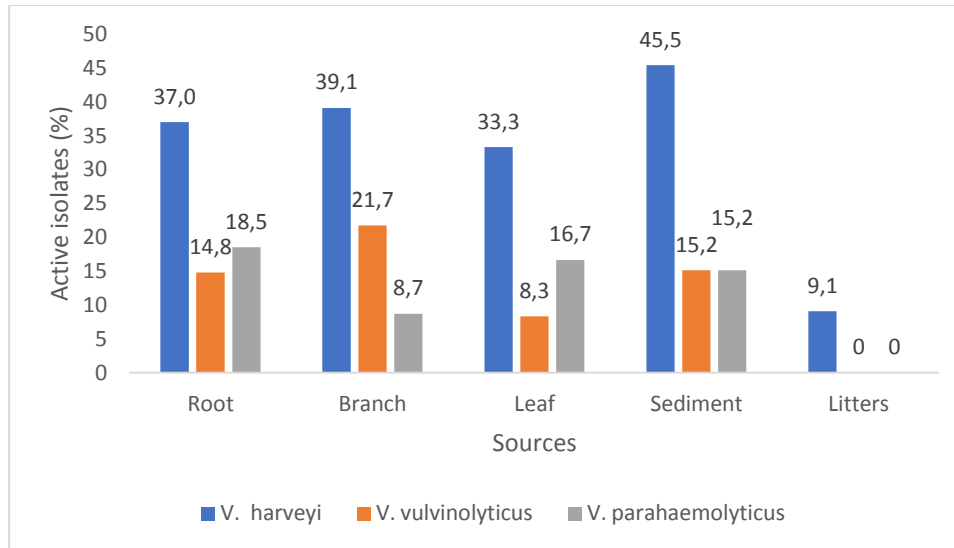
**Fig 5.** The fungal having celluloses and proteolytic activities (%).

#### Antibacterial assay

The result of antimicrobial activity of mangrove-fungal associated against *V. harveyi*, *V. vulnolyticus*, and *V. paramahaemolyticus* has shown in Figure 6 and Figure 7. Based on different location of sources, most of them had antimicrobial activity with the variation of ability. This data reported the highest anti-vibrio activity in each species, which were 47.6% of fungal from Bawoho Restored had antimicrobial activity against *V. harveyi*, 20% of fungal from Likupang Natural had antimicrobial activity against *V. vulnolyticus* and 45.1% of fungal from Tiwoho Natural had antimicrobial activity against *V. paramahaemolyticus*. Then, based on the fungal association of the part of mangrove (Figure 7), we showed that most of them had strong antimicrobial activity against *V. harveyi*, *V. vulnolyticus*, and *V. paramahaemolyticus*. There is no antimicrobial activity against *V. vulnolyticus*, and *V. paramahaemolyticus* was observed on the Litters part. The strongest activity was investigated on fungal from sediment against *V. harveyi* at 45.5%.



**Fig 6.** The active fungal against by site *V. harveyi*, *V. vulnolyticus*, and *V. paramahaemolyticus* (%).



188 **Fig 7.** The active fungal by sources against *V. harveyi*, *V. vulvinolyticus*, and *V. paramahaemolyticus* (%).  
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191 **Biological activity of potential fungal**  
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193 We described the potential fungi, which have proteolytic, celluloses enzyme and anti-vibrio activity. There were  
 194 5 fungi that have proteolytic activity. *Pestalotiopsis theae* and *Fusarium equiseti* were isolated from leaf of *Avicenia*  
 195 sp., then *Fusarium equiseti*, *Pestalotiopsis microspora* PKT2 and *Fusarium equiseti* were isolated from root of  
 196 *Soneratia* sp. The fungus genus *Pestalotiopsis* causes leaf spots, petiole/rachis blights and sometimes a bud rot of  
 197 palms. In other words, unlike the other leaf spot and petiole blight pathogens, which attack either the leaf blade or  
 198 the leaf petiole, *Pestalotiopsis* attacks all parts of the leaf from base to tip (Elliott 2019). *Fusarium equiseti* is the  
 199 species commonly find in tropical and subtropical areas, is considered to be a weak pathogen on cereals and is  
 200 occasionally to be associated with fassarium head blight infected kemels. Isolation of *Fusarium* species in greater  
 201 number and frequency is due to the high nutrient level in the mangrove ecosystem (Selvi and Sivakumar 2013).  
 202

203 **Table 1.** Biological activity of fungal associated mangrove as proteolytic, cellulotic and anti-vibrio.

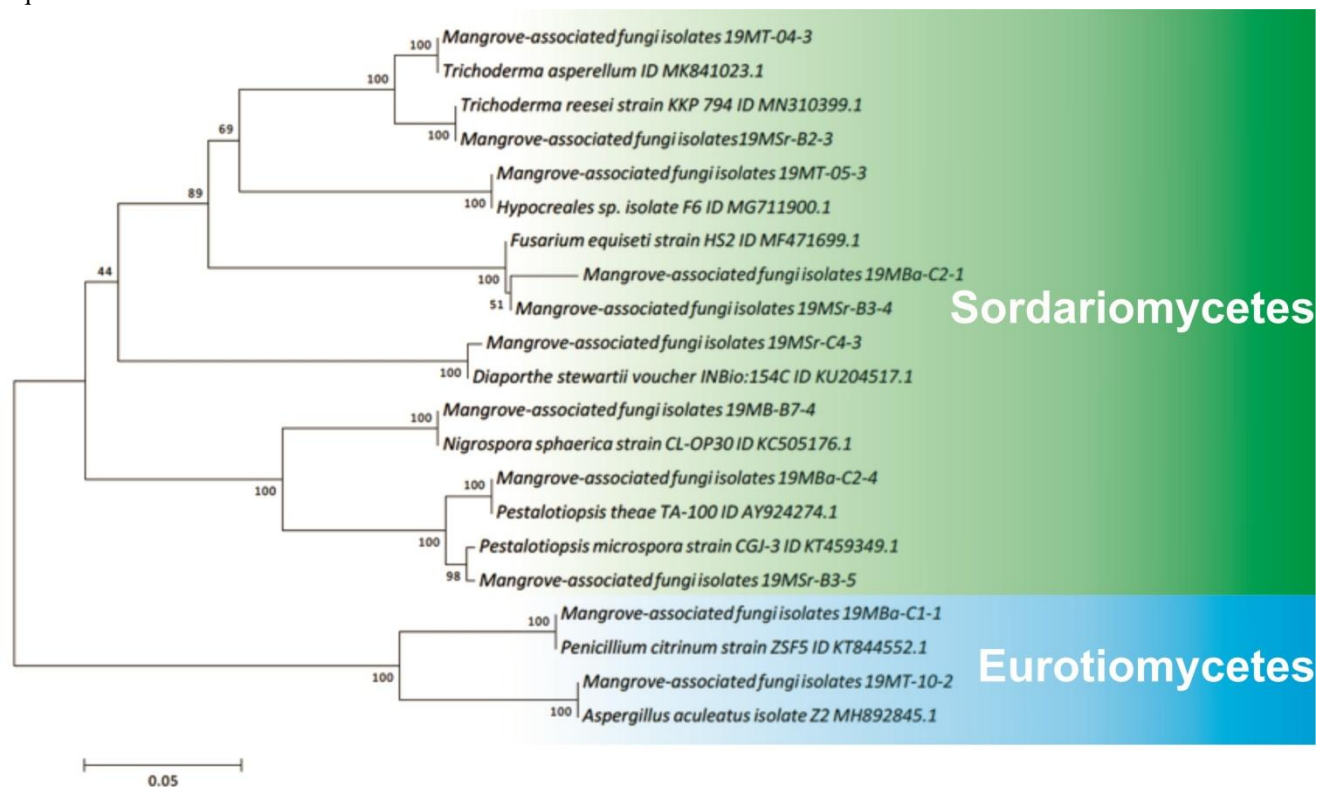
Isolate Code	Source of Mangrove Part	Biological Activity			Identified Species	ACC Number
		Proteolytic	Cellulotic	Anti-vibrio		
19 Mba-C2-4	Leaf of <i>Avicenia</i> sp.	√			<i>Pestalotiopsis theae</i>	AY924274.1
19 Mba-C2-1	Leaf of <i>Avicenia</i> sp.	√			<i>Fusarium equiseti</i>	KT459349.1
19 Mba-C1-1	Branch of <i>Avicenia</i> sp.			√	<i>Penicillium citrinum</i>	KT844552.1
19 MSr-B3-4	Root of <i>Soneratia</i> sp.	√	√		<i>Fusarium equiseti</i>	MF471699.1
19 MSr-B3-5	Root of <i>Soneratia</i> sp.	√			<i>Pestalotiopsis microspora</i>	KT459349.1
19 MB-B7-4	Litters <i>Rhizophora</i> sp.		√		<i>Nigrospora sphaerica</i>	KC505176.1
19 MSr-B2-3	Leaf of <i>Soneratia</i> sp.		√		<i>Hypocrea jecorina</i>	MN310399.1
19 MT-10-2	Leaf of <i>Rhizophora</i> sp.		√		<i>Aspergillus aculeatus</i>	MH892845.1
19 MT-04-3	Root of <i>Rhizophora</i> sp.			√	<i>Trichoderma viride</i>	MK841023.1
19 MSr-C4-3	Sediment		√		<i>Diaporthe stewartii</i>	KU204517.1
19 MT-05-3	Sediment		√		<i>Hypocreales</i> sp.	MG711900.1

204 **Noted:** Likupang Restoration (MSr); Likupang Natural (MSn); Tiwoho Natural (MT); Bawoho Restoration (MBa);  
 205 Buyat Restoration (MB).  
 206  
 207

208 **Molecular identification of active fungi**

209 Phylogenetic trees were build using maximum like hood processing and the number shown of a bootstrap  
 210 replicate of 1000 data with MEGA 7.0.26 bioinformatics software. The tree represents phylogenetic diversity

211 between restoration area and natural area. The accession number of each species was written in bold and italic style  
 212 right after the species name — the scale bar in the bottom of the figure represented the distance of evolutionary  
 213 sequence.



214  
 215 **Fig 8.** Phylogenetic trees of active isolates  
 216  
 217

218 **Discussion**

219 Mangrove ecosystems are a relatively unexplored source of fungal diversity and these fungi represent a  
 220 potential important commercial reservoir of novel enzymes and compounds with novel activities e.g. new  
 221 antibiotics, larvacide etc (Thatoi et al. 2013, Pringgenies et al. 2018, Sibero et al. 2018). Bonugli-Santos et al. 2015  
 222 report that mangrove associated fungi have several benefit of enzyme and biotechnological sources such as to be  
 223 producers of hydrolytic and/or oxidative enzymes, with alginate lyase, amylase, cellulase, chitinase, glucosidase,  
 224 inulinase, keratinase, ligninase, lipase, nuclease, phytase, protease, and xylanase.

225 This work focused on isolating fungi from a variety of mangrove systems in Northern Sulawesi (natural and  
 226 restored) and it was thought that the different sites could harbor different species due to the different mangrove  
 227 species, tidal systems and restoration methods used. We isolated a total of 288 species with the highest diversity  
 228 found in Tiwoho Natural. Fungi in classes Dothideiomycetes, Sordariomycetes and Eurotiomycetes were most  
 229 prevalent which is in agreement (or disagreement) with previous studies e.g. (Lee et al. 2019) found that classes had  
 230 dominated in mangrove environment. In previous study have been reported the diversity of fungal associated  
 231 mangrove in different part such as fruit, leaf, pneumatophore and sediment. They reported that in fruit and leaf is the  
 232 highest amount. The dominant fungal is Ascomycota and class Dothideiomycetes (N. Li et al 2019). On average, our  
 233 study showed that fungi isolate produce protease and cellulose enzymes. The fungal association from leaf showed  
 234 the highest production of proteolytic and cellulolytic enzymes (Figure 5). Mangrove fungal celluloses activity is  
 235 affected by environment conditions (pH, temperature, substrates), fungal community, and culture condition (Hossain  
 236 2016). Fungi that have proteolytic activity have the ability to produce protease enzymes that are secreted into their  
 237 environment. The proteolytic enzyme works to hydrolyze protein compounds into oligopeptides, short-chain  
 238 peptides, and amino acids. The existence of this extracellular protease enzyme is very important for bacterial life

239 because it provides the need for nitrogen compounds that can be transported into cells. The types of fungi that have  
240 the ability to secrete this protease enzyme have great potential to be used as a source of aquaculture probiotics,  
241 especially in shrimp farming (Setyati et al. 2016).

242 Our study showed likupang has complex aquatic dynamics. Likupang-restored mangrove area has abandoned  
243 shrimp pond that rich of protein and cellulose from the waste of shrimp and unconsumed feed. Besides, shrimp  
244 farming is the discharge and deposition of shrimp pond waste left in the pond after harvesting. The abundance of  
245 pollutant sources from the ponds provides a source of nutrients needed by microbes thus resulting Likupang-restored  
246 has a high number of isolat and isolates-samples ratio (Saiya and Katoppo 2015, Ruete et al. 2016). Moreover to the  
247 contrary it was surprising that in Likupang-Natural has the lowest ratio of isolates to samples. Sridhar and  
248 Seetharam (2001) found a loss of over 300 species of the number of aquatic hyphomycete species as negatif effect of  
249 polluted water to fungal diversity. In laboratory experiments, water polutant that contain low concentrations of Cd,  
250 Cu and Zn have been shown to inhibit growth and reproduction of aquatic hyphomycetes and fungi respond by  
251 synthesizing specific stress peptides (Krauss et al. 2011).

252 Leaf, as the part of mangrove, showed the best producer of protease and cellulose enzyme (Figure 5). Previous  
253 research revealed that fungal associated with leaves of the mangrove has a highly diverse fungal community (Chi et  
254 al. 2019). There were total of 110 taxa recovered from the isolation and metabarcoding methods. Among them,  
255 Ascomycota was dominant following by *C. cassicola* (6.90%), *F. oxysporum* (6.40%) and *Guignardia* sp. (6.40%).  
256 Only specifically source from sediment reported that the fungal show anti-vibrio activity. The fungi that live in  
257 seawater sediment grow well than in fresh water. Sediment nutrient content is affected by physiology and  
258 environment industries among the mangrove vegetation. Previous study have been report the fungi from different  
259 location, bottom sediment and depth, identified Macrophytes, Phycomycetes, Ascomycetes and Deuteromycetes  
260 (Sivakumar, 2013).

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

267 An interesting fungi *Hypocrea jecorina* have been found in litters of *Rhizopora* sp. as celluloses enzymes.  
268 *Hypocrea jecorina* anamorphic *Trichoderma reesei* is asaprophyte noted for its ability to abundantly secrete native  
269 hydrolytic enzymes. These enzymes are used in various industrial applications, such as pulp and paper production  
270 and in the food and feed industries and in the textile industry (Steiger et al. 2011). *Penicillium citrinum* and  
271 *Trichoderma viride* have been succesfull isolated from branch of *Avicenia* sp. and root of *Rhizopora* sp.,  
272 respectively.

273 We showed anti-vibrio activity on *Penicillium citrinum* and *Trichoderma viride*. Previous study has been found  
274 four new compounds, penicitrinone E, penicitrinol J, penicitrinol K and citrinolactone D, were isolated together with  
275 six known compounds from the marine-derived *Penicillium* sp. ML226 (Wang et al. 2013). Penicitrinone E,  
276 penicitrinol J and penicitrinol K showed modest selective cytotoxicity against HepG-2 cell line. Citrinolactone D  
277 showed weak cytotoxicity against HepG-2 and HeLa cell lines. penicitrinol J and penicitrinol K showed mild  
278 antimicrobial activity against *Staphylococcus aureus*.

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## ACKNOWLEDGEMENTS

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285 No. 257- 15/UN7.P 4.3/PP/2019.

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## [biodiv] Editor Decision

2021-02-19 04:34 AM

AGUS TRIANTO:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Biotechnological potential of fungi isolated from a mangrove ecosystem in northern Sulawesi, Indonesia: protease, cellulase and anti-microbial capabilities: Fungal isolates producing protease, cellulase and anti-microbial substances."

Our decision is: Revisions Required

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## Manuscript revision 1



### Participants [Edit](#)

Smujo Editors (editors)

Dr. AGUS TRIANTO (atrianto)

DEWI NUR PRATIWI (dewinurpratiwi)

### Messages

Note

From

Dear Dr. Dewi Nur Pratiwi,

atrianto

Herein, we are sending the revised manuscript as suggested by the reviewer.  
Hopefully, the manuscript is matched with the journal requirement.

2021-03-03 04:18  
AM

Best regards,

Agus Trianto, Ph.D.

 [atrianto, B-7791-Article Text-37945-1-18-20210214-AT.doc](#)

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# Biotechnological potential of fungi isolated from a mangrove ecosystem in northern Sulawesi, Indonesia: protease, cellulase and anti-microbial capabilities

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**Abstract.** 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 preliminary 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 have a 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 the discovery of potential biocatalysts for use in hydrolysis of lignocellulosic materials as well as proteic residues. These enzymes can increase and ensure viable production of second generation ethanol from different and alternative sources (Immaculatejevasanta et al. 2011, Ramesh et al. 2014). *Vibrio* is a pathogenic bacteria that is found in environment and community that have a high-risk infection (Igbinsosa and Omoruyi 2016).

Manado is the largest coastal population in North Sulawesi, Indonesia, having 4.6% of 161 km<sup>2</sup> 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 Djamaaluddin 2017, Djamaaluddin 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 in industrial and medical purposes. Previous study has shown that *Aspergillus niger*, *Halocyphina villosa*, and *Lignicola longirostris* are known to produce protease and cellulase (Immaculatejevasanta et al. 2011).

**Comment [Rev1]:** Hypocreales is an Order, not generic epithet. In Pub Med contributors have uploaded it falsely as *Hypocreales* sp., while identifying upto Order level only. Please check

**Comment [AT2R1]:** Thanks for the correction. The genus name should be *Hypocrea*.

**Comment [Rev3]:** Full reference not found

**Comment [AT4R3]:** We have already completed the reference

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

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

Comment [Rev5]: Blood?

Comment [AT6R5]: Yes, a certain protease has significant role in blood clotting

Comment [Rev7]: fingerlings?

Comment [AT8R7]: Fingerling is a common terminology used for fishes or shrimp size.

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## MATERIAL AND METHODS

### 69 Location of sampling sites

70 The natural mangroves are naturally formed mangrove ecosystems, while the restoration mangroves are the mangrove  
71 forests that have been replanted by humans. Samples were collected during 9 to 11 April 2019, from two natural and three  
72 restoration sites viz. Likupang Restoration (MSr), 1°40'33.82"N/125° 3'17.24"E; Likupang Natural (MSn),  
73 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),  
74 1°34'51.69"N/124°49'3.26"E; Buyat Restoration (MB), 0°50'57.01"N/124°42'27.56"E (Fig. 1). The collected samples  
75 (leaf, branch, root, and sediments) were put into sterile plastic bags to avoid the contamination and brought to the  
76 laboratory in a cool-box (4°C) for further treatment.

### 77 Screening for cellulolytic and proteolytic of fungi

78 A total of 288 fungal isolates were tested for their ability to produce cellulolytic and proteolytic enzymes. For  
79 proteolytic screening, the isolates were inoculated on PDA media supplemented with 1 % skimmed milk powder and  
80 incubated for 3-5 days at 30 °C. A clear zone around the colony indicated the presence of protease activity (Bonugli-  
81 Santos et al. 2015). Screening of cellulolytic activity was done by inoculating the fungi on the media (containing 1%  
82 peptone, 0.05% yeast extract, 1% Carboxymethylcellulose (CMC) and 3% bacteriological agar) and then incubated for 3-5  
83 days at 30 °C, and transferred into a refrigerator (4 °C) for overnight. Cellulase activity was detected by the presence of  
84 clear zone around the fungal colony after the addition of Congo Red. The clear zone diameter were classified into weak (<  
85 2 mm), medium (3-4 mm), and strong (>5mm) based on the proteolytic and cellulolytic activities of fungi.

### 86 Antibacterial assay

87 The *Vibrio* strains were chosen for antibacterial assay, which was conducted using the overlay method as used in the  
88 previous study (Trianto et al. 2019). The fungal isolates were inoculated on MEA media in triplicate. After the growth of  
89 isolates which usually takes 1-7 days depending upon the growth rate, the *Vibrio* containing soft agar were poured onto the  
90 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  
91 the indicator strains with concentration of 0.5 McFarland. The following strains were used for antibacterial testing: *Vibrio*  
92 *harveyi*, *V. vulnificus*, *V. vulnificus*, and *V. parahaemolyticus anguillarum*. The plates were incubated at the  
93 optimum temperature for bacterial growth (37 ± 2°C) for 24h. The anti-*Vibrio* activity was defined by the presence of clear  
94 zones around the bacterial isolates. The clear zone diameter formed was also used to classify the antibacterial of fungal  
95 activity into weak (< 2 mm), medium (3-4 mm), and strong (>5mm).

Comment [Rev9]: Results lack statistical analysis e.g., t test for significance out of three replicate

Comment [AT10R9]: The replicate is used for ensuring the activity of the isolates. The statistical analyses usually applied for final test (exp. Pure compound).

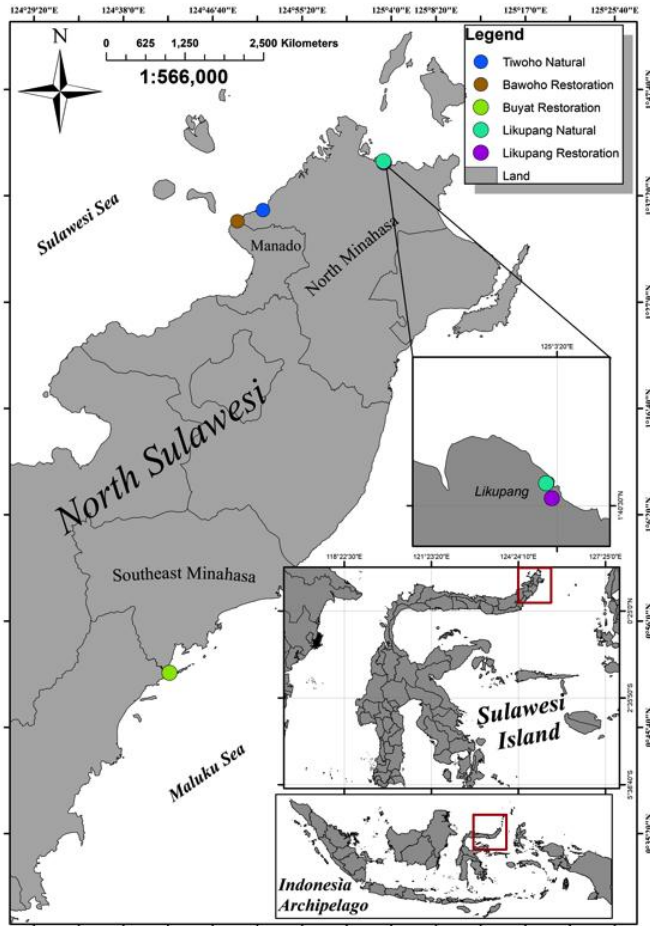
Comment [Rev11]: Its results needs to be written in Results section with more emphasis, in this matter whatever has been mentioned in results needs to be improved

Comment [AT12R11]: We did not put the table of the isolates activity because it up to several pages that contain 228 isolates.

### 96 Molecular identification of the active fungi

97 The DNA of active isolates were extracted using the Zymo DNA kit. The universal primer, internal transcribed spacer  
98 (ITS) was used for the fingerprint region for fungal barcoding using polymerase chain reaction (PCR) thermal cyclers. The  
99 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  
100 1-5 µl, Nuclease-Free Water to total 25µl. The thermal cyclers setting used was denaturation at 95°C for 1 min; 34  
101 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  
102 72°C for 7 min and cooling at 4°C until recovery of the samples. The PCR products were visualized by electrophoresis  
103 process, and sequencing was undertaken at Genetika Science, Jakarta Indonesia, and continued to 1st Base, Malaysia. The  
104 results were compared with other sequences in the NCBI database using BLAST. The phylogenetic tree of sequence  
105 results was constructed by MEGA 7.0 (Kumar et al. 2016).

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

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## RESULTS AND DISCUSSION

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### Fungal isolates

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

**Comment [Rev13]:** Results also need to emphasize statistical significance of bioassay of fungal isolates against different species of *Vibrio*

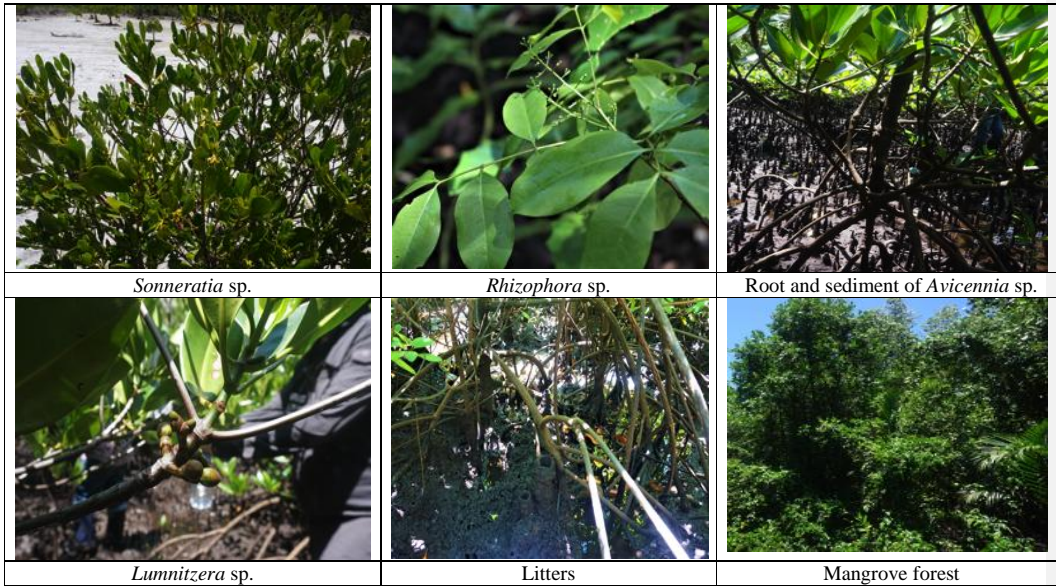


Fig 2. Samples of the study areas in the Manado, North Sulawesi.

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**Isolation of mangrove-associated fungi**

The total number of samples collected was 84 from 5 sites (Figure 1), are shown in Figure 3. The highest number of samples was collected from Tiwoho Natural with 29 samples, followed by Likupang Restoration and Buyat Restored 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. A 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 Restored.

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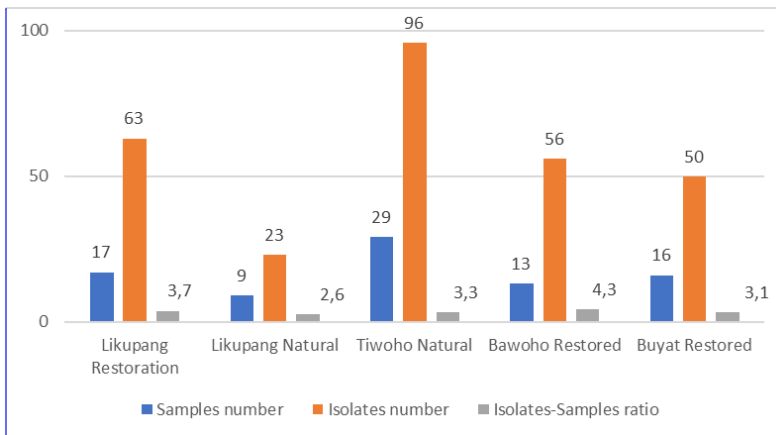


Fig 3. The number of samples and corresponding isolates collected from several locations in North-Sulawesi and their isolate-sample ratio.

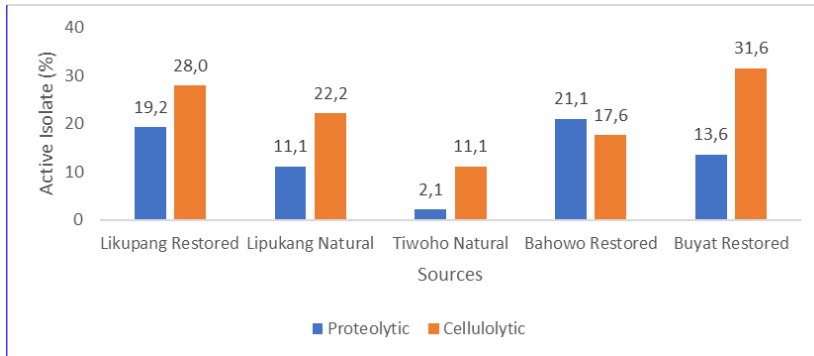
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**Comment [Rev14]:** (,) to be replaced by (.) i.e., 3,7 to be corrected to 3.7, 2,6 to be corrected to 2.6 etc.

Samples number to be corrected as **No. of samples**  
Isolates number to be corrected as **No. of isolates**  
Isolates-Samples ratio to be corrected as **Isolate-Sample ratio**

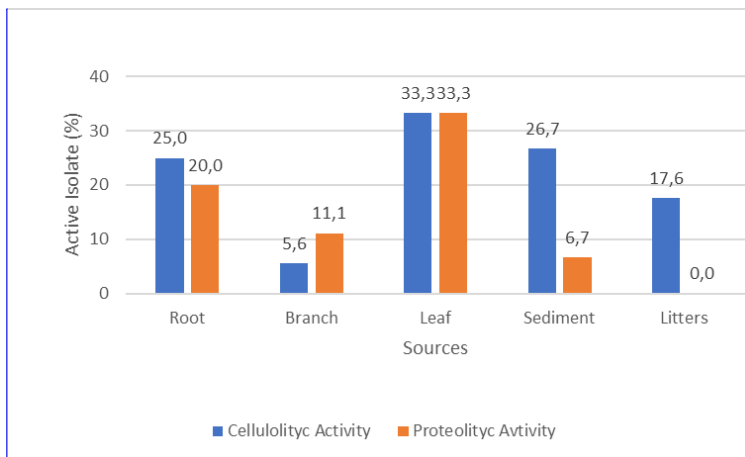
134 **The cellulolytic and proteolytic activities**

135 It was observed that the fungal isolates had cellulolytic and proteolytic activities, as shown in Figure 4. The percentage  
 136 of the proteolytic enzyme was 2.1% to 21.1%. Moreover, the range of the cellulolytic enzyme was identified at 11.1% to  
 137 31.6%. Each location had distinct potential activity in terms of enzyme production. The location in Bahowo restored and  
 138 Buyat Restored had higher production of the enzyme with proteolytic and cellulolytic activities respectively, than other  
 139 places. Tiwoho Natural ranked lowest in terms of enzyme production. Two others locations, Likupang Natural and  
 140 Bahowo Restored had a similar result of proteolytic enzyme in range 11.1 to 21.1% and cellulolytic enzyme in range 17.6  
 141 to 22.2%.  
 142



143 **Fig 4.** The fungal having cellulolytic and proteolytic activities (%).

144 The fungi isolated from leaves were found to be the most potent sources of the enzymes, where 33.3% of them  
 145 produced protease and cellulase (Figure 5). Followed by roots, whose isolates produced 25% cellulases and 20%  
 146 of proteolytic enzyme. Branches produced 5.6% of cellulases and 11.1% of proteolytic enzyme, moreover sediment isolates  
 147 produced 26.7% of cellulases and 6.7% of proteolytic enzyme, while, isolates from litters didn't had any proteolytic  
 148 activity.  
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152 **Fig 5.** The fungal isolates from various samples having cellulolytic and proteolytic activities (%).

153 **Antibacterial assay**

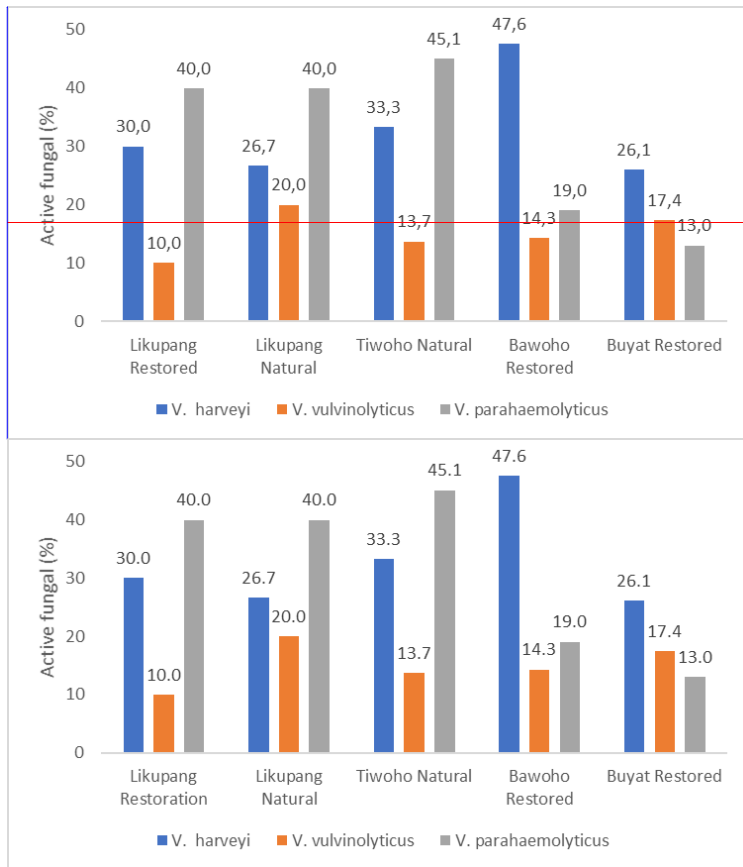
154 The result of antimicrobial activity of mangrove-fungi associated against *V. harveyi*, *V. alginolyticus*, *V. vulnolyticus*  
 155 and *V. parahaemolyticus* was shown in Figure 6 and Figure 7. Based on ~~different the collection sites location~~  
 156 ~~of sources~~, most of them had antimicrobial activity with the varying potential. The highest anti-*Vibrio* activity (47.6%) was  
 157 represented by fungal isolate from from Bahowo Restored which had antimicrobial activity against *V. harveyi*. ~~While,~~  
 158 ~~fungal isolates from Likupang Natural 20% of fungal isolate from Likupang Natural had the highest~~ antimicrobial activity  
 159

**Comment [Rev15]:** All (,) to be replaced by (.)

**Comment [Rev16]:** All (,) to be replaced by (.)  
 Replace Root by **Roots**, Branch by **Branches**,  
 Leaf by **Leaves**, Sediment by **Sediments**,  
 Cellulolytic by **Cellulolytic**, Proteolytic by **Proteolytic**

**Comment [Rev17]:** In Materials and Methods *Vibrio harveyi*, *V. alginolyticus*, and *V. anguillarum* are mentioned to be bacteria used for testing but here *V. parahaemolyticus* is mentioned instead of *V. anguillarum*.  
**Needs a recheck**

161 | (20% )-against *V. vulvinolyticus*. - and- A total 45.1% of fungal isolate from Tiwoho Natural had highest  
 162 antimicrobial activity against *V. parahaemolyticus*. Then, based on the fungal association of the part of mangrove (Figure  
 163 7), it was known that most of them had strong antimicrobial activity against *V. harveyi*, *V. vulvinolyticus*, and  
 164 *V. parahaemolyticus*. There is no antimicrobial activity against *V. vulvinolyticus*, and *V. parahaemolyticus* as  
 165 observed on the fungal isolates from Litters. The strongest activity was investigated on fungi from sediment against *V.*  
 166 *harveyi* at 45.5%.  
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Comment [Rev18]: All (.) to be replaced by (.)

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Fig 6. The active fungal against by site *V. harveyi*, *V. vulvinolyticus*, and *V. parahaemolyticus* (%).

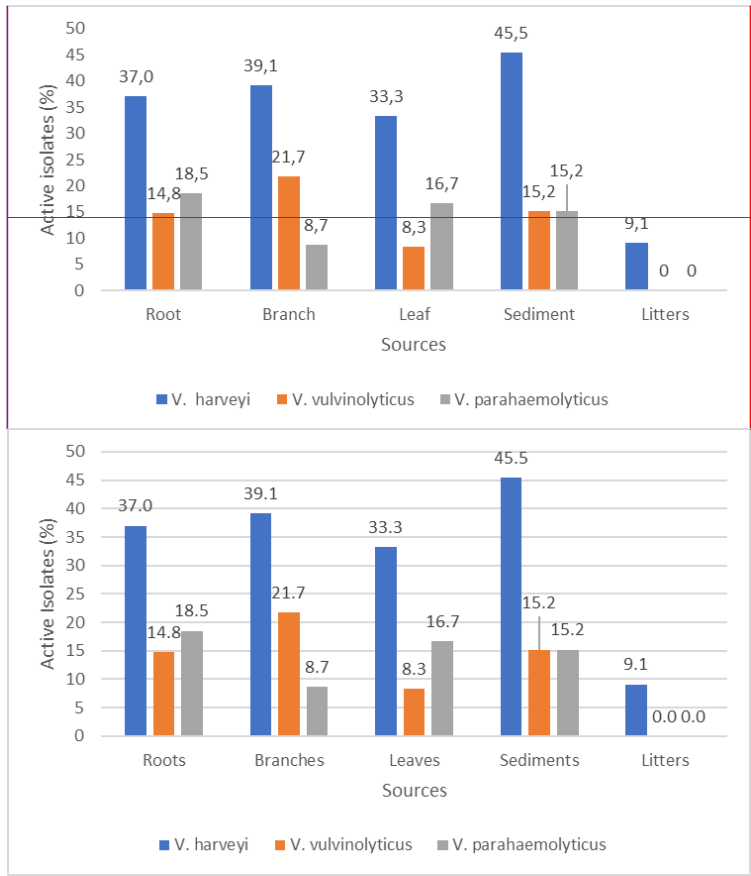


Fig 7. Source-wise activity (%) of fungal isolates against *V. harveyi*, *V. vulvinolyticus* and *V. parahaemolyticus*.

**Comment [Rev19]:** All (,) to be replaced by (.)  
 Replace Root by **Roots**, Branch by **Branches**,  
 Leaf by **Leaves**, Sediment by **Sediments**

Replace *V. vulvinolyticus* with *V. alginolyticus*  
 Recheck *V. parahaemolyticus* with reference to  
*V. anguillarum*

**Comment [AT20R19]:** Thanks for the correction. However, the correct isolates are *V. harveyi*, *V. vulvinicus*, and *V. parahaemolyticus*

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176 **Molecular identification of fungal isolates**

177 Phylogenetic trees were build using maximum like hood method with bootstrap replicates of 1000 in MEGA 7.0.26  
 178 bioinformatics software. The tree represents phylogenetic diversity between restoration area and natural area. The  
 179 accession number of each species was written in bold and italic style right after the species name — the scale bar in the  
 180 bottom of the figure represented the distance of evolutionary sequence.  
 181



Comment [Rev21]: Figure resolution can improve

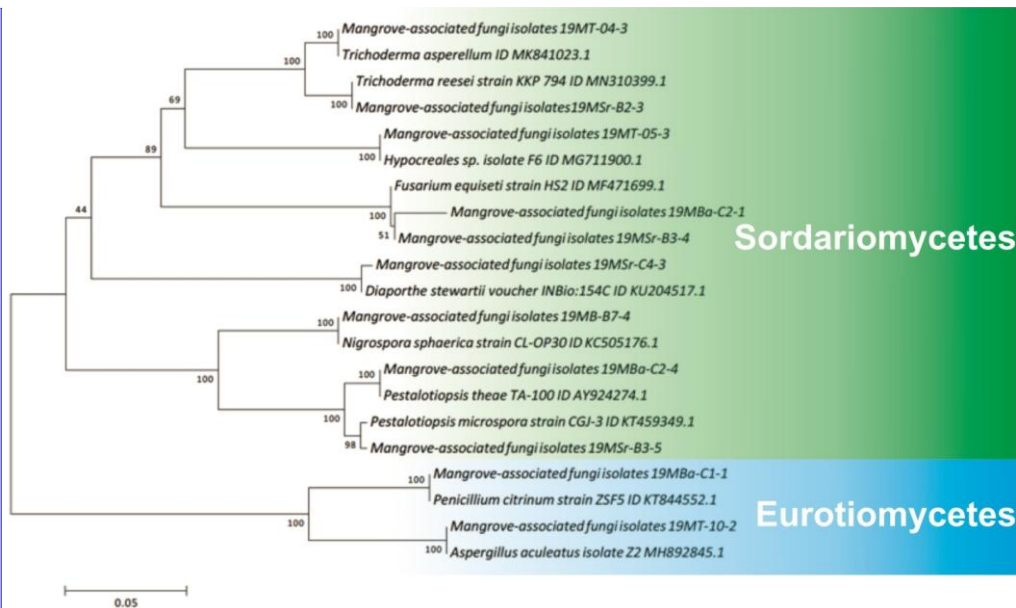


Fig 8. Phylogenetic trees of constructed with sequence of active isolates along with BLAST derived sequences.

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#### 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).

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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- <i>Vibrio</i>		
19 Mba-C2-4	Leaf of <i>Avicennia</i> sp.	√			<i>Pestalotiopsis theae</i>	AY924274.1
19 Mba-C2-1	Leaf of <i>Avicennia</i> sp.	√			<i>Fusarium equiseti</i>	KT459349.1
19 Mba-C1-1	Branch of <i>Avicennia</i> sp.			√	<i>Penicillium citrinum</i>	KT844552.1
19 MSr-B3-4	Root of <i>Sonneratia</i> sp.	√	√		<i>Fusarium equiseti</i>	MF471699.1
19 MSr-B3-5	Root of <i>Sonneratia</i> sp.	√			<i>Pestalotiopsis microspora</i>	KT459349.1
19 MB-B7-4	Litters <i>Rhizophora</i> sp.		√		<i>Nigrospora sphaerica</i>	KC505176.1
19 MSr-B2-3	Leaf of <i>Sonneratia</i> sp.		√		<i>Hypocrea jecorina</i>	MN310399.1
19 MT-10-2	Leaf of <i>Rhizophora</i> sp.		√		<i>Aspergillus aculeatus</i>	MH892845.1
19 MT-04-3	Root of <i>Rhizophora</i> sp.			√	<i>Trichoderma viride</i>	MK841023.1
19 MSr-C4-3	Sediment		√		<i>Diaporthe stewartii</i>	KU204517.1
19 MT-05-3	Sediment		√		<i>Hypocreales</i> sp.	MG711900.1

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

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## 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 source of enzymes with biotechnological application such 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 classes Sordariomycetes and Eurotiomycetes were among the most prevalent in mangrove environment 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 in 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 environment conditions (pH, temperature, substrates), fungal community, and culture condition (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. The existence of 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 have the ability to 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 to fungal diversity. In laboratory experiments, water pollutant 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 found to be the best source of fungal isolates producing protease and cellulase enzyme (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 fresh water. Sediment nutrient content is affected by physiology and environment among the mangrove vegetation. Previous study have report the fungi from different location, bottom sediment and depth, identified Phycomycetes, Ascomycetes and Deuteromycetes (Sivakumar 2013).

244

**Comment [Rev22]:** Hypocreales is an Order, not generic epithet. In Pub Med contributors have uploaded it falsely as *Hypocreales* sp., while identifying upto Order level only. Please check

**Comment [AT23R22]:** Thanks for the correction. It should be *Hypocrea*.

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

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

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

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264

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267 15/UN7.P 4.3/PP/2019.

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## [biodiv] Editor Decision

2021-03-10 02:59 PM

AGUS TRIANTO, OCKY KARNA RADJASA, SUBAGIYO, HARTUTI PURNAWENI, MUHAMMAD SYAIFUDIEN BAHRY, RIGNOLDA DJAMALUDIN, AIYEN TJOA, IAN SINGLETON, KAREN DIELE, DARREN EVAN:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Potential of fungi isolated from a mangrove ecosystem in Northern Sulawesi, Indonesia: Protease, cellulase and anti-microbial capabilities".

Our decision is to: Accept Submission

Smujo Editors  
editors@smujo.id

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## [biodiv] Editor Decision

2021-03-11 01:18 AM

AGUS TRIANTO, OCKY KARNA RADJASA, SUBAGIYO, HARTUTI PURNAWENI, MUHAMMAD SYAIFUDIEN BAHRY, RIGNOLDA DJAMALUDIN, AIYEN TJOA, IAN SINGLETON, KAREN DIELE, DARREN EVAN:

The editing of your submission, "Potential of fungi isolated from a mangrove ecosystem in Northern Sulawesi, Indonesia: Protease, cellulase and anti-microbial capabilities," is complete. We are now sending it to production.

Submission URL: <https://smujo.id/biodiv/authorDashboard/submission/7791>

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