Seaweed-Associated Fungi from Sepanjang Beach, GunungKidul, Yogyakarta as Potential Source of Marine Polysaccharides-Degrading Enzymes

N E B Hutapea¹,², M T Sibero³,⁴, E P Ayuningtyas²,³, E H Frederick²,³, D P Wijayanti², A Sabdono³, D Pringgenies³, O K Radjasa³, D S Zilda³, R Murwani²,⁵

¹ Department of Aquaculture, Faculty of Fisheries and Marine Science, Universitas Diponegoro. Jl. Prof. H. Soedarto, S.H., Tembalang, Semarang 50275, Central Java, Indonesia. Tel.: +62-24-7474698, Fax.: +62-24-7474698
² Natural Product Laboratory, Integrated Laboratory for Research and Services, Universitas Diponegoro, Semarang, Indonesia
³ Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Diponegoro. Jl. Prof. H. Soedarto, S.H., Tembalang, Semarang 50275, Central Java, Indonesia. Tel.: +62-24-7474698, Fax.: +62-24-7474698
⁴ Research and Development Center for Marine and Fisheries Product, Product Processing and Biotechnology St. KS. Tubun Petamburan VI, Jakarta 10260, Indonesia
⁵ Department of Animal Science, Faculty of Animal Science and Agriculture, Universitas Diponegoro. Jl. Prof. Soedarto S.H., Tembalang, Semarang 50275, Central Java, Indonesia

Abstract. Brown algae (Phaeophyceae) and red algae (Rhodophyta) are a group of seaweed that scattered all over the ocean. In addition, previous studies have reported the biotechnological potential of its associated fungi. However, there are only a few studies related to the extracellular enzyme of seaweed-associated fungi. The purposes of this research were to isolated brown algae and red algae associated fungi from Sepanjang Beach, GunungKidul, Yogyakarta, Indonesia, and screen its enzyme production. Padina sp., Asparagopsis sp., and Chondrophycus sp. were collected from Sepanjang Beach, GunungKidul, Yogyakarta. Swab tap method on PDA medium and STD medium was conducted to isolate seaweed-associated fungi, while starch agar medium, agar medium, alginate agar medium, and carrageenan agar medium, was utilized to screen the enzyme activity with addition of povidone-iodine 10% reagent. The presence of clear zone around the colony indicated enzyme.

1. Introduction
Indonesia with a specific geographic location has an impact on biodiversity, including brown algae (Phaeophyceae) and red algae (Rhodophyta). It is known as marine vegetation that has abundant species diversity in sea waters, one of which is on the south coast of GunungKidul Yogyakarta [1]. In
addition, marine microbial community (bacteria, plankton, and fungi) is also considered an important ecological component in marine environment [2-5]. Seaweed associated-fungi has been explored widely for biotechnology purposes. Some studies studied interesting metabolites and extracellular enzyme that is likely to degrade the polymers in the cell walls [6,7]. However, there are only a few studies related to the extracellular polysaccharide-degrading enzymes (amylase, agarase, alginate, and carrageenase) from seaweed-associated fungi [8,9]. The purposes of this research were to isolated brown algae (Phaeophyceae) and red algae (Rhodophyta) associated fungi from Sepanjang Beach, GunungKidul, Yogyakarta, Indonesia, screen its enzyme production with povidone-iodine 10% reagent [6,10-12], and identify the prospective fungal isolates.

2. Methodology and materials research

2.1. Algae sampling
Brown algae (Phaeophyceae) and red algae (Rhodophyta) samples were collected from Sepanjang Beach, GunungKidul, Yogyakarta, Indonesia. The algae were collected namely Padina sp., Asparagopsis sp., and Chondrophycus sp. The samples were put in a sterilized zip-lock plastic and then transferred to Natural Product Laboratory, Integrated Laboratory for Research and Services, Universitas Diponegoro, Semarang, Indonesia for fungal isolation.

2.2. Fungal isolation
The fungi were isolated using swab tap method on potato dextrose agar (PDA) (HIMEDIA® GM096-500G) [13,14] and standard agar (STD) consisted of agar (Difco™ Marine Agar 2216) (37.4 g/l), glucose (5 g/l), yeast extract powder (10 g/l) (HIMEDIA® RM027-500G), beef extract (5 g/l) as the nitrogen source, peptone (5 g/l) and 2% laboratory agar powder (Wako 146-08675) in order to obtain a variety of fungi [15]. The fungal isolation medium was added with 2% chloramphenicol antibiotic to eliminate the possibility of bacterial contamination. Also, during isolation a petri dish with PDA and STD was provided as an environmental control.

2.3. Purification and refresh
Every single colony was purified on PDA medium and incubated at room temperature (27-28 ºC) until growth was initiated [16,17]. In total twenty-nine (29) fungi were isolated from Padina sp., Asparagopsis sp., and Chondrophycus sp.

2.4. Screening of extracellular enzyme
Screening medium for amylase contained 0,5% of peptone (HIMEDIA); 0,1% of yeast extract (HIMEDIA); 0,2% of soluble starch (Merck); and 2% of agar powder (Difco™) [10]. Screening enzyme for alginate contained peptone 0,5%, yeast extract 0,1%; alginate 0,5%; and agar powder 2%; for agarase contained 0,5% of peptone; 0,1% of yeast extract; and 2% of agar powder. The extracellular enzyme screening for carrageenase contained 0,5% of peptone (HIMEDIA); kappa carrageenan 2%; and yeast extract 0,1%. Each isolate grown in the enzyme screening medium was incubated at 27-28 ºC for 5-7 days then tested for the enzyme using GENERIC povidone-iodine solution (SS-K275/R2). The presence of the clear zone around the colony indicated amylase, carrageenase, alginate, and agarase active on all isolates [6]. The fungus as a new single colony with the widest clear zone was to next step (identified through morphology and molecular test).

2.5. Morphology identification
Fungal isolates were characterized through colony and hyphae morphologies. Hyphae morphology was using a microscope with Lactophenol Cotton Blue (LPCB) addition for cell staining [18]. A single colony of fungus was taken from the culture using a loop and transferred gently onto a glass object. Then observed at a magnification of 40 × 10 to characterized the shape of hyphae, the presence of septa, conidiophores, and spores/conidia [19,20].

2.6. DNA extraction, amplification, and PCR sequencing
For molecular analysis, fungi were grown on PDA medium at room temperature (27-28 ºC) for approximately 7 days. Fresh mycelium was taken from the PDA with a sterile loop and then the DNA
was extracted using the Zymo Research Quick-DNA™ Fungal/Bacterial Miniprep Kit according to the manual [21]. The internal transcribed spacer (ITS) region was amplified with the universal primers ITS1 and ITS4 [22]. The amplification process added 12.5 µL of Promega Go Taq™ Master Mixes, 1 µL of primer ITS1 forward (5’ TCCGTAGGTGAACCTGCGG 3’), ITS4 reverse (5’TCCTCCGCTTATTGATGC 3’) each 1 µL, 9.5 µL nuclease-free water, and 1 µL of template DNA into the microtube and homogenized using micropipettes and optimization of annealing temperature [23]. Electrophoresis was carried out with 2% agarose concentration, then PCR sequencing was carried out using UVITEC Cambridge and were pre-denatured at 95 ºC for 3 min. PCR amplification was performed using the following cycling conditions: 34 cycles of denaturation at 95 ºC for 1 min, annealing at 55 ºC for 1 min, and extension at 72 ºC for 1 min; finally, re-extension at 72 ºC for 7 min and stored at 16 ºC before analysis [24].

3. Results and Discussion

3.1 Algae collection
In total, 3 seaweeds were successfully collected from one location in Sepanjang Beach, Gunung Kidul, Yogyakarta.

3.2 Seaweed-associated fungi
In this work, the isolation of fungi was conducted on PDA and STD media due to its rich nutrients, which are needed by fungi to grow and produce spore. The media should contain sources of carbon, nitrogen, and important minerals during the isolation period [25-27]. The swab isolation method plays an important role to obtain 18 isolates on PDA and 11 isolates from STD. The 29 seaweed-associated fungi isolated from 3 seaweed collections are shown by Table 1.

### Table 1. Seaweed-associated fungi from Sepanjang beach, Yogyakarta, Indonesia

| No. | Seaweed origin | Fungi isolates | No. | Seaweed origin | Fungi isolates |
|-----|----------------|---------------|-----|----------------|---------------|
| 1.  | Asparagopsis sp. | MT.F1         | 16. | Padina sp.     | MT.F2.P       |
| 2.  | Asparagopsis sp. | MT.F2         | 17. | Padina sp.     | MT.F3.P       |
| 3.  | Chondrophyccus sp. | MT.F4       | 18. | Padina sp.     | MT.F4.P       |
| 4.  | Chondrophyccus sp. | MT.F5       | 19. | Padina sp.     | MT.F5.P       |
| 5.  | Chondrophyccus sp. | MT.F6       | 20. | Padina sp.     | MT.F6.P       |
| 6.  | Chondrophyccus sp. | MT.F7       | 21. | Padina sp.     | MT.F7.P       |
| 7.  | Chondrophyccus sp. | MT.F8       | 22. | Padina sp.     | MT.F8.P       |
| 8.  | Chondrophyccus sp. | MT.F9       | 23. | Padina sp.     | MT.F9.P       |
| 9.  | Chondrophyccus sp. | MT.F10      | 24. | Padina sp.     | MT.F10.P      |
| 10. | Asparagopsis sp. | MT.F11       | 25. | Padina sp.     | MT.F11.P      |
| 11. | Asparagopsis sp. | MT.F12       | 26. | Padina sp.     | MT.F12.P      |
| 12. | Asparagopsis sp. | MT.F13       | 27. | Padina sp.     | MT.F13.P      |
| 13. | Asparagopsis sp. | MT.F14       | 28. | Padina sp.     | MT.F14.P      |
| 14. | Asparagopsis sp. | MT.F15       | 29. | Padina sp.     | MT.F15.P      |
| 15. | Padina sp.       | MT.F1.P      |     |                |               |

3.3 Fungal morphology observation
Fungal colonies were characterized according to several aspects as follows colour, form, elevation, margin, colony colour, colony texture, mycelium colour, exudate, reverse, soluble pigment, and sclerotia [28]. The result of the macroscopic characterized were presented in Table 2.

### Table 2. Macroscopic characterized of seaweed-associated fungi from Sepanjang Beach

| Isolates | Colour   | Form   | Elevation | Margin  | Colony Colour | Colony Texture | Mycelium Colour | Exudate | Reverse | Soluble Pigment | Sclerotia |
|----------|----------|--------|-----------|---------|---------------|----------------|----------------|---------|---------|----------------|----------|
| MT.F1    | brownish | circular | raised   | entire  | white         | cottony white  | -              | brown   | -       | -               | -        |
| MT.F2    | white    | irregular | flat     | entire  | white         | cottony white  | -              | brown   | -       | -               | -        |
| MT.F4    | glaucous | irregular | flat     | undulate | glaucous      | velvety white  | -              | glaucous | -       | -               | -        |
Simple identification was done by matching the macroscopic and microscopic characteristics of molds with identification books and comparing these characters with the identification keys on monographs (Figure 1) [29].
Figure 1. Fungi cultured on PDA and STD agar medium

3.4 Screening of enzyme activity

Fungi are known to produce a range of extracellular enzymes and other secondary metabolites. All purified fungal isolates have their respective morphological characteristics. Biological properties such as the ability to produce extracellular enzymes can be seen by the presence of a clear zone after the addition of povidone-iodine. Isolation, purification, characterization, and screening enzyme of all isolates were performed to get the highest specific enzyme activity. Soluble starch agar medium, agarase medium, alginate agar medium, and was kappa carrageenan agar medium aseptically prepared and autoclaved for assays of amylase, agarase, alginate, and carrageenase respectively. According to this test, the results showed that there were 22 isolates showing amylase activity (Figure 2), 21 isolates showing agarase activity (Figure 3), 29 isolates showing alginate activity (Figure 4), and 14 isolates showing carrageenase activity (Figure 5).
Figure 2. Clear zone around isolates grown in starch medium
Figure 3. Clear zone around isolates grown in agar medium
Figure 4. Clear zone around isolates grown in alginate medium
Fungi secrete extracellular enzymes to convert complex compounds into compounds that are simpler to digest their food source. The ability of various types of fungi to produce enzymes such as starch, carrageenan, alginate, and agar continues to be studied to complement various studies. Agar plate method is one of the most commonly used enzyme screening methods, using a medium containing polysaccharides with the addition of chromogenic dyes such as povidone-iodine [30]. The use of povidone-iodine is intended to show a positive reaction in the fungus to excrete the enzyme. Based on the results of the tests carried out, some strains did not give clear zones, the level of clearness is different, ranging from those that are not visible to be very clear with different sizes. For fungi that do not produce clear zones according to the composition of the substrate due to the inability to digest the polysaccharides in the substrate itself. The clearest zone with the largest clear zone size is owned by MT.F8 at temperature (27-28 °C). For amylase, carrageenase, alginate, and agarase enzyme activity were calculated by following formula enzyme activity index (EAI) [31,32]. The mean EAI was calculated from twenty-nine (29) isolated observations (Table 3).

### Table 3. Formula enzyme activity index (EAI) values of mold isolates on starch agar medium, agar medium, alginate medium, and κ-carrageenan medium (incubation at 27-28°C °C for 5-7 days)

| Isolates Code | Amylase  | Isolate Codes | Agarase  |
|---------------|----------|---------------|----------|
| MT.F1         | 0.236414 | MT.F1         | 1.024440 |
| MT.F2         | 0.208261 | MT.F2         | 0.791154 |
| MT.F6         | 0.400655 | MT.F6         | 0.462872 |
| MT.F7         | 0.264901 | MT.F9*        | 1.283375*|
| MT.F8*        | 1.170320*| MT.F10        | 1.133967 |
| MT.F9         | 0.621349 | MT.F11        | 0.931291 |
| MT.F11        | 0.759479 | MT.F12        | 0.644942 |
| MT.F12        | 0.655674 | MT.F13        | 0.450976 |
| MT.F13        | 0.734373 | MT.F14        | 0.961165 |
| MT.F14        | 0.357677 | MT.F15        | 0.494885 |
| MT.F15        | 0.496028 | MT.F2.P       | 0.540795 |
| MT.F1.P       | 1.059434 | MT.F2.P       | 0.931858 |
| MT.F2.P       | 0.288885 | MT.F3.P       | 0.723010 |
| MT.F3.P       | 0.904781 | MT.F4.P       | 0.607733 |
| MT.F4.P       | 0.048677 | MT.F7.P       | 0.464687 |
| MT.F5.P       | 0.228968 | MT.F8.P       | 1.092969 |
| MT.F6.P       | 1.230750 | MT.F9.P       | 0.480247 |
| MT.F8.P       | 1.108864 | MT.F12.P      | 0.438156 |
Molecular identification and phylogenetic analysis were carried out on MT.F8 isolates because it showed the largest/prospective clear zone size among all existing isolates using the test media, namely starch agar medium, carrageenan agar medium, alginate agar medium, and agarase medium.

3.5 Molecular identification and phylogenetic analysis

A molecular approach was performed to identify the potential isolate. DNA in \textit{internal transcribed space} (ITS) region was amplified using PCR since it is a conserve region for fungi and usually gives the best result for fungal identification [33,34,35]. The application of ITS1 and ITS4 primers aimed to amplify the ends of the 18S rRNA gene region from DNA, ITS 1, 5.8 S rRNA gene region, and the beginning of the 28S rRNA gene region [22,34,35,36,37,38]. Figure 6 shows the visualization of amplified DNA by ITS primer.

![Figure 6. The presence PCR of fungi MT.F8](image)

The similarities of sequences with other known species were investigated by comparisons with sequence data in the National Center for Biotechnology Information (NCBI). A phylogenetic tree based on the ITS region was constructed using the Molecular Evolutionary Genetics Analysis (MEGA
X) software with the maximum likelihood tree method, and the statistical analysis utilized bootstrapping with 1000 replications [39] (Figure 7). BLAST (basic local alignment search tool) will do to MT.F8 because it showed the biggest clear zone size among all existing isolates. According to the result molecular MT.F8 using the test media was highly similar to *Aspergillus sydowii* NR_131259.1 with 99.80% similarity (Table 4).

**Table 4.** Homology of potential seaweed associated-fungi.

| Fungi isolate | Molecular identification (BLAST closest relatives) | Similarity   |
|---------------|---------------------------------------------------|--------------|
| MT.F8         | *Aspergillus sydowii* NR_131259.1                  | 99.80%       |

![Figure 7. Phylogenetic tree of fungi MT.F8.](image)

Based on various previous studies, it was stated that *Aspergillus sydowii* was able to produce high yields lignocellulosic enzyme source, manganese peroxidase (Mnp) [40], cellulose and Xylanase [41], catalase, urease, sucrase and alkaline phosphatase [42, 43], proteinase [44], β-glucosidase enzyme [45], acetophenone, p-bromoacetophenone and pnitroacetophenone at minor concentrations from isolate *A. sydowii* [46]. Furthermore, previous work showed that *A. sydowii* not only produce extracellular enzyme but also bioactive red pigment with antibacterial activity against MDR pathogens [47].

4. Conclusion

In total 29 fungal isolates were obtained from brown algae (*Phaeophyceae*) and red algae (*Rhodophyta*). There were 11 isolates gave a positive result for amylase, agarase, alginate, and carrageenase enzyme. Molecular identification was carried out on fungus which emitted the largest enzyme extracellular. Fungus MT.F8 as the prospective isolate was identified as *Aspergillus sydowii*.

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