# Isolation, Identification And Screening Antibacterial Activity from Marine Sponge-Associated Fungi Against Multidrug-Resistant (MDR) *Escherichia coli*

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**Abstract.** Irrational used of antibiotic in several decades ago causing resistant in bacteria and decreasing the cure rate of infectious diseases. Multidrug-resistant (MDR) *Escherichia coli* is known to cause several infectious diseases such as urinary tract infection, nosocomial bloodstream infection, meningitis, bacteraemia, and gastrointestinal disease. Marine sponge-associated fungi have potential as source of new compound to combat MDR *E. coli*. The aims of this research were to isolate marine sponge-assosiated fungi, to screen potential fungi against MDR *E. coli*, to identify the potential fungi and its host sponge. There were 29 marine sponge-associated fungi successfully isolated from 9 sponges. Among 29 sponge-associated fungi screened, there were 7 isolates showed antibacterial activity against MDR *E. coli*. The best inhibition zone produced by MPS 14.1/MT 02 and MPS 14.3/MT 04 from sponge PP.SP.16.14. According to fungi identification result fungus MPS 14.1/MT 02 was identified as *Trichoderma asperellum* while MPS 14.3/MT 04 was identified as *Trichoderma reesei*. Sponge identification leaded the PP.SP.16.14 as *Cinachyrella* sp.

**Key words:** Antibacterial, Multi-drug resistant, Sponge associated-fungi, *Trichoderma*

## 1. Introduction

Irrational used of antibiotic for these several decades leads us to multidrug-resistant (MDR) bacteria era. This problem become a big issue for causing the decrease of cure rate thus allowing the increasing of the death rate [1,2,3]. One of bacteria which very common to infect human is *Escherichia coli*. These bacteria causing urinary tract infection, nosocomial bloodstream infection, meningitis, bacteraemia, and gastrointestinal disease [4,5,6,7]. For several cases showed these bacteria resistant to antibiotics such as *amoxilin-clavunalic acid*, *amikacin*, *aztreonam*, *cefazolin*, *cefuroxime*, *ceftazidime*, *cefotaxime*, *cephalosporin*, *ertapenem*, *imipenem*, *fluoroquinolones*, *fosfomycin*, *gentamycin, meropenem* and *piperacillin-tazobactam* [6,8,9]. This condition is known as multi-drug resistant. The

resistance ability of *E. coli* influenced by the mutation or the presence of particular genes such as *mcr-1* and *tetA* [10,11]. Exploration of new antibiotic could be an answer of large number of ineffective antibiotics to cure *E. coli* infections. Indonesia's marine resources have the potential as source of new antibiotic. Sponge was reported to has antibacterial activity to against several pathogenic bacteria [12,13]. But the exploration of antibiotic compounds from sponge was stunted by conservation issue. To solve this problem, the exploration of new antibiotic from sponge-associated fungi is important. The aims of this research were to isolate marine sponge-associated fungi, to screen potential fungi against MDR *E. coli*, to identify the potential fungi and its host sponge.

## 2. Methodology

### *2.1 Sponges sampling*

Sampling was done at February, 12$^{th}$-13$^{th}$ 2016 in Teluk Awur (S 06˚36'57.4" E 110˚38'19.4") and Panjang Island (S 06˚34'35.2" E 110˚37'52.7") Jepara, Central of Java, Indonesia. The sponges were taken at depths between 1-5 m. Approximately 5 cm$^2$ of sponges were taken and put into ziplock plastic contains marine water for the fungi isolation.

### *2.2 Fungi isolation*

Fungi isolation was done according to [14]. The sponges were cut with size 1x1 cm$^2$ then washed with sterile sea water for three times and then rinsed in ethanol (EtOH) 70% for 60 s for surface sterilization and then sprayed with sterile marine water and dried by the wind. After that put the sponge segments on the petri contained marine *Malt Extract Agar* (MEA) medium then incubated at room temperature for 7 days. For this step, an empty petri was opened as the environmental control. Each fungus which growth from the segment was compared to the control, if the morphology was different, the fungus was moved into new petri as single colony.

### *2.3 Screening antibacterial activity against MDR E. coli*

This step performed using plug method [15,16]. The fungi were refreshed for 7 days and then the mycelia with the MEA medium were cut with circular shape with a diameter 1 cm$^2$. Then put on the petri contained *Nutrient Agar* (NA) that has been inoculated MDR *E. coli*. Petri were incubated for 24 h at room temperature. The clear zone was observed as the antibacterial activity from the fungi. Fungus with biggest clear zone were identified through morphology and molecular approaches.

### *2.4 Fungus identification through molecular approach*

Potential fungus were cultivated in 7 days for DNA extraction. The DNA were obtained according to Turan *et al*. [17]. ITS 1 (5′-TCC GTA GGT GAA CCT GCG G-3′) was used as forward primer while ITS 4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) as reverse primer. PCR mix consist of 1µL of ITS 1, 1µL of ITS 4, 12.5 µL of GoTaq Green Master mix, 10 µL of dd$H_2O$ and 0.5 µL of DNA template. Amplification was performed with cDNA preheat at 95$^o$C for 3 min, 30 cycles of denaturation at 95$^o$C for 1 min, annealing at 51,88$^o$C for 1 min and extension at 72$^o$C for 1 min. The post cycling extension was done at 72$^o$C for 7 min. The PCR products were loaded in agarose gel (1%) for electrophoresis then visualized by UVI Doc HD5 (UVITEC Cambridge).

### *2.5 Fungus morphology observation*

Potential fungus was cultivated using slide culture method [18] for 7 days on MEA. Then the mycelia were observed under microscope with addition of lactophenol cotton blue (LPCB). The morphology characteristics were used for fungus identification.

### *2.6 Sponge identification*

Sponge was identified through morphology and spicules identification. Shape, pit and colors were observed for the early identification. The spicules were obtained by immersed the sponge with chlorine for 30 min the centrifuged for 5 min. The supernatant were discard gently the added with

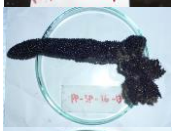
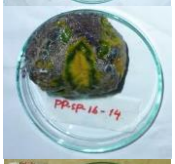
chlorine again. This step were repeated until the transparent spicules seen clearly and then added ethanol (EtOH) 96% for cleaned the residue of the chlorine. After that the spicule were observed under a microscope.

## 3. Result and Discussion

*3.1 Sponges collection*

In total, 9 sponges were successfully collected from two locations. Of these, seven sponges collected from Panjang Island and two sponges collected from Teluk Awur. Mostly the sponges which collected had dark colours such as black, grey with deep yellow colour inside, and deep blue or purple. The colour of sponge usually affected by the light intensity [19]. The shape and characteristic of the sponges could be seen at Tabel 1.

**Tabel 1.** Sponges collection.

| Sample code | Sampling location | Pictures |
|---|---|---|
| PP.SP.16.01 | Panjang island |  |
| PP.SP.16.13 | Panjang island |  |
| PP.SP.16.14 | Panjang island |  |
| PP.SP.16.20 | Panjang island |  |
| PP.SP.16.36 | Panjang island |  |
| PP.SP.16.39 | Panjang island |  |
| PP.SP.16.40 | Panjang island |  |
| TA.SP.16.01 | Teluk Awur |  |
| TA.SP.16.03 | Teluk Awur |  |

*3.2 Sponge-associated fungi*

Sponge-associated fungi were defined as fungi which isolated from sponge where the symbiotic mechanisms between fungi and its host still unknown. In addition, these fungi were very potential for pharmaceutical industry. Sponge-associated fungi such as *Aspergillus similanensis*, *Emericella variecolor, Hypocrea koningii* and, *Trichoderma hazarium* produced new chemical compounds with various biological activities such as antibacteria, antifungi, antioxidant, antitumor, and antihyperlipidemic [20,21,22,23]. There were 29 sponge-associated fungi isolated from 9 sponge collections that shown by Tabel 2.

**Table 2.** Sponge-associated fungi

| No. | Sponge origin | Fungi isolates | No. | Sponge Origin | Fungi isolates |
|---|---|---|---|---|---|
| 1. | TA. SP. 16. 03 | MTS 3.1/MT 01 | 16. | PP. SP. 16. 01 | MPS 1.1/ MT 15 |
| 2. | PP. SP. 16. 14 | MPS 14.1/MT 02 | 17. | TA. SP. 16. 01 | MTS 1.1/ MT 17 |
| 3. | PP. SP. 16. 14 | MPS 14.2/ MT 03 | 18. | TA. SP. 16. 01 | MTS 1.2/ MT 18 |
| 4. | PP. SP. 16. 14 | MPS 14.3/ MT 04 | 19. | TA. SP. 16. 01 | MTS 1.3/ MT 19 |
| 5. | PP. SP. 16. 14 | MPS 14.4/ MT 05 | 20. | PP. SP. 16. 40 | MPS 40.1/ MT 20 |
| 6. | PP. SP. 16. 14 | MPS 14.5/ MT 06 | 21. | PP. SP. 16. 40 | MPS 40.2/ MT 21 |
| 7. | PP. SP. 16. 14 | MPS 14.6/ MT 07 | 22. | PP. SP. 16. 40 | MPS 40.3/ MT 22 |
| 8. | PP. SP. 16. 14 | MPS 14.7/ MT 08 | 23. | TA. SP. 16. 03 | MTS 3.5/ MT 23 |
| 9. | PP. SP. 16. 14 | MPS 14.8/ MT 09 | 24. | PP. SP. 16. 39 | MPS 39.1/ MT 24 |
| 10. | PP. SP. 16. 14 | MPS 14.9/ MT 10 | 25. | PP. SP. 16. 36 | MPS 36.1/ MT 25 |
| 11. | PP. SP. 16. 14 | MPS 14.10/ MT 11 | 26. | PP. SP. 16. 36 | MPS 36.2/ MT 26 |
| 12. | TA. SP. 16. 03 | MPS 3.2/ MT 12 | 27. | PP. SP. 16. 36 | MPS 36.3/ MT 27 |
| 13. | TA. SP. 16. 03 | MPS 3.3/ MT 13A | 28. | PP. SP. 16. 40 | MPS 40.4/ MT 32 |
| 14. | TA. SP. 16. 03 | MPS 3.4/ MT 13B | 29. | PP. SP. 16. 20 | MPS 20.1/ MT 33 |
| 15. | PP. SP. 16. 13 | MPS 13.1/ MT 14 | | | |

The biodiversity of sponge-associated fungi were influenced by the agar media in isolation step. Suitable nutrient in media will give higher biodiversity of isolated associated fungi [24,25]. This research used MEA because this agar medium is the common medium for environmental fungi isolation [14,26].

*3.3 Screening of antibacterial activity*

This research tried to find potential sponge-associated fungi to against pathogenic multi-drug resistant (MDR) *Escherichia coli*. The bacteria was clinical collection from Dr. Kariadi General Hospital Medical Center, Semarang, Central Java. According to antibiotic susceptibility test, this bacteria were resistant to 10 comercial antibiotics such as *Amoxicillin + Clavulanic acid*, *Ceftazidime*, *Ciprofloxacine*, *Cefepime*, *Gentamicin*, *Ampicillin sulbactam*, *Sulfamethoxazol+Trimetropi*, *Tetraciclin*, *Tigeciclin*, and *Piperacillin tazobactam*. Screening of antibacterial activity of 29 sponge-associated fungi was done by plug method. The presence of clear zone around the pluged agar was indicated the antibacterial activity of the fungi [15,16,27]. The result of antibacterial screening is showed by Tabel 3 and Figure 1.

The result of the screening antibacterial activity against MDR *E. coli* showed there were 7 isolates which performed antibacteria activity. Two biggest clear zones were produced by fungi MPS 14.1/MT 02 with inhibition zone was 17.2 $mm^2$ and MPS 14.3/MT 04 with inhibition zone was 16.9 $mm^2$. Furthermore, fungus MPS 14.1/MT 02 and MPS 14.3/MT 04 were identified by macro-microscopic and molecular approaches.

**Table 3**. Screening of antibacterial activity from sponge-associated fungi against MDR *E*. coli

| Fungi isolate | Antibacterial activity | Diameter of clear zone ($mm^2$) |
|---|---|---|
| MTS 3.1/ MT 01 | + | 16.5 |
| MPS 14.1/MT 02 | + | 17.2 |
| MPS 14.2/ MT 03 | - | - |
| MPS 14.3/ MT 04 | + | 16.9 |
| MPS 14.4/ MT 05 | + | 12.2 |
| MPS 14.5/ MT 06 | - | - |
| MPS 14.6/ MT 07 | - | - |
| MPS 14.7/ MT 08 | + | 12.1 |
| MPS 14.8/ MT 09 | - | - |
| MPS 14.9/ MT 10 | - | - |
| MPS 14.10/ MT 11 | - | - |
| MPS 3.2/ MT 12 | - | - |
| MPS 3.3/ MT 13A | - | - |
| MPS 3.4/ MT 13B | - | - |
| MPS 13.1/ MT 14 | - | - |
| MPS 1.1/ MT 15 | - | - |
| MTS 1.1/ MT 17 | - | - |
| MTS 1.2/ MT 18 | - | - |
| MTS 1.3/ MT 19 | - | - |
| MPS 40.1/ MT 20 | - | - |
| MPS 40.2/ MT 21 | - | - |
| MPS 40.3/ MT 22 | - | - |
| MTS 3.5/ MT 23 | + | 16.1 |
| MPS 39.1/ MT 24 | - | - |
| MPS 36.1/ MT 25 | - | - |
| MPS 36.2/ MT 26 | - | - |
| MPS 36.3/ MT 27 | - | - |
| MPS 40.4/ MT 32 | - | - |
| MPS 20.1/ MT 33 | + | 10.9 |

\+ : performed antibacterial activity
\- : not performed antibacterial activity

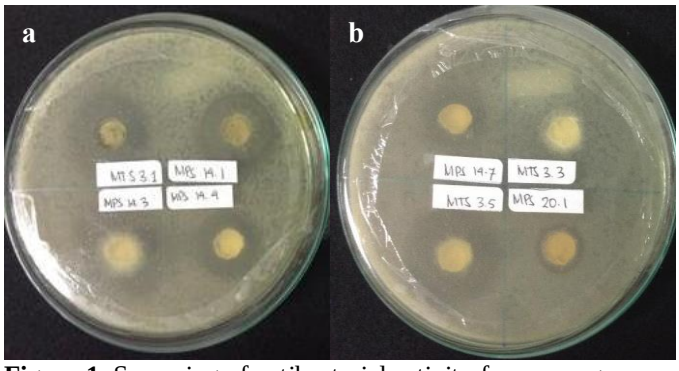



**Figure 1.** Screening of antibacterial activity from sponge-associated fungi.

*3.4 Identification of Potential Fungi*

Fungus MPS 14.1/MT 02 and MPS 14.3/MT 05 had colony distinct. The characteristics of the fungus MPS 14.1/MT 02 colony is shown by Figure 2.

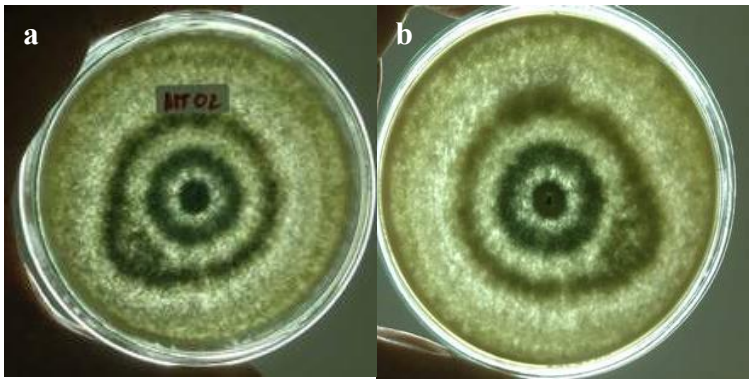



**Figure 2.** Colony of fungus MPS 14.1/MT 02; (a) colony characteristic and (b) reverse side.

Fungus MPS 14.1/MT 02 grew well at room temperature and had green colony with radial growth. This fungus didn't produce pigment in agar media. The microscopic morphology of MPS 14.1/MT 02 could at Figure 3.

Figure 3 shows that fungus MPS 14.1/MT 02 had septa in the hyphae, hyaline conidiophore with primary and secondary branches. The fungus produced sub-globose conidia and chlamydospore. According to the form of colony and its morphology under microscope, fungus MPS 14.1/MT 02 was judged as member of genus *Trichoderma* [28,29]. The colony of fungus MPS 14.3/MT 04 quite different from fungus MPS 14.1/MT 02. This fungus produced white mycelia with greenish yellow radial growth. In addition, fungus MPS 14.3/MT 04 released yellow colour at MEA media (Figure 4).

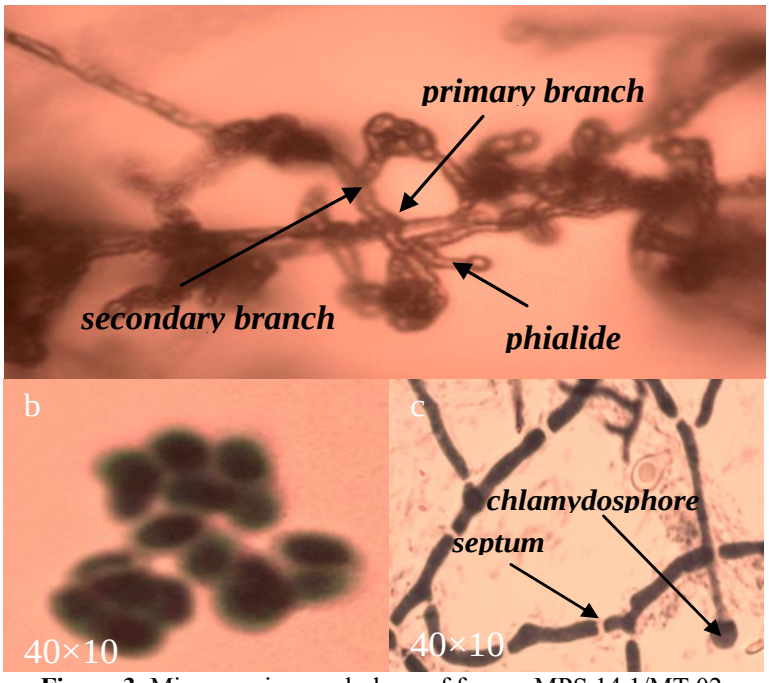



**Figure 3.** Microscopic morphology of fungus MPS 14.1/MT 02; (a) conidiophore, (b). conidia; (c) hyphae

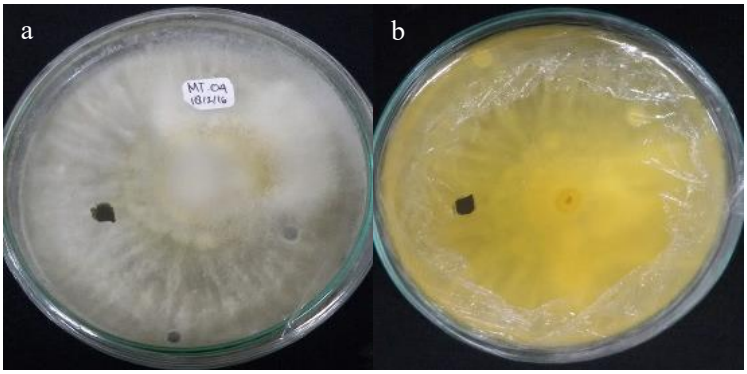



**Figure 4.** Colony of fungus MPS 14.3/MT 04; (a) colony morphology and (b) reverse side.

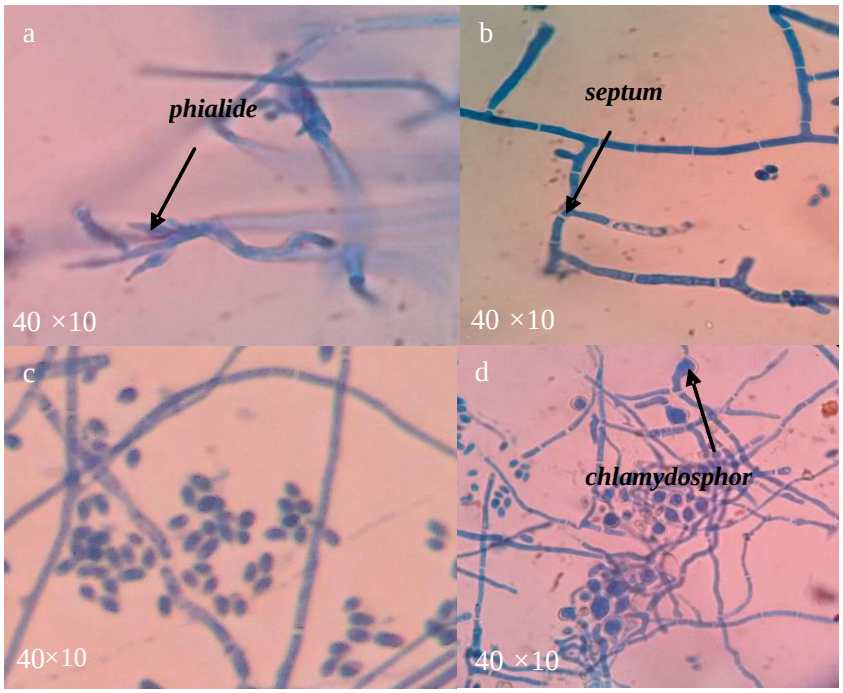



**Figure 5.** Microscopic morphology of fungus MPS 14.5/MT 04; (a) conidiophore, (b) hyphae, (c) conidia, (d) chlamydosphore

Fungus MPS 14.5/MT 04 also had septum and hyaline conidiophore, but braches of this fungus were rarely found. The conidia shape were sub globose and produced chlamydosphore. The characters of MPS 14.3/MT 04 are similar to 14.1/ MT 02 so that this fungus also judge as *Trichoderma*. This judgment were checked using molecular identification. For molecular identification, amplification was

done in *internal transcribed space* (ITS) region because it is conserve region for fungi and usually give best result for fungi identification [30,31,32] The result of BLAST homology could be seen at Tabel 5 while the phylogenetic tree is shown by Figure 6.

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

| Fungi isolate | Molecular identification (BLAST closest relatives) | Similarity |
|---|---|---|
| MPS 14.1/MT 02 | *Trichoderma asperellum* BK261A | 99% |
| MPS 14.3/MT 04 | *Trichoderma reesei* KU377472.1 | 99% |

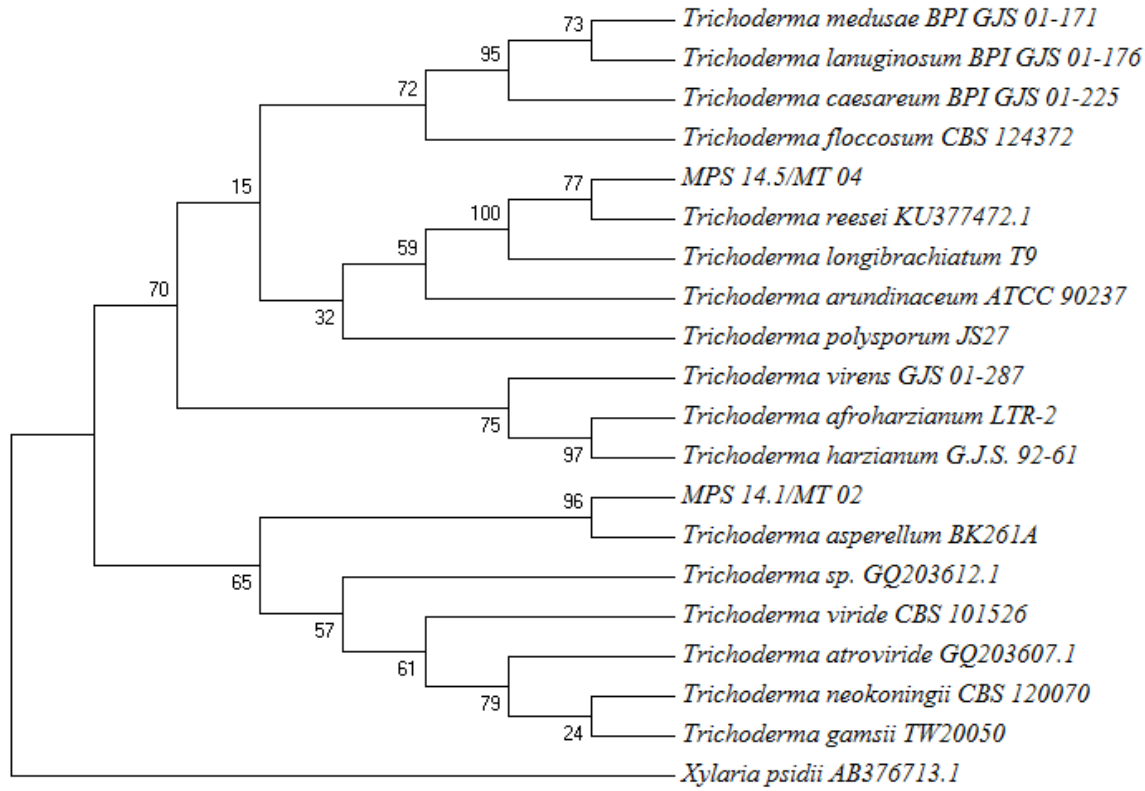



**Figure 6.** Phylogenetic tree of fungi MPS 14.1/MT 02 and MPS 14.5/MT 04

Blast homology search result of the potential sponge-associated fungi identified MPS 14.1/MT 02 as *Trichoderma asperellum* and MPS 14.5/MT 04 as *Trichoderma reesei*. Fungi from genus *Trichoderma* are usually found in soil and terrestrial plant as growth promotor and biocontrol agent to against plant disease [33,34,35,36]. The presence of *Trichoderma* in marine sponge demonstrate the tolerance of this fungi to salinity. Terrestrial fungi with salinity tolerance ability is known as facultative or marine-derived fungi. Several member of this genus were known as marine sponge-associated fungi such as *T. atroviride* and *T. cerinum* [37,38]. Marine-derived *Trichoderma* sp. isolated from Greenland seas produced new antibiotic *pyridones* while *T. longibrachiatum* which isolated from blue mussels produced long-chain peptaibols with antibacterial and antifungal activities [39,40]. *T. asperellum* from marine environment have been studied to produce *asperelines A-F, pertabiols* with antibacterial and

antifungi activities while *trichodermatides A-D* with cytotoxic activity were isolated from marine-derived *T. reesei* [41,42]. Fungi *T. asperellum* MPS 14.1/MT 02 and *T. reesei* MPS 14.3/MT 04 isolated from sponge PP.SP.16.14 so that identification for this sponge was done through morphological and spicules identification.

*3.5. Identification of sponge PP.SP.16.14*

Sponge PP.SP.16.14 was collected from Panjang Island, found in depth less than 5 m and associated with corals. This sponge had characteristics such as globose shape with yellow with green line inside, had pit called as porocalices. This chacteristics owned by family tetillidae [43,44]. The pitures of sponge PP.SP.16.14 under water and outside water could be seen at Figure 7.

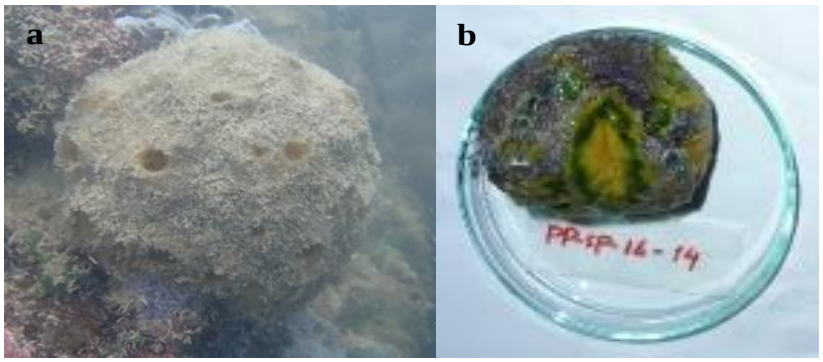



**Figure 7.** Sponge PP. SP. 16.14 (a. under water; b. outside of the water)

For sponge identification, spicules is very important as the specific character. Sponge PP.SP.16.14 had several forms of megascleres which showed by Figure 8.

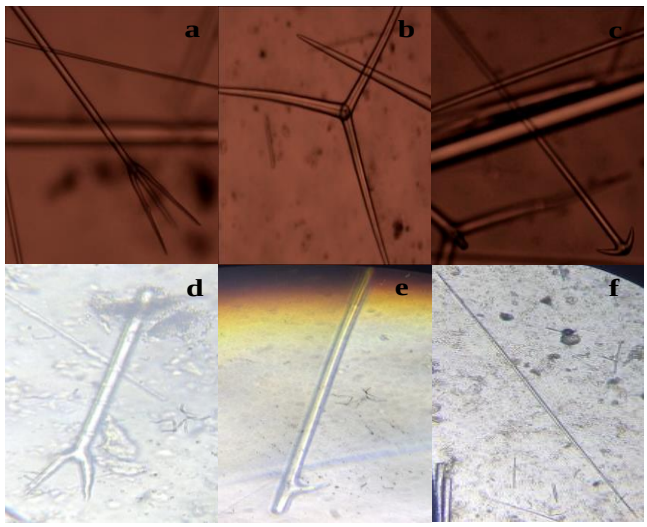



**Figure 8.** Spicule identification; (a) *protriaenes-spicule*, (b) *plagiotriaen-spicule*, (c) *anatriaen-spicule*, (d) *prodiaen-spicule*, (e) *promonoaen-spicule*, (f) *monaxon-spicule*.

In identification guide of sponge from family Tetillidae metion that sponge with specialized pore-bearing pits (porocalices), absence of meganthoxeas and calthrops with no discernible peripheral layer different from that of interior in cross section is member of genus *Cinachyrella* [43,44]. These characteristics are suitable to the characteristics of sponge PP.SP.16.14. So that this sponge was judged as *Cinachyrella* sp. Sponge *Cinachyrella* sp. is known as shallow water sponge and usually founded in tropical countries [45,46].

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