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by Agus Trianto

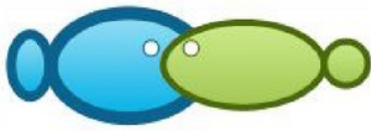
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Antibacterial activity of sponge associated fungi against vibriosis agents in shrimp and its toxicity to *Litopenaeus vannamei*

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Abstract. Whiteleg shrimp (*Litopenaeus vannamei*) is commonly reported to be infected by *Vibrio* spp. which increases the death rate and decreases the productivity. Even though the use of antibiotic is considered effective against the vibriosis, unfortunately, it is prohibited in aquaculture practice. Regarding to this issue, an exploration of biological sources of anti-vibriosis is urgently needed. Our study used sponge associated fungi as source of anti-vibriosis agent. The objectives of this research were to screen the potential of sponge associated fungi against vibriosis agents, to determine the antibacterial activity, to detect the nonribosomal peptide-synthetase (NRPS) gene fragments of potential fungus and to determine the fungal extract lethal concentration (LC₅₀) to *L. vannamei*. There were 8 from 28 of sponge associated fungi which could be revived. From 8 available strains, *Trichoderma asperellum* MT02 showed inhibition zone against *V. harveyi* and *V. alginolyticus*, while *V. parahaemolyticus* was inhibited by *Trichoderma* sp. MT01. Crude extract of *T. asperellum* MT02 showed antibacterial activity against *V. harveyi* and *V. alginolyticus*. The widest inhibition zone was performed against *V. alginolyticus* at concentration 1000 µg mL⁻¹ with LC₅₀ value of 383.70 µg mL⁻¹. The existence of NRPS gene fragments was detected in *T. asperellum* MT02.

Key Words: antibacterial, NRPS, sponge associated fungi, *Trichoderma*.

Introduction. As an archipelago and maritime country, Indonesia has big potential to lead world's aquaculture trade. Tran et al (2017) made several models to forecast Indonesia's aquaculture until 2030. They stated that aquaculture is projected to become the main supplier of fish in 2026. It shows that aquaculture will be an important sector for Indonesia's economy. One of the most favorable aquaculture commodities in Indonesia is whiteleg shrimp (*Litopenaeus vannamei*) because it has high productivity. However, whiteleg shrimp is reported commonly infected by *Vibrio* spp. which increases the death rate and decreases the productivity. In addition, two species from *Vibrio* genus were reported as agents of mass mortality of shrimp in Jiangsu Province, PR China (Zhan et al 2014). Several species of *Vibrio* which ever reported as vibriosis agents were *harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. anguillarum*, *V. campbellii* (Qiao et al 2015; de la Peña et al 2017; Dong et al 2017; Gu et al 2017; Liu et al 2017; Ananda Raja et al 2017). To overcome this problem, several farmers prefer to use antibiotic such as chloramphenicol, enrofloxacin, oxytetracycline and other group of

antibiotic by mixing it with the feed (Chi et al 2017; Maftuch et al 2017). Even though the use of antibiotic is considered effective against the vibriosis, unfortunately, it is prohibited in aquaculture practice.

The usage of antibiotic in aquaculture was scientifically shown the resistance of pathogenic bacteria to the multiple groups of antibiotics (*multidrug-resistant/MDR*) (Igbinsosa 2016). More over, the infections of pathogenic MDR bacteria are more difficult to be handled because they reduce the medical treatment option and increase the death rate of shrimp from previously curable infections. Besides, the use of antibiotic in aquaculture practice could give impact to human health. In their review, Mo et al (2017) stated that the chronic exposure of antibiotic could lead to steatosis, aplastic anemia and leukemia. Regarding this issue, an exploration of a biological source of anti-vibriosis is urgently needed. Marine derived fungi are known as potential source of bioactive compounds. Several studies reported the antimicrobial activity of sponge associated fungi against pathogen bacteria (Sibero et al 2016; Sibero et al 2017a; Trianto et al 2017). However, a study of potential marine derived fungi against vibriosis agents is rarely found. The latest report of this study was done by Wahjuningrum et al (2016). They formulated the crude extract of marine derived fungus *Nodulisporium* sp. KT29 into feed of *L. vannamei* to prevent the infection of *V. harveyi*. This research highlighted the better survival rate of *L. vannamei* after the challenge test treated by the administration of *Nodulisporium* sp. KT 29. It shows that marine derived fungi have a great potential as substitution of antibiotic use in shrimp. Our study used sponge associated fungi as source of anti-vibriosis agent. The objectives of this research were to screen the potential sponge associated fungi against vibriosis agents, to determine the antibacterial activity, to detect the nonribosomal peptide-synthetase (NRPS) genes fragment of potential fungus and to determine the fungal extract lethal concentration (LC_{50}) to *L. vannamei*.

Material and Method. This study was done from April until August 2017. We used culture collection of sponge associated fungi from Division of Marine Fungi, Laboratory of Tropical Marine Biotechnology, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang, Central Java, Indonesia. Vibriosis agents in this study were *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* from Center for Brackish Water Aquaculture (BBPBAP) Jepara, Central Java, Indonesia. *L. vannamei* were caught from the shrimp pond at Japara with range size 10-12 cm.

The preparation of sponge associated fungi. Fungi collection was revived by cultured it in *Potato Dextrose Broth* (PDB) with addition of chloramphenicol (2%) for 4 days with agitation using shaker (110 rpm) (Sibero et al 2016). After that, the mycellium were taken then cut with sterilized cutter and put on PDA for incubation (27°C, 5 days). After 5 days, the fungal collony morphology was compared to the previous morphology. Fungi with same morphology were used for the further steps.

The preparation of bacteria. This research used *Vibrio harveyi* and *Vibrio alginolyticus*. It were revived on trypticase soy agar (TSA) then incubated in incubator for 24 h (32°C).

Screening of anti-vibriosis agent activity. This step was done by agar plug diffusion method based on Balouiri et al (2016). All revived fungi were cultivated on PDA without antibiotic for 7 days. While, *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* were cultivated on TSA agar for 24 h. For antimicrobial test, the bacteria were dilluted in physiological salts to be 0.5 McFarland. After that, the bacteria were inoculated on TSA using sterilized cotton bud with circle pattern until the whole agar surface was covered by the bacteria colony. Then, the fungi were cut with circle shape and put onto the TSA surface for incubation (24 h, 32°C). The presence of clear zone indicated the potential fungi.

Fungal metabolite production. Potential fungus was cultivated in *Malt Extract Broth* (MEB) for 15 days in static condition at 24°C. The mycelium was separated from the broth medium. Ethyl acetate was added into broth medium with ration 1:3

(broth:solvent) then agitated using shaker for 24 h. The solvent was taken for evaporation (35°C).

In vitro antibacterial activity. Fungal extracts were diluted in DMSO to 5 concentrations (50, 125, 250, 500 and 1000 µg mL⁻¹). Chloramphenicol disc 30 µg (Oxoid) was used as positive control. Amount 10 µL of each concentration was injected into paper disc (6 mm) then placed on TSA with tested bacteria. They were incubated for 24 h at 32°C. The antibacterial activity of the fungal extract was shown by the presence of the clear zone (Sibero et al 2017b).

Detection of NRPS genes fragment. Fungal DNA was extracted using Chelex Method (Sibero et al 2016; Sibero et al 2017a, b). NRPS genes fragment was detected using primer A2gamForward (5'-AAG GCN GGC GSB GCS TAY STG CC-3') and A3gamReverse (5'-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3') from MacroGen (Radjasa & Sabdono 2008). Fungal DNA which amplified in ITS region was used as positive control. PCR mix (12.5 µL) for this step consisted of 12.5 µL of GoTaq Green Master mix from Promega Corporation (2800 Woods Hollow Road Madison, WI 53711-5399 U.S.A), 1 µL of primer forward, 1 µL of primer reverse, 10 µL of ddH₂O and 0.5 µL of DNA template (Sibero et al 2017a, b). NRPS genes fragment was amplified using this following condition: preheat at 95°C for 3 min, denaturation at 95°C for 1 min, annealing at 51.88°C for 1 min and extension at 72°C for 1 min while the post cycling extension was done at 72°C for 7 min. Denaturation, annealing and extension were performed 30 cycles (Sibero et al 2017a).

Toxicity to *Litopenaeus vannamei*. Determination of fungal extract toxicity to *L. vannamei* was carried out using method by Ajoy & Padma (2013). *L. vannamei* were caught from the shrimp pond at Japara with range size 10-12 cm. Shrimp acclimatization was done for 1 week in aquarium with temperature ambient and the salinity was 29 ppt. Bubbling air was aimed to produce oxygen content. The shrimp were treated with various fungal extract concentrations (50, 100, 250, 500 and 750 µg mL⁻¹). Chloramphenicol was used as positive control. For each concentration, 20 shrimps were treated with three repetitions. Died shrimps were counted after 24 h. The 50% lethal concentration (LC₅₀) was calculated using the probit analysis.

Data analysis. Data analysis was carried out using SPSS statistical package version 18.0 with confidence interval 95%.

Result and Discussion

Screening of anti-vibriosis agent activity. This research used sponge associated fungi from previous isolation (Sibero et al 2017a). From 28 isolates there were only 8 fungi isolates which able to be revived. They were *Trichoderma* sp. MT01, *Trichoderma asperellum* MT02, unidentified fungus MT06, *Trichoderma* sp. MT07, unidentified fungus MT11, *Penicillium* sp. MT22, unidentified fungus MT26 and *Penicillium* sp. MT27. These fungi were tested against pathogenic *V. harveyi* and *V. alginolyticus*. The result of this assay is shown by Table 1 and Figure 1.

To screen the potential fungi, agar plug diffusion method was carried out. This method is an antagonism assay between microorganisms, in this case was between sponge associated fungus and vibriosis agent. Table 1 and Figure 1 show the result of this assay. It was obviously that only fungus *T. asperellum* MT02 could inhibit *V. harveyi* (1a) and *V. alginolyticus* (1b); in contrast only *Trichoderma* sp. MT01 (1c) inhibited *V. parahaemolyticus*. Fungi produced extracellular metabolites during their growth. Extracellular metabolites are the metabolites which secreted out of the cells (Sibero et al 2016; Sibero 2017b). The presence of clear zone was manifested that the fungus produced extracellular metabolites with antibacterial activity which diffused into the malt extract agar (MEA) medium. When the MEA medium was placed on the inoculated TSA, fungal extracellular metabolites in MEA were diffused onto TSA and inhibited the growth of vibriosis agents (Balouiri et al 2016; Sibero et al 2017a). According to the result of this

step, only fungus *T. asperellum* MT02 was carried to the further steps. Previously, *T. asperellum* MT02 was reported to have antibacterial activity against *E. coli* strain MDR (Sibero et al 2017a). Wu et al (2017) found that *T. asperellum* GDFS1009 produced several primary metabolites which as precursors of antimicrobial compounds and produced secondary metabolite with antibacterial activity including polyketides, alkenes and peptides. This fungus was cultivated in broth medium for extracellular metabolite production.

Table 1
Antibacterial activity of sponge associated fungi against *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* with plug method

Fungal isolates	Vibriosis agent		
	<i>V. harveyi</i>	<i>V. alginolyticus</i>	<i>V. parahaemolyticus</i>
<i>Trichoderma</i> sp. MT01	-	-	+
<i>T. asperellum</i> MT02	+	+	-
Unidentified fungus MT06	-	-	-
<i>Trichoderma</i> sp. MT07	-	-	-
Unidentified fungus MT11	-	-	-
<i>Penicillium</i> sp. MT22	-	-	-
Unidentified fungus MT26	-	-	-
<i>Penicillium</i> sp. MT27	-	-	-

(+) presence of clear zone; (-) absence of clear zone.

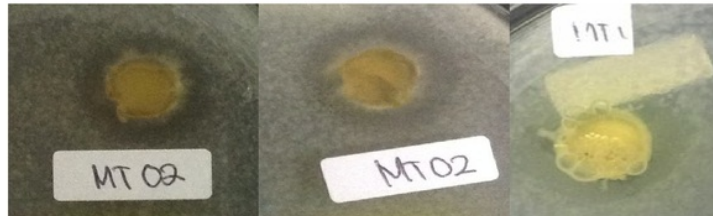


Figure 1. Presence of clear zone for screening of potential fungus against a) *V. harveyi*, b) *V. alginolyticus*, c) *V. parahaemolyticus*

In vitro antibacterial activity against vibriosis agents. Fungal extract was diluted to five different concentrations to know the widest inhibition zone which could performed. The result of this test is shown by Figure 2.

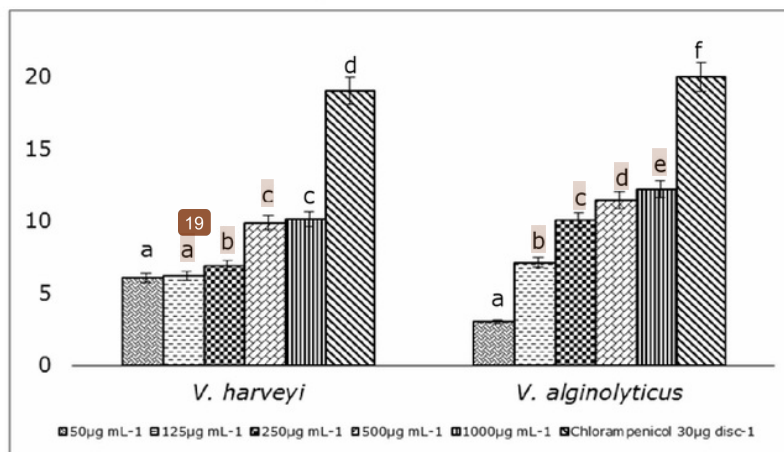


Figure 2. Inhibition zone of *T. asperellum* MT02 in different concentration against vibriosis agent *V. harveyi* and *V. alginolyticus*. Different superscript letters in the same group indicate significant different results ($p < 0.05$).

Figure 2 shows that each concentration of fungal crude extract inhibited *V. harveyi* and *V. alginolyticus*. In *V. harveyi*, there was no significant difference of inhibition zone diameter between 500 $\mu\text{g mL}^{-1}$ (9.87 ± 0.10 mm) and 1000 $\mu\text{g mL}^{-1}$ (10.15 ± 0.11 mm). On the other hand, all concentrations showed significant different in diameter of inhibition zone against *V. alginolyticus*. The widest diameter of inhibition zone was performed by concentration 1000 $\mu\text{g mL}^{-1}$ with value 12.20 ± 0.15 mm then followed by 500 $\mu\text{g mL}^{-1}$ with value 11.43 ± 0.04 mm. However, there was none concentration which performed wider inhibition zone than Chloramphenicol. We highlight that fungus *T. asperellum* had strongest antibacterial activity, and it was performed against *V. alginolyticus*. Sponge associated fungi are known as potential source of antibacterial compounds. Özkaya et al (2017) extracted 70 strains of sponge associated fungi to combat several pathogenic bacteria in aquaculture. In their study, they found there were six fungi showing antibacterial activity against *V. anguillarum*. In addition, crude extract of *Trichoderma* species was reported to have antimicrobial activity against *V. harveyi* and *V. parahaemolyticus* with MIC 150 $\mu\text{g mL}^{-1}$ and 200 $\mu\text{g mL}^{-1}$ (Narendran & Kathiresan 2016). Marine-derived *Trichoderma* is commonly reported as peptaibols producer. Peptaibols are biological active peptides with great antimicrobial activity (Mohamed-Benkada et al 2016; Arinbasarova et al 2017). Moreover, Ren et al (2013) reported 38 peptaibols from marine-derived fungus *T. asperellum*. We proved that fungus *T. asperellum* MT02 produced antimicrobial peptaibols to inhibit *V. harveyi* and *V. alginolyticus*. To support this allegation, we detected NRPS genes fragment in this fungus. Figure 3 shows the result of NRPS genes fragment detection in fungus *T. asperellum* MT02.

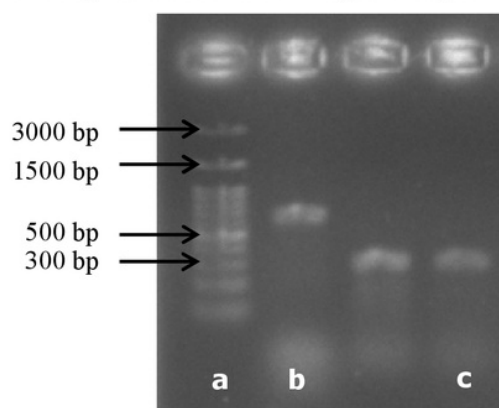


Figure 3. NRPS genes fragment of *T. asperellum* MT02
a) marker, b) control c) NRPS genes fragment of *T. asperellum* MT02.

Figure 3 shows the existence of NRPS genes in *T. asperellum* MT02. The NRPS gene fragments were amplified and showed band at 300 bp (3c). Tambadou et al (2014) reviewed several primers used for amplification of NRPS gene fragments. They stated that set primers A2gamF/A3gamR will amplify fragments of 300 bp. Other primers which amplified fungal NRPS gene fragments are MTR (5'-GCNNGYGGYGCNTAYGTNCC-3') and MTF (5'-CCNCGDATYTTNACYTG-3') (Vizcaino et al 2005). NRPS genes fragments will encode the multi-enzyme complex called non-ribosomal peptide synthetases (NRPSs). This multi-enzymes synthesize various secondary metabolites include peptaibols with antimicrobial potential, extracellular siderophore and toxins (Brito et al 2014; Bansal & Mukherjee 2016). This result supported the allegation that fungus *T. asperellum* MT02 produced peptaibols compounds. However, further analyses are needed to prove this allegation, such as mass spectrometry.

Toxicity to *Litopenaeus vannamei*. The toxicity (LC_{50}) of fungal crude extract was done to *L. vannamei*. The purpose of this step was to know the potential of fungal crude extract from *T. asperellum* MT02 to be shrimp feed to prevent vibriosis in *L. vannamei*.

Lethal concentration 50 (LC₅₀) will show the minimum concentration which able to kill 50% of the treated *L. vannamei*. The result of this assay is shown in Table 2.

Table 2
Toxicity (LC₅₀) of *T. asperellum* MT02 crude extract to *L. vannamei*

	Concentration ($\mu\text{g mL}^{-1}$)						LC ₅₀ ($\mu\text{g mL}^{-1}$)
	0	10	100	250	500	750	
Mortality (%)	6.66	11.66	18.33	38.33	55.00	70.00	383.70

According to toxicity test, *T. asperellum* MT02 was known to have LC₅₀ value 383.70 $\mu\text{g mL}^{-1}$ (Table 2). It means, fungal crude extract killed 50% of *L. vannamei* population with concentration 383.70 $\mu\text{g mL}^{-1}$. We compared the LC₅₀ value to its antimicrobial activity (Figure 2). Previously, we highlighted that 1000 $\mu\text{g mL}^{-1}$ was the best concentration to inhibit *V. harveyi* and *V. alginolyticus*. Unfortunately, according to toxicity test more than 383.70 $\mu\text{g mL}^{-1}$ of extract could kill more than 50% population of *L. vannamei*. So that, we concluded that crude extract from *T. asperellum* MT02 was not potential to be formulated as shrimp feed to prevent vibriosis infection in *L. vannamei*. The high toxicity of crude extract from *T. asperellum* MT02 was judged as result of accumulation of various compounds including mycotoxins. *Genus Trichoderma* also reported as toxins producer such as trichothecene and gliotoxin (Cardoza et al 2011; Zeilinger et al 2016; Zhang et al 2017). Moreover, *T. asperellum* was also reported to produce toxin named Trichotoxins (Brito et al 2014). These toxins are compounds which are produced by NRPS also (Bansal & Mukherjee 2016; Zeilinger et al 2016). The existence of NRPS gene fragments in *T. asperellum* MT02 was alleged not only to produce peptaibols as antimicrobial but also mycotoxin which influenced the toxicity of fungal crude extract to *L. vannamei*.

Conclusions. There were 8 from 28 of sponge associated fungi which could be revived. From 8 available strains, *T. asperellum* MT02 showed inhibition zone against *V. harveyi* and *V. alginolyticus* while *V. parahaemolyticus* was inhibited by *Trichoderma* sp. MT01. Crude extract of *T. asperellum* MT02 showed antibacterial activity against *V. harveyi* and *V. alginolyticus*. The widest inhibition zone was performed against *V. alginolyticus* at concentration 1000 $\mu\text{g mL}^{-1}$ with LC₅₀ value of 383.70 $\mu\text{g mL}^{-1}$. The existence of NRPS gene fragments was detected in *T. asperellum* MT02.

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