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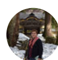

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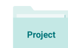

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Antibacterial Activity of Indonesian Sponge Associated Fungi Against Clinical Pathogenic Multidrug Resistant Bacteria

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ABSTRACT

Exploration of new source of antibiotics to combat multidrug-resistant bacteria is urgently needed. Indonesia as archipelago country has a wide variety of marine organisms with potential as source of new antibacterial compounds against MDRO. Aims of the study were to isolate sponge-associated fungi from sponge *Cinachyrella* sp. collected from Pandang Island, North Sumatera, Indonesia, to screen potential fungi against clinical pathogenic MDR bacteria, to identify the potential fungi; and to determine the best cultivation time for antibacterial production. Nine sponge-associated fungi were successfully isolated. Result of agar plug method showed fungus PDSP 5.7 was the most potential candidate which inhibited ESBL *Escherichia coli*, *Salmonella enterica* ser. Typhi, MRSA, and *Staphylococcus haemolyticus* strain MDR. This fungus had 100% similarity to Trichoderma reesei. In malt extract broth, *T. reesei* PDSP 5.7 had stationary phase from day 12 to day 18. In addition, the widest antibacterial was performed by extract from day 15. Furthermore, fungal extract showed best antibacterial activity against *S. enterica* ser. Typhi strain MDR with inhibition value of 14.72 ± 0.07 mm^2.

INTRODUCTION

The massive number of multidrug-resistant organisms (MDRO) infections nowday is caused by the irrational use of antibiotic in several decades ago (Alanis, 2005; de Simone *et al*., 2016; Cansizoglu and Toprak, 2017). Several pathogenic bacteria such as extended-spectrum beta-lactamase (ESBL) *E. coli*, methicillin-resistant *S. aureus* (MRSA), *Staphylococcus haemolyticus*, and *Salmonella enterica* ser. Typhi have been isolated from patients and identified as infectious MDRO (Hosseinkhani *et al*., 2016; Goudarzi *et al*., 2017; Lugito and Cucunawangsih, 2017; Shakya *et al*., 2017). As MDRO, these bacteria are resistant to antibiotics so that the urgency of finding the new antibiotic candidate has become very important.

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Previous studies discovered new antibacterial compounds from marine organisms such as algae, coral and sponge (Liu *et al*., 2014; Abdel-Raouf *et al*., 2015; Nguyen *et al*., 2017). Among all marine organisms, sponge is the most studied as source of bioactive compounds (Mehbub *et al*., 2014; Kumar and Pal, 2016). Beesoo *et al*. (2017) reported extract from *Neopetrosia exigua* contained beta-sitosterol and cholesterol. This extract inhibited *S. aureus* and *Bacillus cereus* with MIC and MBC values of 0.039 mg/mL and 0.078 mg/mL. Furthermore, Wright *et al.* (2017) isolated dragmacidin G from *Spongosorites sp*. with broad spectrum antibacterial activity against *S. aureus* and *Mycobacterium tuberculosis*. At the same year, Nguyen *et al*. (2017) successfully discovered langcoquinone C and smenospongorine from *Spongia sp*. which had significant antibacterial against *B. subtilis* and *S. aureus* with MICs ranging from 6.25 to 25 µM. Admittedly, exploration of new antibiotics candidate from sponges leads to environmental issue. So that, the utilization of their associated microorganisms is commonly applied.

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Marine sponge-associated fungi are also known as a potential source for discovery of new antibiotic candidate (Handayani and Artasasta, 2017; Sibero *et al*., 2017). In addition, several discoveries were successfully isolate new compounds from sponge-associated fungi with biological activities (Zin *et al*., 2016; Noinart *et al*., 2017). Furthermore, Liu *et al*. (2017) isolated 11 compounds from sponge-derived fungus *Aspergillus sydowii* J05B-7F-4. Among 11 compounds, there were 5 diphenylethers which had antibacterial activity against human pathogen *S. aureus*. Fungus *Pestalotiopsis heterocornis* which isolated from sponge *Phakellia fusca* was reported to produce isocumarins 1-3. These compounds had antibacterial activity against *S. aureus* and *B. subtilis* with MIC values ranging from 25 to 100 μg/mL (Lei *et al*. 2017). Unfortunatelly, there are a few researches which study antibacterial activity of sponge-associated fungi against multidrug-resistant bacteria. Aims of the study were to isolate sponge-associated fungi from sponge *Cinachyrella* sp. collected from Pandang Island, North Sumatera, to screen potential fungi against clinical pathogenic MDR bacteria, to identify the potential fungi, and to determine the best cultivation time for antibacterial production.

MATERIAL AND METHODS

Sponge *Cinachyrella* sp.

Sponge *Cinachyrella* sp. was collected from Pandang Island, North Sumatera, Indonesia from a depth of 2 m by snorkeling. After the collection, approximietly 5 × 5 cm^2 sample was cut and kept in sterile zip lock plastic for fungal isolation.

Clinical pathogenic MDR bacteria

The clinical pathogenic MDR bacteria used in this study were clinical collections from Dr. Kariadi General Hospital Medical Center, Semarang, Central Java, Indonesia. ESBL *E. coli*, MRSA, *S. haemolyticus* and *S. enterica* ser. Typhi were used as test bacteria. ESBL and *S. enterica* ser. Typhi were re-cultured on MacConkey M081B agar from HiMedia while MRSA and *S. haemolyticus* were re-cultured on Nutrient Agar CM0003 from Oxoid Ltd. at 37°C for 24 h.

Fungal isolation and purification

This study applied surface sterilization methods from Kjer *et al.* (2010) and Sibero *et al.* (2017) for fungal isolation with several modifications. Firstly, sponge was cut into 3 pieces in size 2 × 1 cm^2 and washed using sterile marine water then was immersed in alcohol 70% for 60 s. After that, sponge was washed using sterile marine water to clean the alcohol residue. Lastly, each piece was tapped on a Malt Extract Agar (MEA) M137 HiMedia as a control of quality of surface sterilization method before placed on other MEA and incubated for 7 days (27°C). During fungal isolation, a petri dish with MEA was opened as an environmental control. The fungal growth was observed daily. Each mycelium which grew on sponge was isolated and transferred to a new medium for further step.

Antibacterial activity screening of sponge-associated fungi

Agar plug method was carried out for antibacterial activity screening. All fungal isolates were refreshed on MEA for 7 days at 27°C while MDR bacteria were re-cultured for 24 h at 37°C and diluted to be 0.5 McFarland in physiological saline solution. After that, MDR dilution was inoculated on Mueller Hinton Agar (MHA) from Merck KGaA. Each fungus with its agar medium were cut in circle shape then placed on an inoculated MHA medium and incubated for 24 h at 37°C. Antibacterial activity was indicated by the presence of clear zone around fungal colony (Rahaweman *et al*., 2016; Sibero *et al*., 2017). Fungal isolate which able inhibited all tested bacteria was used for the further step as potential fungus.

Table 1: Antibacterial activity of sponge-associated fungi.

Fungal Isolate	Presence of inhibition zone			
	ESBL *E. coli*	*S. enterica* ser. Typhi	MRSA	*S. haemolyticus*
PDSP 5.1	-	-	+	+
PDSP 5.2	-	-	-	-
PDSP 5.3	-	-	-	-
PDSP 5.4	+	-	-	-
PDSP 5.5	-	-	-	-
PDSP 5.6	-	-	-	-
PDSP 5.7	+	+	+	+
PDSP 5.8	+	-	-	+
PDSP 5.9	+	-	-	+

(+: presence of inhibition zone; -: absence of inhibition zone).

Fungal identification

Identification through macro-microscopic and molecular approaches was carried for this study. Fungus was grown on MEA for 7 days. Microscopic characterization was done using slide culture method (Qiu *et al*., 2005). Furthermore, mycelia were taken for DNA extraction using Chelex 100 method. For DNA amplification, PCR mix was consisted of 12.5 μL of GoTaq Green Master mix from *Promega Corporation*, 1 μL of ITS 1, 1 μL of ITS 4, 10 μL of ddH2O and 0.5 μL of DNA template. In addition, primer ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) from Macrogen were applied. Fungal DNA was amplified with these following conditions: cDNA preheat at 95°C for 3 m, 30 cycles of denaturation at 95°C for 1 min, annealing at 51.80°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. PCR product was sequenced by 1st BASE DNA Laboratories Sdn Bhd, Malaysia. Lastly, phylogenetic three of potential fungus was reconstructed using MEGA.7 software package (Sibero *et al*., 2017).

Fungal cultivation in broth medium for antibacterial assay and growth curve

Potential fungus was cultivated in 250 mL of *Malt Extract Broth* (MEB) from Difco™ for 21 days at 27°C. Fungus was harvested every three days. Fungal mycelia were separated using filter paper (Advantec 7, Ø 125 mm). Filter paper had been dried in oven at 50°C for 24 h and weighed before used as blank (W_o). After separation, filter papers contained mycelia were dried

in oven (50°C) for 24 h then weighed (W_t). Mycelial weights was obtained using following simple mathematical formula and used to construct fungal growth curve while the broth media was used for further step. pH of media in each harvesting day was measured using pH meter.

$$W_m = W_t - W_o$$

Extraction of fungal metabolite

Ethyl acetate was used for extraction of fungal metabolite. The ratio of media to solvent was 1:2 (v/v). Solvent were separated from media using separatory funnel then the solvent were evaporated using rotary evaporator at 37°C.

Table 2: Antibacterial activity of fungal extract from different harvesting time.

Bacteria	Day	Inhibition Zone (mm)
ESBL *E. coli*	3	0.00 ± 0.00[a]
	6	3.25 ± 0. 07[b]
	9	4.20 ± 0.28[c]
	12	5.45 ± 0.49[d]
	15	9.09 ± 0.14[g*]
	18	8.20 ± 0.00[f]
	21	7.58 ± 0.16[e]
S. enterica ser. Typhi	3	0.00 ± 0.00[a]
	6	2.40 ± 0.09[b]
	9	3.37 ± 0.72[c]
	12	10.76 ± 0.04[d]
	15	14.78 ± 0.07[f*]
	18	14.64 ± 0.06[f]
	21	13.47 ± 0.04[e]
MRSA	3	0.00 ± 0.00[a]
	6	3.05 ± 0.28[b]
	9	4.56 ± 0.14[c]
	12	5.28 ± 0.07[d]
	15	7.40 ± 0.14[f*]
	18	7.25 ± 0.04[f]
	21	7.09 ± 0.02[e]
S. haemolyticus	3	0.00 ± 0.00[a]
	6	2.20 ± 0.01[b]
	9	3.38 ± 0.01[c]
	12	3.89 ± 0.14[d]
	15	6.81 ± 0.11[g*]
	18	6.48 ± 0.07[f]
	21	6.24 ± 0.02[e]

(Data are mean ± SD. Values with different shared letters in each MDR denote significant difference at $P < 0.05$. Values with * denote the highest antibacterial activity in each MDR bacteria).

***In vitro* antibacterial assay of fungal extract**

Fungal extract was diluted to 50 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL then tested against ESBL *E. coli*, MRSA, *S. haemolyticus* and *S. enterica* ser. Typhi according to CLSI (2016). The bacteria were inoculated on MHA using sterile cotton bud then 10 µL of each concentration was injected into the paper disc (Ø 6 mm Oxoid™) and placed onto the MHA medium then incubated for 24 h at 32°C. Amoxicillin + Clavulanic acid (30 µg/disc, Ø 6 mm Oxoid™) was used as positive control. The presence of clear zone indicated the antibacterial activity. Determination of antibacterial activity was done with two replications.

Data analysis

Data were analyzed using factorial design in SPSS software package version 18.0 for Windows with confidence interval 95% ($P < 0.05$).

Table 3: Antibacterial activity of fungal extract at 15 days of cultivation.

Bacteria	Day	Inhibition Zone (mm)
ESBL *E. coli*	50	4.13 ± 0.06[a]
	125	5.30 ± 0.28[b]
	250	8.72 ± 0.07[c]
	500	8.83 ± 0.08[c]
	1000	9.10 ± 0.14[c]
	Amoxicillin + Clavulanic acid	12.50 ± 0.14[d*]
S. enterica ser. Typhi	50	8.19 ± 0.02[a]
	125	8.49 ± 0.04[a]
	250	11.90 ± 0.42[b]
	500	14.40 ± 0.15[c]
	1000	14.72 ± 0.07[c*]
	Amoxicillin + Clavulanic acid	12.20 ± 0.28[b]
MRSA	50	5.03 ± 0.02[a]
	125	7.59 ± 0.14[b]
	250	7.75 ± 0.21[b]
	500	8.40 ± 0.14[c]
	1000	8.97 ± 0.09[d*]
	Amoxicillin + Clavulanic acid	8.86 ± 0.04[d]
S. haemolyticus	50	5.09 ± 0.16[a]
	125	7.60 ± 0.23[b]
	250	8.10 ± 0.14[c]
	500	8.21 ± 0.00[c]
	1000	9.04 ± 0.20[d]
	Amoxicillin + Clavulanic acid	10.25 ± 0.07[e*]

(Data are mean ± SD. Values with different shared letters in each MDR denote significant difference at $P < 0.05$. Values with * denote the highest antibacterial activity in each MDR bacteria).

RESULT AND DISCUSSION

There were 9 sponge associated fungi isolated from *Cinachyrella* sp. Each fungus had been screened against four clinical pathogenic MDR. During its growth on agar medium, fungus PDSP 5.7 secreted extracellular metabolites into medium. In agar plug method, fungal MEA medium contained extracellular

metabolite were plugged on agar medium inoculated with tested MDR bacteria. In incubation time, fungal extracellular metabolites diffused from plug to the agar medium to kill tested MDR bacteria. The appearance of inhibition zone around the agar plug indicated the antibacterial activity (Balouiri *et al*., 2016; Rahaweman *et al*., 2016; Sibero *et al*., 2017). This study used ESBL *E. coli* and *S. enterica* ser. Typhi as representatives of gram negative bacteria while MRSA and *S. haemolyticus* as representatives of gram positive bacteria. The result of the screening with agar plug method is presented by Table 1.

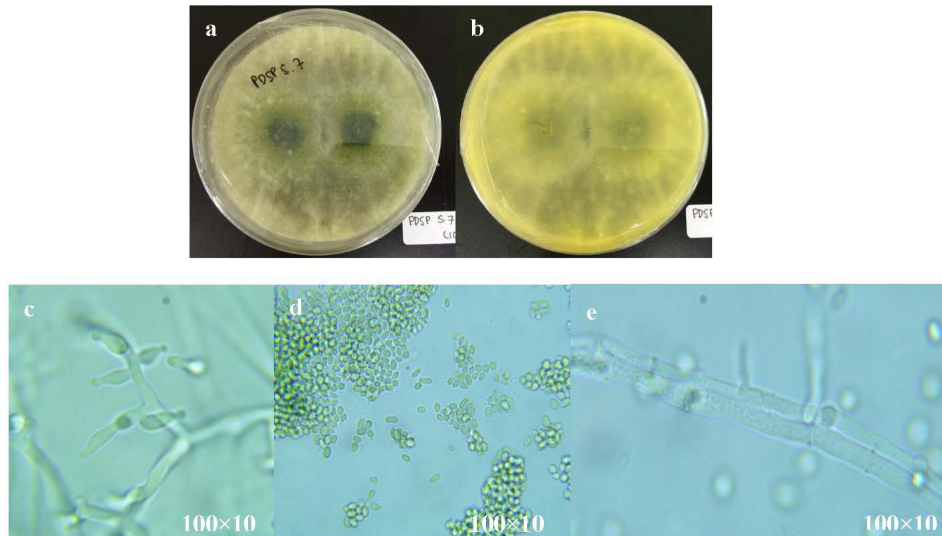

Fig. 1: Morphological characteristics of fungus PDSP 5.7. (a. top side, b. reverse side, c. *conidiophore*, d. conidia, e. *hyphae*).

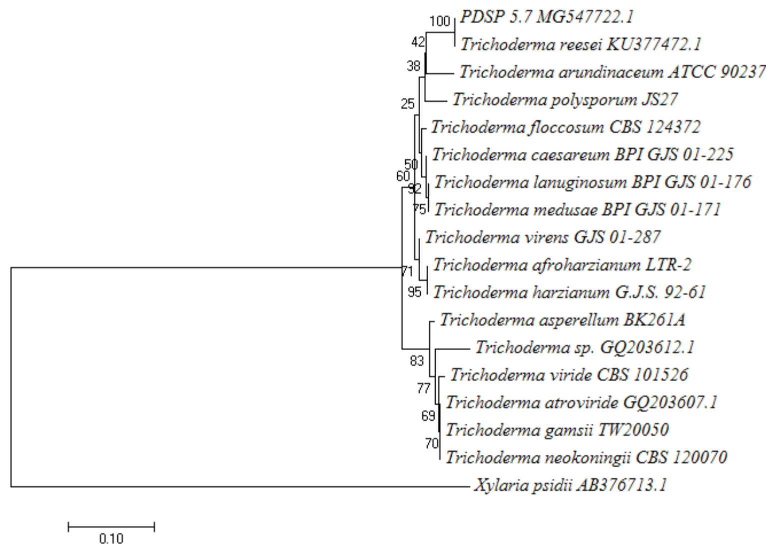

Fig. 2: Phylogram of genetic distance of fungus PDSP 5.7 using ITS rRNA region.

Table 1 shows 5 fungi which had antibacterial activity against clinical pathogenic MDR bacteria. Fungus PDSP 5.1 showed antibacterial activity against gram positive MDR, fungus PDSP 5.4 only showed antibacterial activity against ESBL *E. coli*, fungus PDSP 5.8 and PDSP 5.9 inhibited ESBL *E. coli* and *S. haemolyticus*. By contrast, fungus PDSP 5.7 had antibacterial activity against all tested MDR bacteria. Therefore, fungus PDSP 5.7 was selected as potential isolate and used further steps.

As a potential isolate, fungus PDSP 5.7 was identified with macro-microscopic and molecular approaches. Figure 1 (a-b) show fungal colony on MEA. It had white colony with green circular pattern on the middle of it. At room temperature (27°C), this fungus produced yellow extracellular pigment which secreted into the agar medium. Figure 1 (c-e) show fugal microscopic morphology of fungus PDSP 5.7. This fungus had branching conidiophore with lageniform phialides, conidia ellipsodial nearly to oblong and septum in hyphae. These characteristics leaded

fungus PDSP 5.7 to be judge as member of genus *Trichoderma* (Rahman *et al*., 2011; Qin and Zhuang 2016; Sibero *et al*. 2017). Molecular analysis was done for the further identification. The phylogenetic tree of fungus PDSP 5.7 is shown by Figure 2.

The phylogram shows the homology comparison of fungus PDSP 5.7 to several *Trichoderma* species. This fungus had 100% similarity to *Trichoderma reesei* KU377472.1. Fungi from genus *Trichoderma* are commonly isolated as sponge-associated fungi (Sibero *et al*., 2016; Mohamed-Benkada *et al*., 2016; Pang *et al*., 2017). *T. reesei* MPS 14.5/MT 04 was also found as fungal associate in *Cinachyrella* sp. from Panjang Island, Jepara, Indonesia with antibacterial activity (Sibero *et al*., 2017). Antibacterial activity from microorganisms is strongly related to its life phases (Tarman *et al*., 2013; Indarmawan *et al*. 2016; Ukhty *et al*., 2017). Fungus PDSP 5.7 has been registered in GeneBank as *Trichoderma reesei* with accession number MG547722.1. A growth curve is important to determine fungal life phase. Growth curve of *T. reesei* PDSP 5.7 is presented by Figure 3.

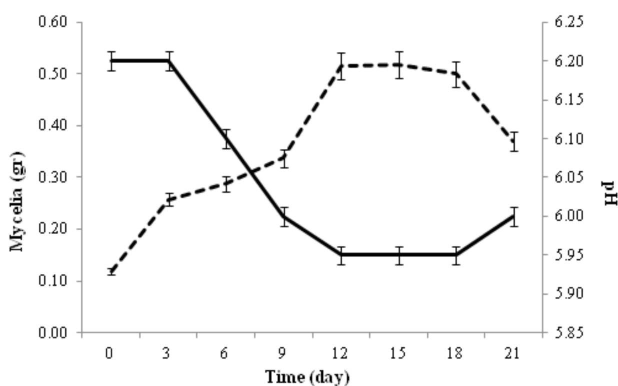

Fig. 3: Growth and pH curves of fungus PDSP 5.7 in MEB for 21 days of cultivation.

Fungus PDSP 5.7 passed log phase at day 3 to day 9, while stationary phase started at day 12 to day 18. The highest biomass of dry mycelia was produced at day 15 (0.5180 ± 0.001 gr) Usually, microorganisms produce secondary metabolites in stationary phase. Tarman *et al*. (2013) stated that endophytic fungi from *Rhizophora mucronata* produced the highest bioactive compounds on day 15 in PDB-based medium. Meanwhile, marine algicolous fungus *Xylaria psidii* KT30 produced the highest metabolite activity during stationary phase on day 21 in Hagem-based medium. Nutrient content in broth medium related to the fungal growth phase (Ukhty *et al*., 2017). Malt extract broth (MEB) used in this study as fermentation medium contain malt extract as carbon source and mycological peptone as nitrogen source. Sánchez *et al*. (2010) noted that carbon source has important role on antibiotic production. The pH of broth medium decreased during log phase then stable at stationary phase. It was caused by the fermentation done by the fungi (Indarmawan *et al*., 2016). Antibacterial activity of fungal extract in each harvesting time is shown by Table 2. For this step, fungal extracts were diluted to 1000 µg/mL.

Diameter of inhibition zone from fungal extracts increased from day 3 to day 15 (Table 1). Day 15 had the highest inhibition zone against all clinical pathogenic MDR bacteria so that, we proposed 15 days as the optimum cultivation time for fungus *T. reesei* PDSP 5.7. As shown in Figure 2, day 15 was known as stationary phase of fungus *T. reesei* PDSP 5.7. We noted that, this fungus strongly inhibited *Salmonella enterica* ser. Typhi followed by ESBL *E. coli*, then *S. haemolyticus* and MRSA. The ability of fungus against gram negative and positive MDR bacteria indicated a broad spectrum antibacterial activity. Several studies reported that fungi demonstrated widest antibacterial activity in stationary phase (Tarman *et al*., 2013; Indarmawan *et al*., 2016; Ukhty *et al*., 2017). In stationary phase, fungi produce particular bioactive compounds to protect themselves from competitors (Manavathu and Vazquez, 2014). Furthermore, fungal extract from day 15 was dilluted to be 50 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL then tested against ESBL *E. coli*, MRSA, *S. haemolyticus* and *S. enterica* ser. Typhi. The result of this assay is presented by Table 2 and Figure 4.

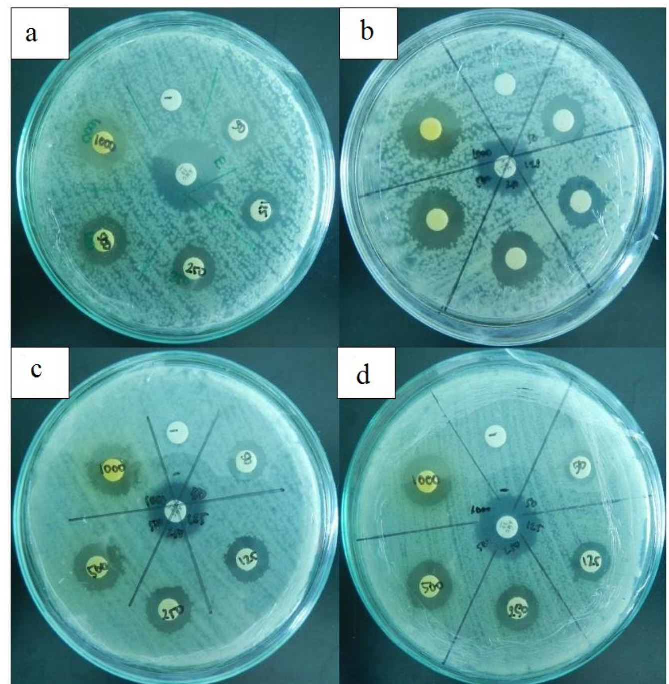

Fig. 4: Inhibition zone from fungal extract *T. reesei* PDSP 5.7 against (a) ESBL *E. coli*, (b) *S. enterica* ser. Typhi, (c) MRSA, (d) *S. haemolyticus*.

The range of inhibition zone value from fungal extract which harvested at day 15 was 4.13 ± 0.06 to 14.72 ± 0.07 (Table 2). The widest inhibition zone was performed by concentration 1000 µg/mL against *S. enterica* ser. Typhi, moreover this inhibition zone was wider than Amoxicillin + Clavulanic acid as positive control. In addition, this extract showed no significantly different ($P < 0.05$) against MRSA compared to positive control. The genus *Trichoderma* were known as potential source of polypeptide which has antibacterial activity. Panizel *et al*. (2013) stated that *T. atrovorode* isolated from sponge *Axinella* produced peptaibols with antibacterial activity against environmental bacteria such as *Sporosarcina* sp. (NB90); *Bacillus* sp. (NB36), *Shewanella* sp. (III.07) and *Microbacterium* sp. (PII.14). Fungus

T. longibrachiatum MMS151 from blue mussels produced six long-chain peptaibols. Two of six long-chain peptaibols showed noticeable cytotoxic activity against KB cells, antibacterial activity against gram positive bacteria and antifungal activity against human pathogenic *Aspergillus fumigatus* (Mohamed-Benkada *et al*., 2016). Moreover, *T. parareesei* from Indonesian marine sponge produced yellow pigment which inhibited *E. coli* and *S. enterica* ser. Typhi strain MDR (Sibero *et al*., 2016). Beside polypeptides, *Trichoderma* members also produced polyketide compounds such as trichodermatides A-D, trichoderpyrone and trichoketides A and B with various biological activities (Sun *et al*., 2008; Yamazaki *et al*., 2015; Chen *et al*., 2017). For the further research, isolation of lead compounds from fungus *T. reesei* PDSP 5.7 is suggested to obtain the next generation of antibiotic.

CONCLUSION

There were nine sponge-associated fungi isolated from sponge *Cinachyrella* sp. Result of agar plug method showed fungus PDSP 5.7 was the most potential fungi which inhibited all tested MDR bacteria. Macro-microscopic and molecular identification judged this fungus as *Trichoderma reesei* MG547722.1. Fifteen day of cultivation was proposed as optimal time for cultivation of fungus *T. reesei* PDSP 5.7 MG547722.1. Fungal extract showed best antibacterial activity against *S. enterica ser*. Typhi, followed by ESBL *E. coli*, *S. haemolyticus* then MRSA.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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