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ANTIMICROBIAL ACTIVITY OF SPONGE-ASSOCIATED FUNGI FROM PANDANG ISLAND, NORTH SUMATERA AGAINST CLINICAL PATHOGENIC MICROORGANISMS

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Abstract - As an archipelago and maritime country, Indonesia is consisted by numerous small islands with abundance of marine organisms. Pandang Island is one of the outer islands in Indonesia, located in Batu Bara District, North Sumatera Province. The aims of this study were to isolate the marine sponge associated fungi, obtain potential strain against clinical pathogenic fungi and MDR bacteria, determine the best cultivation time for antimicrobial production, characterize potential fungus through macro-microscopic and molecular approaches. The result showed there were 6 fungi which associated with sponge PPD.SP.7 and only fungus PDSP 7.1 could inhibit all pathogenic microorganisms. This fungus produced widest inhibition zone against *S. enterica ser*. Typhi in stationary phase. According to the result of identification, fungus PDSP 7.1 was judge as *Trichoderma saturnisporum* MG644629. Antimicrobial activity of this fungus was expected as the influence of the presence of NRPS gene fragments. This research is first report which studied sponge associated fungi and its antimicrobial activity from Pandang Island, North Sumatera, Indonesia.

INTRODUCTION

Indonesia is known as the biggest archipelago and maritime country in South East Asia with enormous marine organisms. Some new species of marine invertebrates a were found in Indonesia such as sponge *Acanthotetilla celebensis* from North Sulawesi, coral *Euphyllia baliensis* sp. nov. from Bali Island,crustacea *Leucothoeeltoni* sp. from Raja Ampat, and mollusca *Plakobranchus papua* from West Papua (de Voogd *et al*., 2007; Turak *et al*., 2012; Thomas, 2015; Meyers-Muñoz, 2016). Among the marine invertebrates, sponges are widely known as the producer of bioactive compounds (Abdul *et al*., 2017; Santalova *et al*., 2017). Nowadays the bioprospecting of new candidate of antibiotics from marine organisms are increasing. Moreover, the emergence of multidrug resistant (MDR) bacteria and fungi have challenged the natural product scientists to take part in this topic. Many studies have successfully isolated bioactive compounds from sponges with antibacterial activity against infectious diseases agents (Nguyen *et al*., 2017; Chu *et al*., 2017; Kubota *et al*., 2017). The exploration of

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new bioactive compounds from sponges for particular purposes leads to the extinction of those organisms. To prevent that issue, study on sponge-associated microorganisms as alternative source of antibiotics is reasonably needed.

Sponge-associated fungi had been reported to have antimicrobial activity against pathogenic bacteria. Sibero *et al*., (2017) successfully isolated and identified sponge associated fungi *Trichoderma asperellum* and *Trichoderma reesei* from a sponge *Cinachyrella* sp. with antibacterial activity against *Escherichia coli* strain MDR. An associated fungus *Aspergillus sydowii* from a sponge *Axinella* sp.was characterized by Trianto *et al*., (2017) and reported to have antibacterial activity against MDRS *Staphylococcus aureus* and *E. coli*. In addition, antibacterial compounds such as 4-carboxy-diorcinal, diorcinol, violaceol I, cordyol E, and cyclo-(L-Phe-L-Trp) were isolated from sponge associated fungi *A. sydowii* against *S. aureus* (Liu *et al*., 2017). These studies showed the potential of sponge associated fungi to combat the pathogenic bacteria. As a mega-biodiversity country, Indonesia has enormous marine organisms which have not been fully explored. Pandang Island is a small island located in North Sumatera, Indonesia. This study is the first report of marine sponge associated fungi from this location. The aims of this study were to isolate the marine sponge associated fungi, obtain the potential strain against pathogenic micro-organisms, determine the best cultivation time for the antibacterial production, and characterize the potential fungus through both macro-microscopic and molecular approaches.

MATERIALS AND METHODS

Sample collection

Sponge PPD.SP.7 was collected on January 2017 from coastal zone (3°25'13.6"N 99°45'24.6"E) in Pandang Island, North Sumatera, Indonesia. The sponge was cut and kept into Zip Lock plastic bag and stored in a cooling box at 4 °C for further isolation (Sibero *et al*., 2017).

Fungal isolation and purification

Sponge associated fungi were isolated using the modified method which used by Sibero *et al*. (2017) and Kjer *et al*. (2010). The collected sponge was washed by using sterilized sea water then rinsed in ethanol 70% for 1 min to sterilize the surface then washed again by using sterilized sea water. The sponge was then cut 1×1 cm^2 in size then tapped for 5 sec on Potato Dextrose Agar (PDA) medium to check the quality of surface sterilization. After that, each part of the sponge was laid on a new PDA medium and incubated in room temperature (23 °C) until the mycelia grew. The hyphae which grew on sponge were transferred to new PDA plates to obtain the pure isolates.

Screening of antimicrobial activity

This step was done according to Sibero *et al*., (2017). The fungi were tested to clinical pathogenic multi drug resistant (MDR) bacteria (*Salmonella enterica ser*. Typhi, Extended Spectrum Betalactamase (ESBL) *Escherichia coli*, *Klabsiella pneumonia*) and skin diseases pathogens (*Candida albicans*,*Malassezia furfur* and *Trichophytonrubrum*). The pathogens were obtained from Dr. Kariadi General Hospital Medical Center, Semarang, Central Java, Indonesia and Diponegoro National Hospital, Semarang, Central Java, Indonesia. The most potential fungus was selected for further analyses.

Fungal characterization

Colony and morphology observation

The active fungus was grown on PDA agar in 7 days for colony observation. While morphological observation was done by using slide culture method (Sibero *et al*., 2017; Qiu *et al*., 2005).The fungal morphology was observed under micro-scope with addition of Lactophenol Blue.

Molecular identification

The fungal DNA was amplified by using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') with same PCR condition with amplification of NRPS gene fragments. PCR mix was consisted of 12.5 µL of GoTaq Green Master master mix, 1µL of ITS 1, 1µL of ITS 4, 10 µL of ddH2O and 0.5 µL of DNA template (Sibero *et al*., 2017). PCR product was sent to 1st BASE DNA Laboratories SdnBhd, Malaysia. The DNA sequencing was compared for homology to the BLAST database. A phylogenetic tree was reconstructed using MEGA.7 software package.

NRPS gene fragments identification

Mycelia of seven days old fungus was immersed in saponin solution and kept for overnight at 4 °C. The DNA were extracted using Chelex methods (Sibero *et al*., 2017; Turan *et al*., 2015). Amplification

of gene fragments of non-ribosomal peptide synthetase (NRPS) was done using primers A2gamF (5′-AAG GCN GGC GSB GCS TAY STG CC-3′) and A3gamR (5′-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3′) (Radjasa *et al*., 2008). PCR mix composition was 1µL of A2gamF, 1µL of A3gamR, 12.5 µL of GoTaq Green Master master mix, 10 µL of ddH2O and 0.5 µL of DNA template. The gene fragment was amplified with PCR 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 extention was done at 72 °C for 7 min (Sibero *et al*., 2017).

Growth curve

The potential fungus was cultured on Malt Extract Broth (MEB) medium for 21 days. The harvesting time was done for once in three days, on day 3, 6, 9, 12, 15, 18 and 21. The acidity of the medium was measured in every harvesting timeby using pH meter. Filter papers were prepared and dried in desiccator for 2×24 h then it was weighed (W_o). The mycelia were separated from the MEB using filter paper then it were dried in desiccator for 2×24 h. After that, the filter papers with mycelia were weighed (W_t). The weight of dry mycelia (W_m) were obtained by subtraction of W_t-W_o. The dry weight of mycelia was used for construction of growth curve to determine the fungal life phases (Tarman *et al*., 2013; Indarmawan *et al*., 2016).

Bioactive extraction

Bioactive substances were extracted using ethyl acetate from MEB medium in 2:1 ratio (v/v). Agitation using shaker was applied for maceration (24 h) then the solvent was evaporated by using rotary evaporator.

Antimicrobial activity

The fungal extract (500 µg/mL) was tested against clinical pathogenic bacteria strain MDR according to CLSI(2015). The bacteria were inoculated on Mueller Hinton Agar (MHA) plates while the pathogenic fungi were inoculated on PDA plates.The extract (10 µL) was loaded into paper disc. Amoxicillin+Clavulanic acid (antibacterial) and Nystatin (antifungal) were used as positive control for antimicrobial assay. The plates were incubated for 24 h in incubator (32 °C). The presence of clear zone indicated the antibacterial activity.

Data analysis

Data were analyzed using SPSS package version 18.0 for Windows with Split Plot In Time design and factorial design with confidence interval 95% (P<0.05).

RESULTS AND DISCUSSION

Pandang Island is located in Batu Bara District, North Sumatra with coordinate 3°25'17.924"N 99°45'27.775"E (DP3K, 2012). The sponge sample was collected from intertidal zone when low tide in the morning and labelled as PPD.SP.07.

The sponge PPD.SP.07 (Figure 1) was found living as coral-associated sponge. Its characteristics were thick encrusting (1-3 cm thick), the color of the sponge was light green outside and dark green inside. The surface wasvery porous and rough.

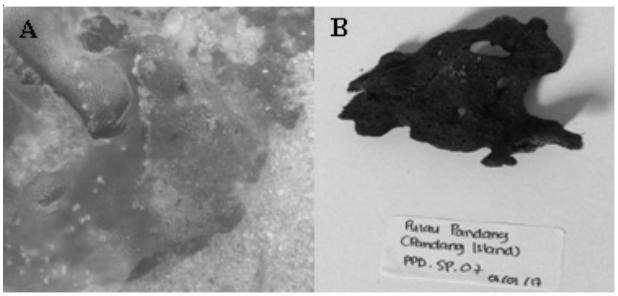


Fig. 1 Underwater (A) and out of water (B) images of spongePPD.SP.07

Oscules dispersed with 1-4 mm wide.

A total of 6 associated fungi were isolated from this sponge. The fungal colonies are shown in Figure 2. The isolation technique and growth medium have important role to the diversity of the isolated associated fungi. This study used the tapping method in PDA plates to isolate the fungi. Kossuga *et al*., (2012) mentioned that inoculation of marine macroorganisms fragments in petri dish gave the higher diversity of marine associated microorganisms. Sibero *et al*., (2017) also used the same method with Malt Extract Agar (MEA) as the growth media to isolate sponge-associated fungi and obtained 29 sponge-associated fungi from nine sponges. In addition, growth media which content 3% malt extract gave the largest number of marine microorganism strains. On the other hand, Zhang *et al*., (2012) showed that PDA media yielded the highest diversity of associated fungi from marine

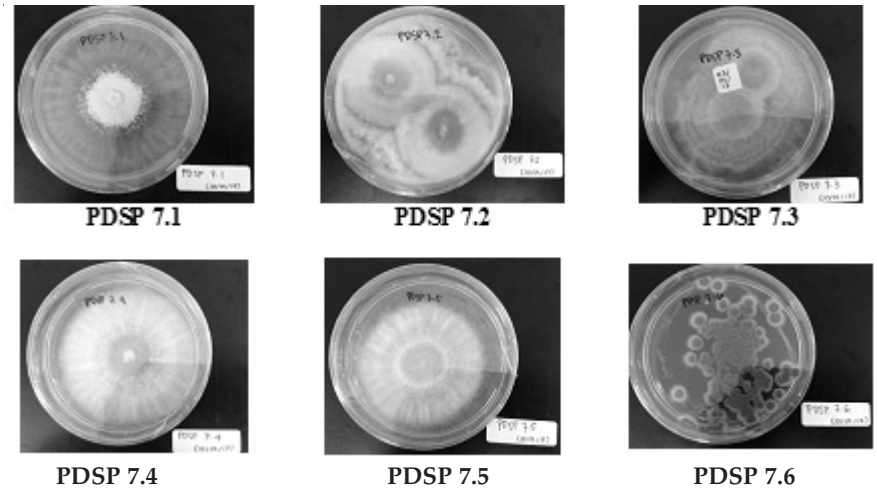


Fig. 2 Sponge associated fungi from PPD.SP.07

Table 1. Screening of the potential fungi against pathogenic microorganisms

Isolate	*S. entericaser.* Typhi	ESBL	*K. pneumoniae*	*C. albicans*	*M. furfur*	*T. rubrum*
PDSP 7.1	+	+	+	+	+	+
PDSP 7.2	-	-	-	-	+	+
PDSP 7.3	-	-	-	-	+	-
PDSP 7.4	-	-	-	-	-	+
PDSP 7.5	-	-	-	-	+	+
PDSP 7.6	-	-	-	-	-	-

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

invertebrates.

The initial sreening was done to select the most potent fungal isolate. The associate fungi were tested against three pathogenic fungi and three MDR bacteria. Agar plug method was chosen according to previous reports which screen the potential of antimicrobial activity from fungi (Sibero *et al.*, 2017; Rahaweman *et al.*, 2016; Cristianawati *et al.*, 2017; Sabdaningsih *et al.*, 2017). The result of the antibacterial screening was shown Table 1.

Table 1 shows that only fungus PDSP 7.1 could inhibit all the pathogens. It means, fungus PDSP 7.1 was the most potential fungus and used for the next steps. The fungus PDSP 7.1 was cultivated in broth media until 21 days while the bioactivities were monitored every three days. The aim of this step was to determine the best time period of fungal cultivation with the most active secondary metabolites. The growth curve of PDSP 7.1 is presented by Figure 3.

The lag phase started at day 0 to day 3 followed by the log phase at day 12. The stationary phase

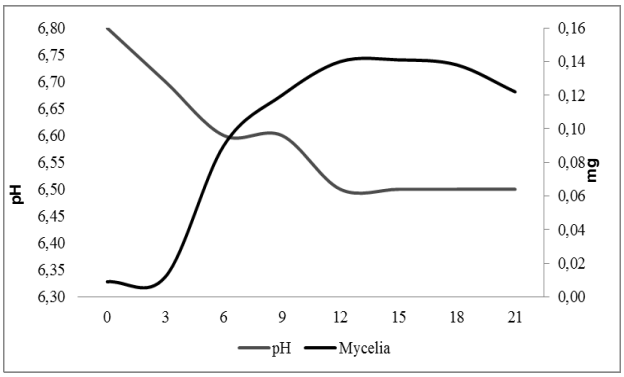


Fig. 3 Growth curve of PDSP 7.1

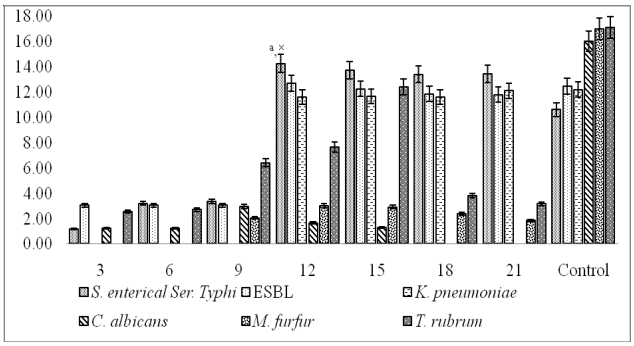
was begun at day 12 until day 18 and the death phase was reached at day 18 to 21. The curve on figure 3 shows the decreasing of pH in each cultivation day. Indarmawan *et al.*, (2016) got similar growth curve for *Xylaria psidii* in Hagem broth media. *X. psidii* had stationary phase at day 12 until day 15 then reached the death phase at day 18 to 21. The pH medium was decreasing along with the length of cultivation time. This condition was

allegedly caused by the fermentation that occurs in the broth media.

Antibacterial activity of PDSP 7.1 in each harvesting time were evaluated to combat pathogenic microorganisms. The aim of this evaluation was to know the optimum day of fungal cultivation to produce the biggest antimicrobial activity. Figure 4 shows the result of antimicrobial activity assay.

Crude extract of fungus PDSP 7.1 showed antimicrobial activity against all MDR bacteria and clinical pathogenic fungi with different performance. According to statistical analysis, this research proved that the cultivation time influenced antimicrobial activity of fungal extract ($P<0.05$). Fungal crude extract had the best antibacterial activity against *S. enterica* ser. Typhi with inhibition zone was 14.2±0.05 mm. This antibacterial activity was produced by fungus PDSP 7.1 in 12 days of cultivation. It was significantly different compared to Amoxicillin+ Clavulanic acid 10 µg/disc ($P<0.05$) with greater antibacterial activity against *S.enterica ser*. Typhi. Antibacterial activity of fungal extract against *S. enterica ser*. Typhi and ESBL enhanced with an incubation period 12 days, however it declined after day 12. Meanwhile, inhibition zone against *K. pneumoniae* was getting wider until day 18. Day 12 and 18 were known as the stationary phase of PDSP 7.1. Therefore, previous researches showed that bioactive compounds were usually produced by fungi during stationary phase and after stationary phase (Demain, 2000; Elias *et al*., 2006; Swathi *et al*., 2013).

The most potential fungus was identified both phenotypically and genotypically. Based on the morphological characterization, the colony of PSDP 7.1 in PDA plate (23 °C) had white to yellowish cushion-shaped color in the center of the colony the hyphae barely visible on agar surface, the mycelia grown in 2 weeks or less and produced



Control: Antibacterial : Amoxicillin + Clavulanic acid 10 µg/disc
Antifungal : Nystatin 50 µg/disc
* : Widest inhibition zone
[a] : Significantly different ($P<0.05$)

Fig. 4 Antimicrobial activity of PDSP 7.1 in every harvesting time

yellow pigment. The microscopic characteristic of this fungus could be seen in Figure 5.

According to Figure 5, PDSP 7.1 had characteristics such as hyaline hyphae with septum, branching conidiophore with lageniform phialide, produced conidia with oval to cylindrical form and had chlamydosphores. These characteristics lead fungus PDSP 7.1 to the genus *Trichoderma* (Atanasova *et al*., 2010; Rahman *et al*., 2011; Qin *et al*., 2016; Sibero *et al*., 2017).

Fungal DNA was isolated using Chelex 100 method (Sibero *et al*., 2017; Qiu *et al*., 2005; Cristianawati *et al*., 2017). Walsh *et al*. (2013) mentioned Chelex 100 is able to prevent DNA damage by inhibiting the activity of DNAse. To amplify fungal DNA in polymerase chain reaction(PCR), ITS1 and ITS4 were applied as primers. Those primers amplify the fungal DNA in the internal transcribed spacer (ITS) region.

DNA sequences of fungus PDSP 7.1 were compared to the closest relatives of fungal strains according to BLAST search. The result of

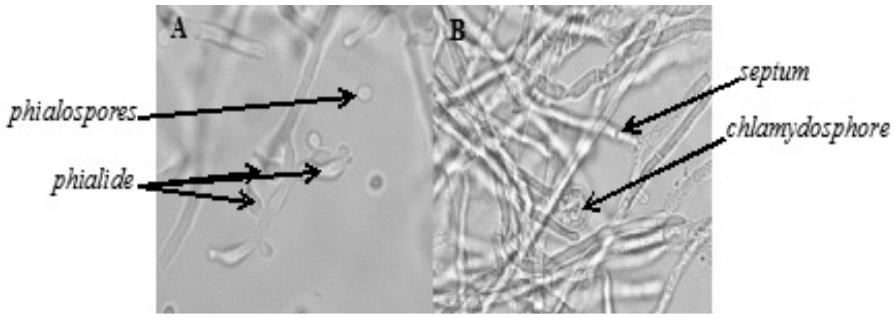


Fig. 5 Microscopic morphology of PSDP 7.1 conidiophores (A); hyphae (B)

Tabel 2. Identification of fungal strains based on morphological and molecular approaches

Isolate code	Morphological characterization	Closest relatives (BLAST)	Acc. No.	Query cover	Similarity
PDSP 7.1	*Trichoderma* sp.	*Trichoderma saturnisporum* isolate T-61	KC884818.1	100%	99%
		Trichoderma capillare TW22166 isolate	KT862523.1	98%	98%

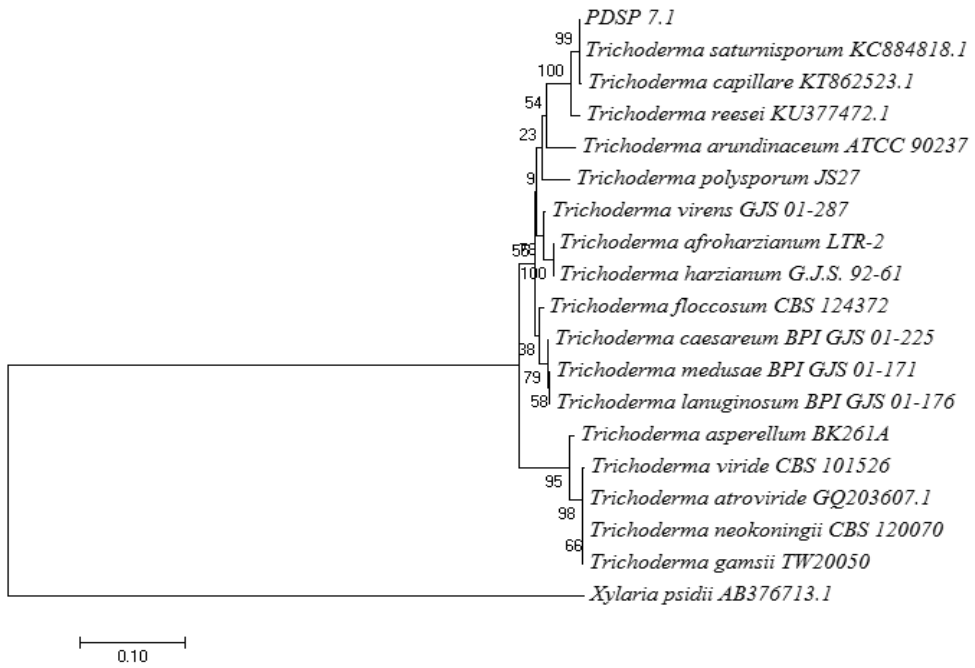


Fig. 6 Phylogenetic tree of fungus PDSP 7.1

comparison between morphological characteristics and genetic analysis in the ITS region is presented by Table 2 and the phylogenetic three is shown in Figure 6.

The molecular identification data supported that the fungus PDSP 7.1 was the member of *Trichoderma* and had 99% and 98% similarity to *Trichoderma saturnisporum* KC884818.1 and *Trichoderma capillare* KT862523.1 respectively. In the phylogenetic tree, fungus PDSP 7.1 was closer to *T. saturnisporum* KC884818.1. Therefore, fungus PDSP 7.1 had been registered in GeneBank with accession number MG644629. The genus *Trichoderma* is known as marine derived fungi which can be isolated from various organisms. Mohamed-Benkada *et al*. (2016) utilized *T. longibrachiatum* which isolated from blue mussles as producer of long-chain peptaibols for several biological activities assays. Onderoet *al*. (2017) also successfully isolated two species of *Trichoderma* fungi from a marine sponge *Cinachyrella* sp.. Sabdaningsih *et al*. (2017), working with *T. longibrachiatum*, isolated from soft coral, was reported to have antibacterial activity against MDR *Staphylococcus haemolyticus*. In addition, Martínez *et al*. (2016) applied *T. saturnisporum* as growth promotor and biological control against *Phytophthora capsici* and *P. parasitica*.

The ability to produce bioactive compound is usually influenced by the presence of particular gene which encode the producing of nonribosomal peptide synthetases (NRPS). The role of this enzyme is to synthesize nonribosomal peptides (NRP) which has biological activities (Tambadou *et al*., 2014; Amoutzias *et al*., 2016). Uncharacterized antimicrobial compounds from sponge associated fungus *T. saturnisporum* was thought as group of NRP. Thus, we detected the presence of gene fragments which encode NRPS enzyme by using PCR with specific primers. The result of genes detection is presented in Figure 7.

PCR product electropherogram shows single band of NRPS gene in *T. saturnisporum* PDSP 7.1 located at 300 bp at marker. It casued by the set primer of A2gamF/A3gamR amplify DNA count

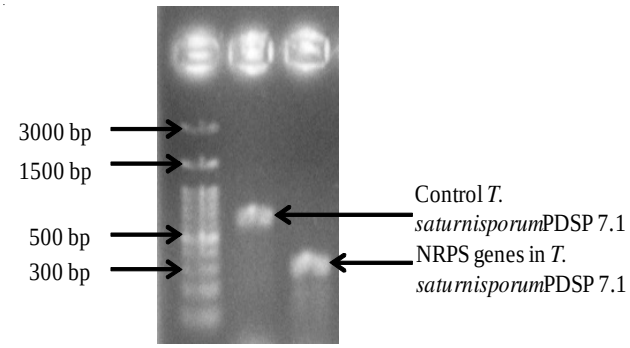


Fig. 7 PCR amplification of NRPS gene fragments in *T. saturnisporum* PDSP 7.1

300 bp (Tambadou *et al*., 2014). The presence of NRPS genes fragments in *T. saturnisporum* PDSP 7.1 could be an assumption as reason of its antimicrobial activity.

The present results demonstrate the potential of sponge-associated fungi for the biological control of human pathogens.

CONCLUSION

Six sponge-associated fungi were isolated from an unidentified sponge originated from coastal region in Pandang Island, North Sumatera. Fungus PDSP 7.1 performed strongest antimicrobial activity in compared to other isolates. According to diameter of inhibition zone, fungal extract from 12 days of cultivation was noted as the best time of cultivation to produce antimicrobial substances. We highlighted that day 12 to 18 were the stationary phase of fungus PDSP 7.1. Based on morphological and molecular approaches, fungus PDSP 7.1 was identified as *Trichoderma saturnisporum* MG644629. This fungus had NRPS gene fragments which assumed as the reason of its wide spectrum antimicrobial activity. It proves that this sponge-associated fungi can be utilized as an alternative source of antimicrobial substances against human pathogens.

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