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Isolation and Characterization of Antimicrobial Substance from Marine *Streptomyces* sp.

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Isolation and purification of antimicrobial active substance produced by marine *Actinomycetes* has been carried out. Marine sediment samples were obtained from six different places at Banten West Coast. Isolation was conducted using two pretreatment methods, acid and heat shock pre-treatment. A total of 29 *Actinomycetes* isolates were obtained from the various sediment samples collected, then tested for antimicrobial activity against *Escherichia coli ATCC 25922*, *Staphylococcus aureus ATCC25923*, *Pseudomonas aeruginosa* ATCC27853, *Bacillus subtilis* ATCC 66923, *Candida albicans* BIOMCC00122, and *Aspergillus niger BIOMCC00134*. Among the isolates, isolate A11 was the most activity to Gram-positive and Gram-negative bacteria, and morphological observation and identification using 16S rRNA showed that the isolate was similar to *Streptomyces* sp. Production of active compound from A11 isolate used yeast peptone medium. Purification of active compounds was carried out using silica-gel-column chromatography and preparative HPLC. A single peak of active compounds was detected by HPLC, which showed a retention time of 8.35 min and maximum absorbance in UV visible at 210 nm and 274.5 nm respectively.

Key words: marine *Actinomycetes*, isolation, screening, antimicrobial activity, purification

Actinomycetes are the most widely distributed group of microorganisms in nature and which primarily inhabit the soil (Goodfellow and Williams 1983; Locci and Sharples 1983). Almost 70% of the world's antibiotics are known to come from *Actinomycetes*, mostly from the genera *Streptomyces* and *Micromonospora* (Berdy 2005; Fenical and Jensen 2006). In the past, researcher more focuses to explore the terrestrial *Actinomycetes*. Nevertheless, over time new antibiotics have been discovered from marine *Actinomycetes* (Fiedler *et al.* 2005; Lam 2006; Bull and Stach 2007).

Although the exploitation of marine *Actinomycetes* as a source to discover novel secondary metabolites is at an early stage, numerous novel metabolites have been isolated in the past few years. For examples, proximicin A, B, and C are novel antibiotic and anticancer compounds isolated from marine actinomycete Verrucosispora (Fiedler et al. 2008). Valinomycin, staurosporine, butenolide are anti-parasitic compounds from marine *Streptomyces* sp. (Elardo *et al.* 2010). Lodopyridone is cytotoxic to HCT-116 human colon cancer cells produced by a marine *Saccharomonospora* sp. (Maloney *et al*. 2009). L-glutaminase is enzyme therapy for cancer especially for acute lymphocytic leukemia produced by marine *Streptomyces* sp. (Balagurunathan *et al*. 2010). Abyssomicin C is a novel polycyclic polyketide antibiotic produced by a marine *Verrucosispora* strain (Riegdlinger *et al.* 2004), and possesses potent activity against Gram-positive bacteria, including clinical isolates of multiple-resistant strains. Diazepinomicin is a unique farnesylated dibenzodiazepinone produced by a *Micromonospora* strain (Charan *et al.* 2004) with antibacterial, anti-inflammatory and antitumor activities.

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Indonesia is an archipelago country having a wide area of sea, which is more than 3.1 million km^2. The characteristics of the sea showed a high level of biodiversity in microorganisms, plants, animals. Nevertheless this potential has not been exploited. Currently exploration of *Actinomycetes* in Indonesia is still limited to terrestrial *Actinomycetes*. The objective of this research were to isolate and to purify of active compounds produced by marine *Actinomycetes* such as A11 isolate.

MATERIALS AND METHODS

Sample Collection. Sediments were obtained from six marine sites in Banten West Java. From each location, six sediment samples of 5 g each were collected from 10−15 cm below the surface. Each of the sediment samples for each site was placed in small pre-labeled plastic bags and were tightly sealed. Hereinafter each sample was given code in accordance to sampling location.

Isolation of *Actinomycetes*. All sediment samples were processed in laboratory as soon as possible after collection. The samples were suspended in sterilized water and were made serial dilution. Pretreatment were conducted by using acid and heat-shock treatments. Acid treatment was conducted by the acidifying the samples to pH 2 were obtained for 3 h. Heat-shock treatment was conducted by the heating the samples at 60ºC for 4 h (Pisano *et al.* 1986). Treated samples were then inoculated onto starch agar medium (1% w/v starch, 0.4% w/v yeast extract, 0.2% w/v peptone, natural seawater, and 2% w/v agar) and incubated for 4-8 weeks at room temperature. One hundred gram per mL of nalidixic acid and 5 g mL^{-1} of rifampicin were added to reduce the number of unicellular bacteria (Pisano *et al.* 1989). The antifungal agent cycloheximide (100 g mL^{-1}) and 25 g mL^{-1} nystatin were added to all isolation media. *Actinomycetes* colonies were recognized by the presence of

branching, vegetative filaments and the formation of tough, leathery colonies that adhered to the agar surface. Morphologically diverse *Actinomycetes* were repeatedly transferred to the same media until pure cultures were obtained. All pure strains were grown in yeast extract-malt extract (YEME) broth and cryopreserved at -80°C in 10% v/v glycerol solution.

Identification Based on 16S rRNA Analysis. The DNA was isolated using FastPrep kit for DNA isolation. The pellet was lysised using a lysing matrix, combined with 1 mL, and homogenized using a FastPrep instrument for 40 sec at 4500 rpm.

Polymerase chain reaction was done for DNA amplification using 8F and 1492R primers. The PCR mixture containing 8F and 1492R primers was added to the DNA solution. The PCR product was then purified using a Gel/DNA extraction kit.

The 16S rRNA gene obtained was submitted to the DNA sequencing facility, Genetic Laboratory, Biotech Centre. A big Dye® terminator V 3.1 cycle sequencing kit was used to sequence the DNA. The DNA was then run in an automated DNA sequencer using capillary electrophoresis (ABI 300 genetic analyzer). The sequence was compared to a database available at NCBI using the BLAST search engine.

Liquid Culture and Extraction of Active Substance. An established slant of *isolate w*as inoculated into a 250 mL flask containing 100 mL of vegetative medium (YEME medium) consisting of : bacto peptone 5 g L^{-1}, yeast extract 3 g L^{-1}, malt extract 3 g L^{-1}, glucose 3 g L^{-1}, demineral water 25 mL, and sea water 75 mL. pH value of the medium was adjusted to 7.6 before sterilization. The flask was incubated at 30ºC for 2 d in an incubator-shaker. Fifty mL of the culture was transferred to 1000 mL of the fermentation medium. Fermentation medium consisted of bacto peptone 15 g L^{-1}, yeast extract 3 g L^{-1}, Fe (III) citrate hydrate 0.3 g L^{-1}, demineralised water 250 mL, and sea water 750 mL (Nedialkova and Mariana 2005). The pH value of the medium was adjusted to 7.6 before sterilization. The fermentation was carried out at 30ºC for 5 d in incubator-shaker.

For extraction of active substance, the culture broth was centrifuged at 14 000 x g for 15 min. The broth supernatants were divided and extracted using ethyl acetate as solvent. Supernatant and organic solvent were mixed thoroughly by shaking them in 1 L capacity separating funnel and allowed to stand for 30 min. Two layers were separated; the aqueous layer and the organic layer, which contained the solvent and the antimicrobial agent. The organic layer was concentrated by evaporation under vacuum to the least possible volume, after dehydration with anhydrous Na_2SO_4. The aqueous layer was re-extracted and the organic layer added to the above organic layer. The organic layer was a concentrated by repeated cycle of evaporation under vacuum.

The dry extract of the supernatant was purified using silica gel column chromatography. Dry extract was injected onto the column and then eluted stepwise with chloroform-methanol solvent system as follows: First the column was eluted with 100% chloroform (Fraction 1). Then repeated with reducing the chloroform by 10% in each fraction while the methanol was increased by 10% in each fraction, until the percentage of methanol was 100%. Thirty fractions were collected (each of 20 mL) and then concentrated and dried for testing their antimicrobial activities. The active fractions obtained from chromatography column were further purified by preparative HPLC.

Preparative HPLC. Purification by preparative HPLC was conducted using a Waters 2695 HPLC, photodiode array detector (PAD), and Column puresil 5 C18 4.6x150 mm. The volume injected was 100 uL per injection under conditions of average pressure of 1267 psi, and the flow rate was 1 mL min^{-1} where the mobile phase was 0-45% methanol-water and time period was 25 min. (Kazakevich and Lobrutto 2007).

Antimicrobial Activity Assay. Antimicrobial activity was monitored by the agar diffusion paper-disc (6 mm) method. Discs were dripped with methanol solution of extract, dried and then placed over the agar surface plates freshly inoculated with either *Escherichia coli ATCC 25922*, *Staphylococcus aureus ATCC25923*, *Bacillus subtilis* ATCC 66923, *Pseudomonas aeruginosa* ATCC27853*, Candida albicans* BIOMCC00122, and *Aspergillus niger BIOMCC00134* as test organisms. Suspensions of test organisms were adjusted to 10^6 cfu mL^{-1}. The most potent isolates were noted for each test microorganism, based on the mean diameter of inhibition zones (Bonev *et al*. 2008).

Analysis HPLC. Analysis was performed using HPLC with an analytical Sunfire C18 column (4.6 x 250 mm, Shiseido Co. Ltd., Tokyo, Japan). Mobile phase used methanol-water (0-100% linear gradient for 25 min and then isocratic elution with 100% methanol over 10 min), at a flow rate of 1 mL min^{-1}, volume of injection 10L per injection, and detection was at a of 210 nm (Kazakevich and Lobrutto 2007).

RESULTS

Isolation and Screening of *Actinomycetes* from Marine Samples. Twenty nine of actinomycete isolates were successfully isolated from 6 sediment samples. Eight of them showed antimicrobial activity, 2 isolates were active against *E. coli ATCC 25922*, 4 isolates were active against *S. aureus ATCC25923*, 2 isolates were active against *B. subtilis* ATCC 66923, 3 isolates were active against *P. aeruginosa* ATCC27853, 3 isolates were active against *C. albicans* BIOMCC00122, and 2 isolates were active against *A. niger BIOMCC00134* (Table 1).

From 8 active isolates, A11 was chosen for further study due to highest activity against *Gram−positive and Gram-negative bacteria. I*dentification using 16S rRNA revealed that isolate A11 was 100% homology to *Streptomyces* J22, class *Actinobacteria*, order *Actinomycetales*, family *Streptomycetaceae* and genus *Streptomyces*. Morphologically observation showed bold white color, glossy surface and circular with folding hyphae with some antenna (aerial hyphae) arising out in vertical. In the beginning of growth single globular colony was formed, and hereinafter the hyphae further expanded.

The phylogenic tree (Fig 1) indicated that A11 has close contiguity with *S. tanashiensis* subsp. *cephalomyceticus*. An isolate of *S. tanashiensis* subsp. *cephalomyceticus* was recognized which could synthesize TAK-637 (tachykinin-receptor-antagonist) (Tarui 2001).

Liquid Culture and Purification of Active Substance. Liquid culture of A11 was carried out for 5 d by using yeast-peptone medium. On the last day of culture, the medium color was darker and more viscous than on the first day. Many white granules were observed in the bottom of the flask. From a 5 L volume of culture we obtained 4.72 g of dry biomass was obtained and after extraction by methanol 2.72 g of extract was obtained. On the other hand, 0.33 g of ethyl acetate extract was obtained from supernatant. Antibacterial activity assay of the both extract against *B. subtilis* ATCC 66923 *showed* that the extract of supernatant

Table 1 Eight isolates of *Actinomycetes* (Banten West Java Coast) producing antimicrobial active

Isolate	Sample treatment	Diameter of inhibition (mm)					
		Escherichia coli	*Staphylococcus aureus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Candida albicans*	*Aspergillus niger*
A64	HS	-	-	-	-	-	15.00
A610	A	-	12.00	-	-	-	-
A11	HS	18.00	15.00	14.00	14.00	-	-
A21	HS	-	-	-	7.00	-	9.00
A32	HS	-	12.00	-	-	7.00	-
A43	A	10.16	-	8.67	9.51	-	-
A44	A	-	-	-	-	10.61	-
A54	HS	-	8,56	-	-	8.67	-
Positive control rifamp icin 500 ppm		23.52	9.16	15.98	15.18	-	-
nystatin 1000 ppm		-	-	-	-	14.84	16.38

HS, heat shock treatment; A, acid treatment; Diameter of paper disc 6 mm

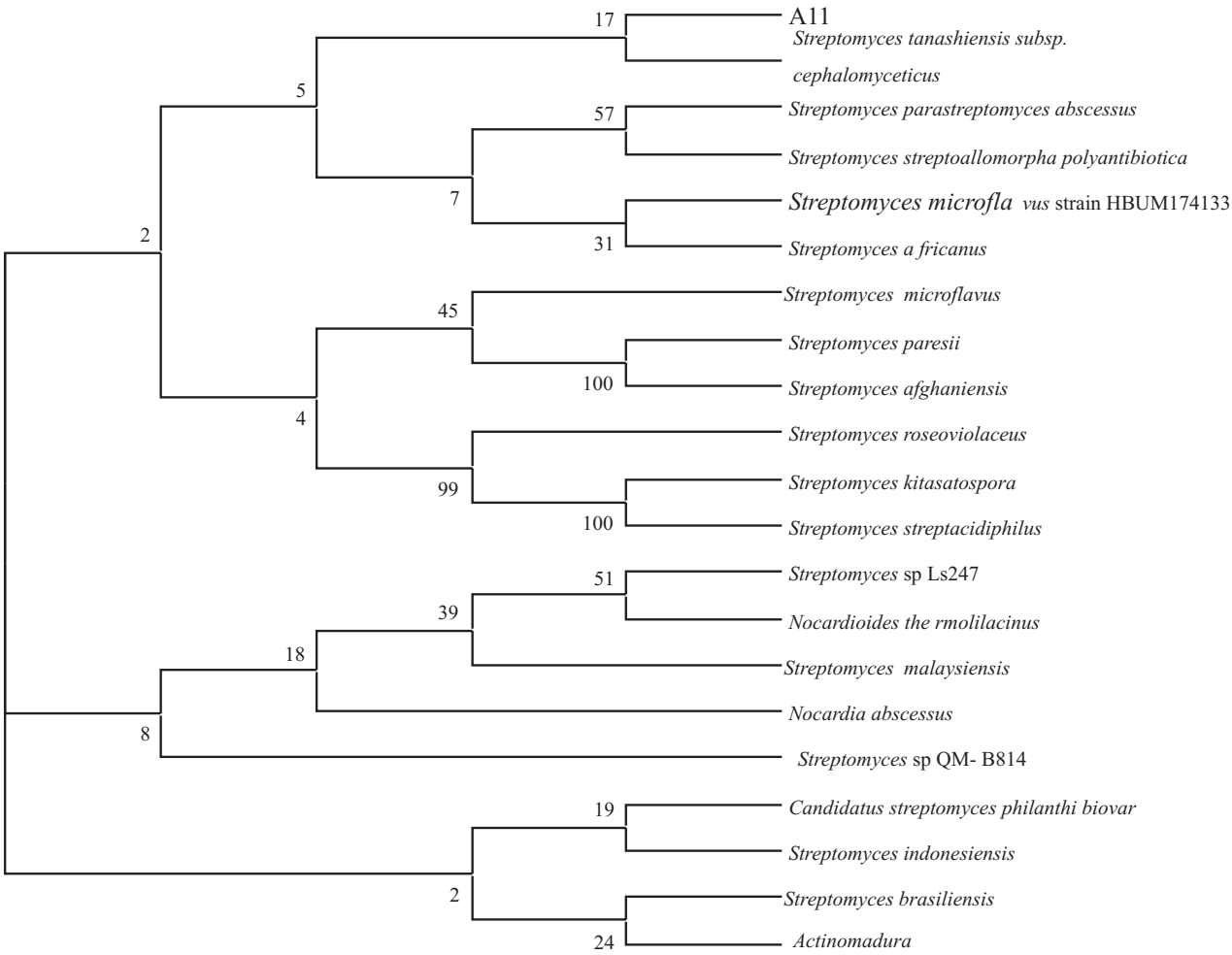

Fig 1 Polygenetic tree of isolate A11 shown as *Streptomyces* sp.

was active, but no activity with the extract of biomass (Table 2). This was also confirm in HPLC chromatogram of both extracts, in where more peaks were in supernatant extract rather than in biomass extract (Figs 2 and 3).

Further purification of the supernatant extract was than carried out using silica gel column and preparative HPLC. As shown in Fig 4, several peaks were still observed in the chromatogram of HPLC preparative.

The antibacterial activity test applied to all fractions of HPLC showed that peak retention 10.1 min contained the active fraction. The fraction was then collected and used for HPLC analysis. Chromatogram of active fraction shows that active fraction had retention time of 8.623 min at gradient elution methanol-water 0-100% using analytical HPLC (Fig 5). Purification using preparative HPLC obtained a single peak with maximum absorbance of UV visible at 210 nm and 274.5 nm (Fig 6). This compound easy dissolves in methanol-chloroform 50%-50%, but cannot dissolve in 100% chloroform.

DISCUSSION

We obtained six sediment samples derived from 29 isolates of *Actinomycetes*. Some of sediment samples obtained many isolate of *Actinomycetes*, but some of them did not contain *Actinomycetes*. It indicates that *Actinomycetes* are distributed unevenly in Banten, West Java Coast. When compared with brackish *Actinomycetes*, the population of marine *Actinomycetes* was less. *Actinomycetes* are less common in marine sediments relative to brackish environments (Goodfellow and Williams 1983; Parungao *et al.* 2007). Another study (Goodfellow and Haynes 1984) suggested that *Actinomycetes* represent only a small component of the total bacterial population in marine sediments. They observed that most of the isolates were of terrestrial and brackish origin. Terrestrial soils have been the main reservoir of *Actinomycetes*. They comprise a large part of the microbial population of the soil (Parungao *et al.* 2007). Table 1 shows that many *Actinomycetes* had antibacterial activity rather than anti fungal activity, same as reported by Berdy (2005). In the group of antibiotics, 66% are antibacterial (Gram-positive and Gram-negative), and 34% are antifungi including yeast.

Selected isolate for further study (A11) which isolates showed high activity against G*ram–positive and Gram-negative bacteria* indicated that isolate was *Streptomyces* sp. Morphology of A11 is the same like genus of *Streptomyces* (Chater 2006; Antonova-Nikolova *et al*. 2007). Surface looked glossy and circular with folding hyphae that length and formed some antenna (aerial hyphae) arising out in vertical were characteristic of *Streptomyces* morphology

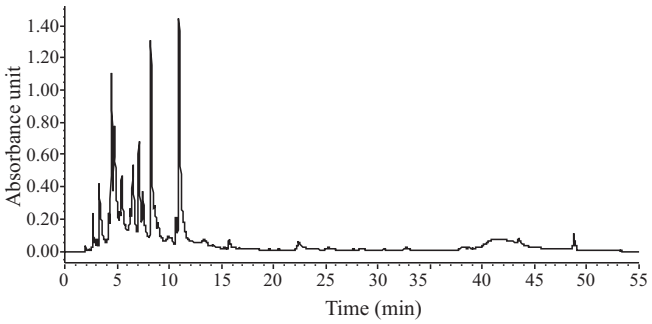

Fig 2 Analysis HPLC chromatogram of supernatant extract.

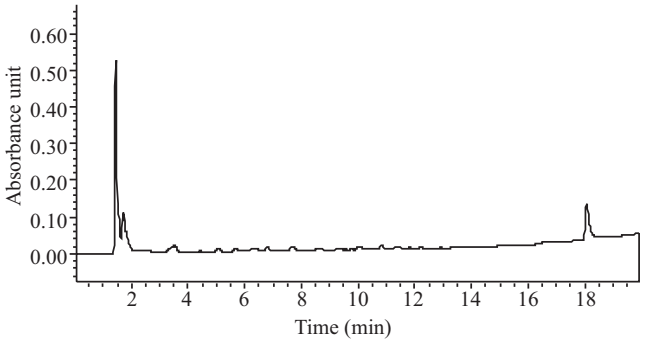

Fig 3 Analysis HPLC chromatogram of biomass extract.

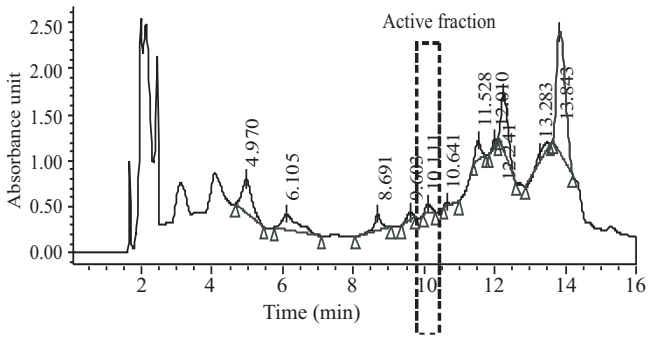

Fig 4 Chromatogram of preparative HPLC.

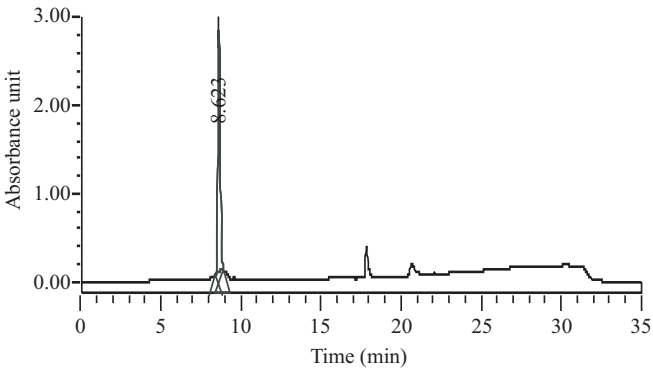

Fig 5 Analysis HPLC chromatogram of active fraction.

Table 2 Biological activity of biomass and supernatant extract from isolate A11

Sample	Diameter of inhibition/clear zone (mm)					
	Staphylococcus aureus	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicans*	*Aspergillus niger*
Biomass extract	-	-	-	-	-	-
Supernatant extract	10.39	24.43	9.64	9.55	-	-
Positive control (rifampicin 500 ppm)	21.27	44.57	10.08	10.12	-	-

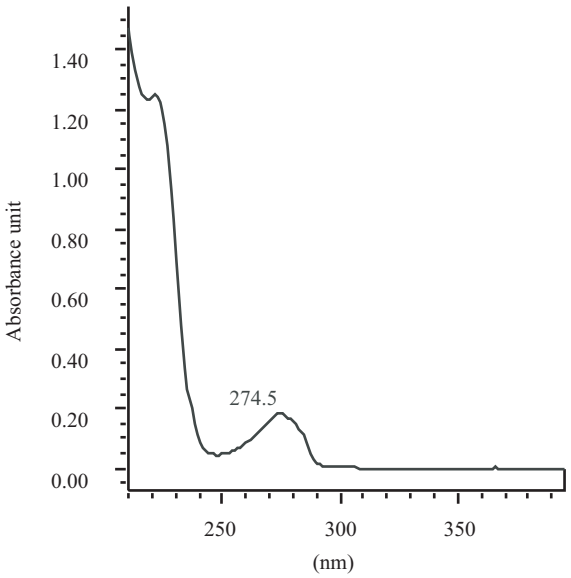

Fig 6 UV visible spectrum of active fraction.

(*Flardh and Buttner 2009)*. *Streptomyces* are the one a genus of *Actinomycetes* that morphologically resemble fungi and physiologically resemble bacteria. Subsequent growth of *Streptomyces* colonies as they spread over the agar surface is thought to follow similar kinetics to filamentous fungi (Bushell 1988). The colony growth of the *Streptomyces* is initiated when a spore germinates, giving rise to one or more long multinucleoid filaments. These filaments elongate and branch repeatedly, originating a vegetative mycelium (substrate mycelium) that develops over, and into the culture medium (Miguelez *et al.* 1999).

Extract preparation of broth culture was carried out by using organic solvent. Table 2 showed that there were strong antibacterial activities on supernatant extract, but no in the biomass extract. This indicates that A11 produced antibacterial substance by extracellular secretion. The pattern of HPLC chromatogram (Fig 2 and 3) for supernatant extract and biomass extract in were different. Fig 2 showed many peaks, indicating that many compounds were produced by A11 and extractable from the supernatant. On the other hand, there were not many compounds showing up in extracts of the biomass (Fig 3). After further purification with silica gel column and preparative HPLC, the single active compound could be obtained. Fig 5 shows the polarity of active compounds, indicating semi polar and a high purity. UV maximum absorbance at 210 nm and 274.5 nm (Fig 6) indicated that this compound is colorless or white powder. It is reported that the most of peptide antibiotic exhibit maximum absorbance at 210-230 nm and 270-280 nm. An absorbance at 220-230 nm is corresponding to characteristic absorption of peptide bonds (Kumar *et al.* 2009).

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