BIODIVERSITAS
Volume 11, Number 4, October 2010
Pages: 176-181

ISSN: 1412-033X (printed edition)
ISSN: 2085-4722 (electronic)
DOI: 10.13057/biodiv/d110402

Marine Actinomycetes screening of Banten West Coast and their antibiotics purification

ROFIQ SUNARYANTO♥, BAMBANG MARWOTO
Center of Biotechnology BPPT, PUSPIPTEK, Serpong, Tangerang Selatan 15340, Banten, Indonesia Tel./Fax.+62 21 7560208, ♥email: rofiqsn@yahoo.com

Manuscript received: 18 May 2010. Revision accepted: 2 November 2010.

ABSTRACT

Sunaryanto R, Marwoto B (2010) Marine Actinomycetes screening of Banten West Coast and their antibiotics purification. *Biodiversitas 11: 176-181*. Isolation and purification of active compounds produced by marine Actinomycetes has been carried out. Marine sediment samples were obtained from six different places at Anyer, Banten West Coast in October 20, 2007. Isolation was carried out using two methods pretreatments, acid treatment and heat shock treatment. A total of 29 Actinomycetes isolates were obtained from the various sediment samples collected, then tested for antimicrobial test against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853, *Bacillus subtilis* ATCC 66923, *Candida albicans* BIOMCC00122 and *Aspergillus niger* BIOMCC00134. Identification of potential isolate was carried out using 16S rRNA. Purification of active compound was carried out using silica gel column chromatography and preparative HPLC. Result of this research showed that isolate A11 produced the most active compound against Gram-positive and Gram-negative bacteria. Morphology and identification test using 16S rRNA gen showed that isolate A11 is *Streptomyces* sp. Production of active compound from isolate A11 used yeast peptone medium. The single peak of active compound was detected by HPLC and showed retention time on 8.35 min and maximum absorbance UV visible of antibiotic was 210 nm and 274.5 nm. Active purified compound showed inhibition activity to Gram-positive and Gram-negative bacteria. Minimum inhibitory concentration (MIC) to *E. coli* ATCC 25922 was 27 µg/mL, *P. aeruginosa* ATCC 27853 68.7 µg/mL, *S. aureus* ATCC 25923 80.2 µg/mL, and *B. subtilis* ATCC 66923 73.7 µg/mL.

Key words: marine Actinomycetes, isolation, screening, antimicrobial activity, minimum inhibitory concentration.

INTRODUCTION

Actinomycetes are the most widely distributed group of microorganisms in nature which primarily inhabit the soil (Goodfellow et al. 1983; Locci et al. 1983). Almost 70% of the world antibiotics are known to come from Actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora* (Berdy 2005; Goodfellow et al. 1988). Previously, researchers are more focused on explore at terrestrial Actinomycetes. Now days, new antibiotics has been found from marine Actinomycetes (Fiedler et al. 2005; Ghanem et al. 2000; Lam 2006).

Although the exploitation of marine Actinomycetes as a source for discovery of novel secondary metabolites is at early stage, numerous novel metabolites have been isolated in past few years. As example, abyssomicin C is novel polycyclic polyketide antibiotic produced by a marine *Verrucosispora* strain (Riegdlinger et al. 2004). Abyssomicin C possesses potent activity against Gram-positive bacteria, including clinical isolates of multiple-resistant. Diazepinomicin is a unique farnesylated dibenzodiazepinone produced by a *Micromonospora* strain (Charan et al. 2004). It possesses antibacterial, anti-inflammatory and antitumor activity. Salinosporamide A is a novel β-lactone-γ-lactam isolated from fermentation broth of new obligate marine Actinomycetes, *Salinispora tropica* (Feling et al. 2003).

Indonesia is archipelago country that has wide sea area that is more than 3.1 million km^2. The high characteristic of sea showed a high of biodiversity such as microorganism, plant, and animal. Nevertheless this potency has been not exploited. Currently exploration of Actinomycetes in Indonesia still limited to terrestrial Actinomycetes. The objective of this research are isolation and purification active compound which produced by marine Actinomycetes (isolate A11).

MATERIALS AND METHODS

Sample collection and processing

Sediments were obtained from six locations of marine site in Anyer, Banten West Coast in October 20, 2007. From each location, six sediment samples of 5 g each were collected from 10 to 15 cm below the surface. Each of the sediment samples for each site was placed in small pre-labeled plastic bags which were tightly sealed. Serial dilutions up to 10^{-6} were then prepared for each of the six samples. Hereinafter each sample is given code in accordance 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 using acid and heat-shock treatments. Acid treatment was conducted by the acidifying the samples to pH 2 were obtained for 3 hours. Heat-shock treatment was conducted by the heating the samples at 60^{o} C for 4 hours (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 milliliter of nalidixic acid and 5 g/mL of rifampicin were added to reduce the number of unicellular bacteria (Pisano et al. 1989). The antifungal agent cycloheximide (100 g/mL) and 25 g/mL 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.

Actinomycetes identification based on 16S rRNA analysis.

DNA isolation. The DNA was isolated using FastPrep kit for DNA isolation. The pellet was lysised using lysing matrix, added with 1000 µL and homogenized using FastPrep instrument for 40 second at 4500 rpm.

DNA amplification and purification. PCR 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 Gel/DNA extraction kit.

16S rRNA gene sequencing. 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 system, ABI 300 genetic analyzer. The sequence was compared to a database available at NCBI using BLAST search.

Liquid culture of active substance

A well grown agar slant of isolate was inoculated into a 250 mL flask containing 100 mL of the vegetative medium (YEME medium) consisting of: bacto peptone 5 g/L, yeast extract 3 g/L, malt extract 3 g/L, glucose 3 g/L, demineral water 250 mL, and sea water 750 mL. pH value of the medium was adjusted at 7.6 before sterilization. The flask was incubated at 30º C for 2 days in incubator shaker. Fifty milliliter of this culture was transferred to 1000 milliliter of the fermentative medium (Nedialkova et al. 2005). Fermentative medium consisting of bacto peptone 15 g/L, yeast extract 3 g/L, Fe (III) citrate hydrate 0.3 g/L, demineral water 250 mL, and sea water 750 mL. pH value of the medium was adjusted at 7.6 before sterilization. The fermentation was carried out at 30ºC for 5 days in incubator shaker (Kanoh et al. 2005).

Extraction and purification

The culture broth was centrifuged at 14000 x g for 15 min. The dark filtrate was divided and extracted using ethyl acetate solvent. Filtrate and organic solvent was mixed thoroughly by shaking them in 1000 mL 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 volume, after the dehydration with anhydrous Na_2SO_4. The aqueous layer re-extracted and the organic layer added to the above organic layer. The organic layer was concentrated by evaporation under vacuum again.

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

Preparative HPLC

Purification by preparative HPLC was conducted using a Waters 2695 HPLC, photodiode array detector (PDA), and Column puresil 5µ C18 4.6x150 mm. The volume injected was 100 µL per injection under conditions of average pressure of 1.267 psi, and the flow rate was 1 mL/min 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), which are dripped by extract solution, dried and then placed over the agar surface plates freshly inoculated with the *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 66923, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* BIOMCC00122 and *Aspergillus niger* BIOMCC00134 as test organism. Suspensions of test organisms were adjusted to 10^6 cfu/mL. The most potent isolates were noted for each test microorganism, based on the mean diameter of inhibition zones.

Analysis HPLC

HPLC analysis was performed using an analytical column Sunfire (4.6 x 250 mm, Shiseido Co. Ltd., Tokyo, Japan), elution using methanol-water (0-100% linear gradient for 25 min and then isocratic elution with 100% methanol until 10 min), at a flow rate of 1 mL/min and detection at 210 nm.

Determination of the minimum inhibitory concentration (MIC)

MIC determinations were performed using the agar-dilution methods according to modified methods of Bonev et al. (2008) and Andrews (2001). Active purified compound was dissolved in methanol (6500 µg/mL concentration) were taken as standard stock. A series of two fold dilutions of each extract in the final concentration of 25 µg/mL were dripped on paper disc 6mm, dried and then placed over the agar surface plates freshly inoculated with either *E. coli* ATCC 25922, *S. aureus* ATCC25923, *B. subtilis* ATCC 66923, and *P. aeruginosa* ATCC27853 as test organisms. The value of logarithm of MIC (Log MIC) was determined as the zero intercept of a linear regression of logarithm of concentration Log[C] as Y axis versus the squared size of clear zones diameter (X^2) as X axis. MIC is antilogarithm the intercept.

RESULTS AND DISCUSSION

Isolation and screening of Actinomycetes from marine

The five sediment samples of the sampling area yielded 29 Actinomycetes isolates. Eight of the 29 Actinomycetes isolates showed antimicrobial activity, 2 isolates active against *E. coli* ATCC 25922, 4 isolates active against *S. aureus* ATCC25923, 2 isolates active against *B. subtilis* ATCC 66923, 3 isolates active against *P. aeruginosa* ATCC27853, 3 isolates active against *C. albicans*, and 2 isolates active against *A. niger* (Table 1).

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, western 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 anti fungi including yeast.

From eight isolates which active against bacteria test, only one was chosen to next study. A11 isolate showed high activity against Gram-positive and Gram-negative bacteria. A11 isolate was selected for next study. From identification using 16S rRNA was obtained the information that isolate A11 was *Streptomyces* sp., homology 100% to *Streptomyces* sp. J22, class *Actinobacteria*, order *Actinomycetales*, family Streptomycetaceae, and genus *Streptomyces.* 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 was characteristic of *Streptomyces* morphology (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).

Table 1. Eight isolates of Actinomycetes (Banten, western Java coast) producing antimicrobial active compound.

Name of isolate	Sample pre-treatment	Antimicrobial (clear zone diameter in mm)					
		E. coli	*S. aereus*	*B. subtilis*	*P. aeruginosa*	*C. albican*	*A. niger*
A61	HS	0	0	0	0	0	0
A62	HS	0	0	0	0	0	0
A63	HS	0	0	0	0	0	0
A64	HS	0	0	0	0	0	**15**
A65	HS	0	0	0	0	0	0
A66	HS	0	0	0	0	0	0
A67	A	0	0	0	0	0	0
A68	A	0	0	0	0	0	0
A69	A	0	0	0	0	0	0
A610	A	0	**12**	0	0	0	0
A611	A	0	0	0	0	0	0
A11	HS	**18**	**15**	**14**	**14**	0	0
A12	HS	0	0	0	0	0	0
A21	HS	0	0	0	**7**	0	**9**
A23	A	0	0	0	0	0	0
A24	A	0	0	0	0	0	0
A31	HS	0	0	0	0	0	0
A32	HS	0	**12**	0	0	**7**	0
A33	HS	0	0	0	0	0	0
A41	HS	0	0	0	0	0	0
A42	HS	0	0	0	0	0	0
A43	A	**10.16**	**0**	**8.67**	**9.51**	0	0
A44	A	0	0	0	0	**10.61**	0
A45	A	0	0	0	0	0	0
A51	HS	0	0	0	0	0	0
A52	HS	0	0	0	0	0	0
A53	HS	0	0	0	0	0	0
A54	HS	0	**8.56**	0	0	**8.67**	0
A56	A	0	0	0	0	0	0

Note: HS: Heatshock treatment, A: Acid treatmeant, Diameter of paper disc: 6 mm.

The phylogenic tree (Figure 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).

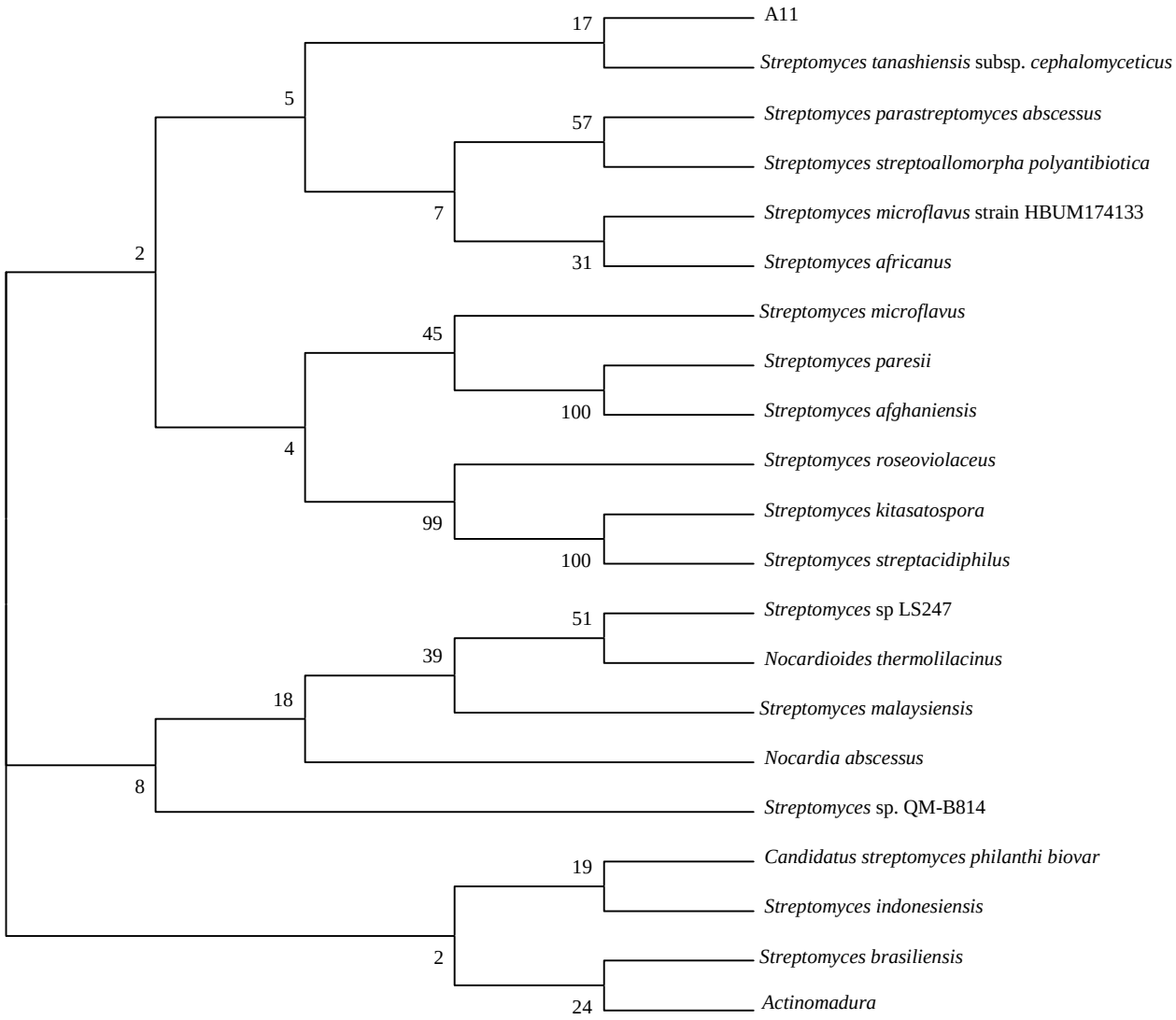

Figure 1. Polygenetic tree of isolate A11 shown as *Streptomyces* sp.

Fermentation and purification

Fermentation of isolate A11 was carried out for 7 days with yeast-peptone medium. At the last day of fermentation, the medium color became dark and more viscous than first day. It was looked many white granular in the bottom of flask. From 5 liters volume of fermentation was obtained 4.72 g of dry biomass after extracted by methanol, and methanol extract of biomass was obtained 2.72 g, extract of supernatant was obtained 0.33 g. Antimicrobial bioassay

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

Sample	Diameter of inhibition/clear zone (mm)					
	S. aureus	***B. subtilis***	***P. aeruginosa***	***E. coli***	***C. albicans***	***A. 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	-	-

Note: Diameter of paper disc: 6 mm.

Table 3. Minimum inhibitory concentration (MIC) of active purified compound.

Sample	Minimum Inhibitory Concentration (MIC) µg/mL			
	E. coli **ATCC 25922**	***S. aureus*** **ATCC 25923**	***B. subtilis*** **ATCC 66923**	***P. aeruginosa*** **ATCC 27853**
Active purified compound	27	80.2	73.7	68.7
Tetracycline (positive control)	64.0	256	128	12.5

showed that extract of supernatant active to bacterial test, but extract of biomass have no activity to bacterial test. The data of biological activity extract fermentation from isolate A11 is presented in Table 2. Table 2 showed that there were strong antibacterial activities on supernatant extract, but no in the biomass extract. This indicates that isolate A11 produced antibacterial substance by extracellular secretion.

Further purification of the antibiotic has been carried out using column chromatography and preparative HPLC. Antibacterial test to all fraction of preparative HPLC showed that peak retention 10.1 min was active fraction. Active fraction was collected and test to analysis HPLC. Analysis HPLC chromatogram of active fraction and UV visible spectrum was presented at Figures 2 and 3.

Figure 2 showed that active fraction of antibiotic has retention time 8.6 min at gradient elution methanol-water 0-100% using column sunfire. Purification using preparative HPLC obtained single peak with maximum absorbance UV visible was 210 nm and 274.5 (Figure 3). This compound indicated that was colorless or white powder.

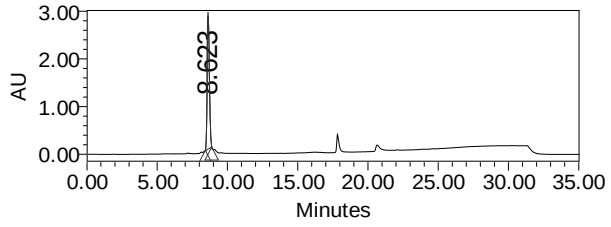

Figure 2. Analysis HPLC chromatogram of active fraction

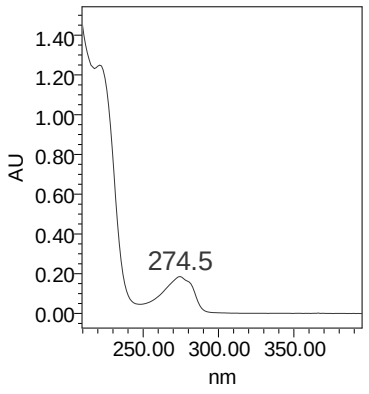

Figure 3. UV visible spectrum of active fraction.

Isolate A11 was chosen to be subjected for minimum inhibitory concentration (MIC) assay since it exhibited the larger zone of inhibition. Table 3 showed that active compounds produced by isolate A11 was highly active against *E. coli* ATCC 25922, *S. aureus* ATCC25923, *P. aeruginosa* ATCC27853, *B. subtilis* ATCC 66923, with respective MIC value 27, 80.2, 68.7, and 73.7 µg/mL. This indicates that this active compounds highly active against Gram-positive and Gram-negative bacteria. It was compared tetracycline, this active compound was stronger active against *E. coli* ATCC, *S. aureus* ATCC25923, and *B. subtilis* ATCC 66923, but rather weaken against *P. aeruginosa* ATCC27853.

CONCLUSION

Actinomycetes (isolate A11) was isolated from sediment in Anyer, Banten produced antibiotic active against to *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853, *Bacillus subtilis* ATCC 66923. Identification using 16S rRNA showed that isolate A11 is *Streptomyces* sp. Purification of antibiotic using column chromatography and preparative HPLC produce single peak of chromatogram at retention time 8.623 min and max UV absorbance was 210 nm and 274.5 nm. Minimum inhibitory concentration (MIC) to *E. coli* ATCC 25922 was 27 µg/mL, *P. aeruginosa* ATCC 27853 68.7 µg/mL, *S. aureus* ATCC 25923 80.2 µg/mL, and *B. subtilis* ATCC 66923 73.7 µg/mL.

ACKNOWLEDGMENTS

We thank to Anis Mahsunah (Head of Downstream Processing Laboratory) and Hardaning Pranamuda (Head of Industrial Biotechnology Division, Biotech Center BPPT, South Tangerang) for their valuable and critical comments on this research. We also thank IDB (Islamic Development Bank) for supporting scholarship of our study.

REFERENCES

Andrews JM (2001) Determination of minimum inhibitory concentration. J Antimicrob Chemother 48: 5-16.

Antonova-Nikolova S, Stefanova V, Yocheva L (2007) Taxonomic study of *Streptomyces* sp. strain 34-1. J Cult Collect 5: 10-15.

Berdy J (2005) Bioactive microbial metabolites (review article). J Antibiot 58 (1): 1-26.

Bonev BH, James, Judicael P (2008) Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. J Antimicrob Chemother 61: 1295-1301.

Bushell ME (1988) Growth, product formation and fermentation technology in Goodfellow M, Williams ST, Mordarski M (1988) Actinomycetes in biotechnology. Acad Press, London.

Charan RD, Schlingmann G, Janso J, Bernan V, Feng X, Carter GT (2004) Diazepinomicin, a new antimicrobial alkaloid from marine *Micromonospora* sp. J Nat Prod 67: 1431-1433.

Chater K (2006) *Streptomyces* inside-out: a new perspective on the bacteria that provide us with antibiotics. Phil Trans R Soc B 361: 761-768.

Feling RH, Buchanan GO, Mincer TJ, Kauffman CA, Jensen PR, Fenical W (2003) Salinosporamide A: a highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus *Salinospora*. Angew Chem Int Ed Engl 42: 355-357.

Fiedler HP, Christina B, Alan TB, Alan CW, Michael G, Olivier P, Carsten P, Gerhard H (2005) Marine Actinomycetes as a source of novel secondary metabolites. Antonie Leeuwenhock 87: 37-42.

Flardh K, Buttner MJ (2009) *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. J Nat Rev Microbiol 7: 36-50.

Ghanem BN, Soraya AS, Zeinab ME, Gehan AAE (2000) Isolation and enumeration of marine Actinomycetes from seawater and sediments

in Alexandria. J Gen Appl Microbiol 46 (45): 105-111.
Goodfellow M, Williams ST (1983) Ecology of Actinomycetes. Ann Rev Microbiol 37: 189-216.
Goodfellow M, Haynes JA (1984) Actinomycetes in marine sediment. P.453-472 In Ortiz-ortiz L, Bojalil LF, Vakoleff V (ed). Biological, Biochemical, and Biomedical aspect of Actinomycetes. Academic Press Inc, Orlando.Fla.
Goodfellow M, William ST, Mordarski M (1988) Actinomycetes in biotechnology. Academic Press, New York.
Kanoh K, Matsuo Y, Adachi K, Imagawa K, Nishizawa M, Shizuri Y (2005) Mechercharmycins A and B, cytotoxic substances from marine-derived *Thermoactinomyces* sp. YM3-251. J Antibiot 58 (4): 289-292.
Kazakevich Y and Lobrutto R (2007) HPLC for pharmaceutical scientists. A John Wiley & Sons Inc, New Jersey.
Lam KM (2006) Discovery of novel metabolites from marine actinomycetes . Curr Opin Microbiol 9: 245-251.
Locci R, Sharples GP (1983) Morphology of Actinomycetes in Goodfellow M, Mordarski M, Williams ST (1984) The biology of the Actinomycetes. Academic Press, London.
Miguelez EM, Hardisson C, Manzanal MB (1999) Hyphal death during colony development in *Streptomyces antibioticus*: Morphological evidence for the existence of a process of cell deletion in a multicellular prokaryote. J Cell Biol 145: 515-525.
Nedialkova D, Mariana N (2005) Screening the antimicrobial activity of Actinomycetes strains isolated from Antarctica. J Cult Collect 4: 29-35.
Parungao MM, Maceda EBG, and Villano MAV (2007) Screening of antibiotic-producing Actinomycetes from marine, brackish and terrestrial sediments of Samal Island, Philippines. J Res in Sci Comp Eng 4: 329-338.
Pisano MA, Michael JS, Madelyn ML (1986) Application of pretreatments for the isolation of bioactive Actinomycetes from marine sediments. Appl Microbiol Biotechnol 25: 285-288.
Pisano MA, Sommer MJ, and Brancaccio L (1989) Isolation of bioactive Actinomycetes from marine sediments using rifampicin. Appl Microbiol and Biotechnol 31: 609-612.
Riegdlinger J, Reicke A, Zahner H, Krismer B, Bull AT, Maldanado LA, Ward Ac, Goodfellow M, Bister B, Bischoff D (2004) Abyssomicins, inhibitors of the para-aminobenzoic acid pathway produced by the marine *Verrucosispora* strain AB-18-032. J Antibiot 57: 271-279.
Tarui N, Yoshinori I, Hideaki, Kazuo N (2001) Microbial synthesis of three metabolites of a tachykinin receptor antagonist, TAK-637. J Biosci Bioeng 92: 285-287.