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Isolation and Screening of Antimicrobial Producing-Actinomycetes Symbionts in Nudibranch

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Abstract

The aims of this study were to isolate and to screen actinomycetes associated with sea slug which have the ability to produce antimicrobial compound, especially against MDR strains. Actinomycetes were isolated from nudibranchs collected from Bandengan coastal waters and the Panjang island, Jepara, Central Java. Actinomycete isolates were assayed for their antimicrobial activity against MDR strains (MDR 6 *E. coli*, MDR 7 *Enterobacter* sp., MDR 13 *Proteus* sp., MDR 14 *Staphylococcus sp.*). The genetic diversity of the active isolates was analyzed by using repetitive DNA fingerprinting. Antimicrobial activity was also performed on the ethyl acetate bacterial extract. The amplification of Polyketide Synthase-I (PKS-I) and Non-Ribosomal Peptide Synthetase (NRPS) genes was carried out to estimate the genetic potency of actinomycetes. The most active actinomycete isolate was sequenced based on 16S rDNA approach. General profile of antimicrobial substances was analyzed by using Thin Layer Chromatography (TLC). A total 27 isolates were obtained from nudibranchs *Jorunna* sp. and 12 isolates from *Chromodoris* sp. Ten isolates exhibited antimicrobial activity. Five representative isolates were selected based on rep-PCR analysis. Three ethyl acetate extracts exhibited antimicrobial activity against MDR 7, MDR 13, and MDR 14, except MDR 6. NPC 8 isolates significantly inhibited the growth of the tested strain and amplified NRPS gene fragment. Molecular identification revealed that isolate NPC 8 closely related to *Streptomyces* sp with a high homology of 96%.

Keywords : sea slugs (nudibranch), actinomycetes, MDR strain, 16S rRNA gene, *Streptomyces* sp.

Introduction

More than 70% of our planet's surface is covered by oceans and life on Earth originated from the sea (Proksch *et al.*, 2003; Lam, 2006). In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than in the tropical rainforests (Lam, 2006). Marine invertebrates are sources of a diverse array of bioactive metabolites with great potential for development as drugs and research tools (Haygood *et al.*, 1999). Nudibranch have long been recognized as producers of biologically active secondary metabolites (Fahey and Carroll, 2007). The supply problem with regard to drug development and sustainable production lies in the limited amounts of biomass of most marine invertebrates available from wild stocks.

Marine microorganisms are excellent source for antimicrobial compounds. Serious attempts to reach the vast potential of marine organisms as sources of bioactive metabolites that may be directly utilized as drugs (El-sersy dan Abou-Elela, 2006; Lam, 2006). In many cases, microorganisms are known or suspected to be the biosynthetic source of marine invertebrate natural products (Haygood *et al.*, 1999; Kelecom, 2002). Actinomycetes are the most economically and biotechnologically

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valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics, anti-tumor, immunosuppressive agents and enzymes (Lam, 2006; Vasavada *et al.*, 2006). Actinomycetes, are prolific producers of antibiotics and other industrially useful secondary metabolites (Mincer *et al.*, 2005; El-Sersy dan Abou-Elela, 2006).

Actinomycetes have often been exploited because of their ability to biosynthesize an impressive array of novel metabolites particularly polyketides. As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds (Lam, 2006).

Antimicrobial resistance has become a major health problem worldwide, both in the hospitals and the community. The emergence of antimicrobial resistance is correlated with selective pressure from, for instance, inappropriate use of antimicrobial agents, and results in increased mortality, morbidity and health care costs (Lestari *et al.*, 2007). Moreover, the rise in the number of drug-resistant pathogens and the limited success of strategies, such as combinatorial chemistry in providing new agents indicates an uncertain forecast for future antimicrobial therapy (Magarvey *et al.*, 2004). Thus, it is critical that new groups of actinomycetes from nudibranch could be pursued as the sources of novel antibiotics and other small-molecule therapeutic agents.

Materials and Methods

Sample collection

The actinomycete strains were isolated from nudibranch samples collected from Bandengan coastal waters and the Panjang island (3 and 10 m in depth), Jepara, Central Java by scuba diving.

Isolation of marine-derived actinomycete

Nudibrach samples were rinsed with sterile seawaters 3 times to clean all the debris and non-symbiont microbial community. Only those that firmly attached were left behind in the nudibranchs. Nudibranchs were cut into pieces and then were homogenated with blender. The mixture was then serially diluted by putting into 9 ml sterile seawaters and was proceeded until dilution of 10^{-4} was obtained. A 100 ml of each dilution was then spread on to a starch nitrate solid medium previously prepared. The inoculated medium was then incubated at room temperature for 7 d until the colonies appeared. Purification of the colonies was performed until single and pure isolate was obtained.

DNA extraction

Mycelia (5 ml) grown in a starch nitrate were centrifuged, rinsed with TE and resuspended in 0.4 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Lysozyme was added about of 50 ml (10 mg / ml) and incubated at 37°C for 1 h. Then 50 ìl 10% SDS was added and incubated at 65°C with occasional inversion for 1 h. About of 50 ml 5 M NaCl was added and incubated at 65°C for 1 h. Chloroform 400 ml was added and incubated at room temperature for 30 min with frequent inversion. The mixture was centrifuged at 13.000 rpm for 10 min and the aquoeus phase was transfered to a new tube. Chromosomal DNA was precipitated by the addition of 1 vol 2-propanol with gentle inversion. The DNA was transferred to a new tube, rinsed with 70% ethanol, dried and dissolved in a suitable volume of TE buffer. Samples were extracted in the same volume of phenol / chloroform / isoamyl alcohol (25:24:1) and precipited with 0.1 vol 3M

sodium acetate. The pellets were washed with 70% ethanol, dried and dissolved in TE buffer.

Repetitive extragenic palindromic-PCR (rep-PCR)

Repetitive DNA fingerprinting was performed on all the isolates following the method of Sadowsky *et al.*, (1996). The PCR primer BOXA1R derived from the repetitive sequences (5'-CTACGGCAAGGCGACGCTGACG -3') was used to amplify the DNA samples. The PCR mixture contained 3 µl H_2O, 1 µl DNA genome of actinomycetes as template (50 ng / µl), 1 µl BOX A1R primer (15 pmol), and 5 µl Mega Mix Royal (MMR). The following PCR condition was performed: Pre-denaturing (95°C, 5 min), 30 cycles of 94ºC for 1 min, 53ºC for 1.5 min, 68°C for 1 min, and a final extension step at 68ºC for 10 min. Amplification products were analyzed by electrophoresis in 1% agarose gels stained with ethydium bromide.

Extraction of secondary metabolites of isolates

Loopful of selected actinomycetes on slant agar were inoculated into 5 ml soluble Starch nitrate broth. Then it was incubated in a rotary shaker at 150 rpm for 7 days at room temperature. The culture was inoculated into 50 ml starch nitrate broth in 500 ml Erlenmeyer flask. The flask was incubated on shaker at room temperature for 7 days. The culture was transferred into 50 ml sterile conical tubes and centrifuged at 3000 rpm for 15 min. Furthermore, the supernatant was used as a source of antimicrobial metabolites.

Extraction of antimicrobial metabolites was performed by using ethyl acetate. The supernatant was transferred to a separating flask. Ethyl acetate was added with a ratio of 1:1(v / v) and shaked vigorously for 10 min. The top layer was transferred by Pasteur pipette to a clean glass tube. Ethyl acetate extraction was done twice. The supernatant was collected and passed throughout a column containing sodium sulphate and the filtrate was evaporated until the volume becomes 1ml and kept in the refrigerator until further experiments.

Determination of antibacterial activity

Isolates of actinomycetes were screened for their antibacterial activity against MDR 6 *Escherichia coli*, MDR 7 *Enterobacter* sp., MDR 13 *Proteus* sp. and MDR 14 *Staphylococcus* sp. by using swab method. Zobell 2216E was used as medium. Five microliter of ethyl acetate extract was diluted in 10 ml ethyl acetate and then loaded into paper disk. The paper disk was placed into petridish which has been previously swabbed with each of the bacteria. The plates were incubated at temperature 37°C for 24 h. The diameter of inhibition (clear) zone was measured to determine the extent of antibacterial activity.

PCR Amplification of NRPS and PKS-I Sequences

The PCR primers A3F(5'-GCSTACSYSATSTACACSTCSGG-3'),A7R (5'-SASGTCVCCSGTSCGGTAS-3'), K1F (5'-TSAAGTCSAACATCGGBCA-3'), and M6R (5'-CGCAGGTTSCSGTACCAGTA-3') was used to amplify the DNA samples. The PCR mixture contained 2 µl H_2O, 1 µl DNA genome of actinomycetes as template (50 ng / µl), 0,5 µl each primer (15 pmol), and 5 µl MMB. The following PCR condition was performed in 5 min at 95°C and 35 cycles of 30 s at 95ºC, 2 min at 58ºC, 4 min at 72°C, followed by 10 min at 68ºC. Amplification products were analyzed by electrophoresis in 2% (agarose gels stained with ethidium bromide) (Ayudo-Sacido and Genilloud, 2005).

16S rRNA gene amplification and DNA sequencing

The PCR primers F8 (5'-AGAGTTTGATC(A/C)TGGCTC-3') and R1492 (5'-TACGG[A/T/C]TACCTTGTTACGACTT-3') was used to amplify the DNA samples. The PCR mixture contained 2 µl H_2O, 1 µl DNA genome of actinomycetes as template (50 ng/µl), 1 µl each primer (25 pmol), and 5 µl Mega Mix Blue(MMB). The PCR conditions consisted of an initial denaturation at 80°C for 5 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and a final 7-min extension at 72°C. Amplification products were analyzed by electrophoresis in 2% (agarose gels stained with ethidium bromide) (Magarvey *et al*,, 2004).

The nearly complete 16S rRNA gene was sequenced by using the PCR products directly as sequencing templates with the following primers: F8, F357, F530,F790 and F981. All sequencing reactions were carried out with an ABI 3700 automated DNA sequencer ABI PRISM® 310 Genetic Analyzer,University of Islamic Sunan Kalijaga.

Molecular taxonomy determined by sequences and phylogenetic analysis

The nearly complete 16S rRNA gene sequences (1,445 nucleotides) were used to search the GenBank database with the BlastN algorithm to determine relative phylogenetic positions. Sequences then were aligned by using ClustalW software .

Profiling the secondary metabolites by Thin Layer Chromatography (TLC)

Fifteen microliters of ethyl acetate extract were spotted on the Silica gel 60F254 (Merck) with solvent system ethyl acetate : n-hexane (1:2) (v / v).

Results and Discussion

Isolation of actinomycetes

The decreasing rate of discovery of novel drugs from established terrestrial sources has motivated the evaluation of new sources of chemically diverse bioactive compounds. The oceans represent an under-explored environment for microbial discovery, and although new methods are under development, relatively few have been applied to reveal the microbial diversity of the ocean environment.

Marine microbial symbionts in nudibranch, in particular, have been largely overlooked. The data presented here provided conclusive evidence for the persistent occurrence of actinomycete populations in nudibranchs. It has been frequently suggested that microorganisms should be the true producers of a number of secondary metabolites (Kelecom, 2002).

A total 27 isolates were obtained from sea slugs *Jorunna* sp. and 12 isolates from *Chromodoris* sp. Actinomycetes grew well on the starch nitrate agar medium. Starch nitrate gave high counts and high ratios to the total viable actinomycetes. Moreover, it supported the isolation of different types of actinomycetes as judged by the different morphological characters and pigmentation. The colonies first appeared on the 4th until 7th days. The colonies were recognized by pigmentation range from brown, white, yellow and orange. According to Mincer *et al.* (2005), the colonies first appeared after 3 weeks of incubation but sometimes took as long as 6 weeks to appear, depending upon the medium and isolation method .

Marine bacteria have proven to be uneasy to be cultured. Special media had to be developed, and it seems logical to admit that the most difficult organisms to grow well and produce the most original new metabolites. Isolating microorganism from host and culturing it, metabolic changes may occur probably due to partially unsatisfied micro-nutrients in the culture medium.

Antagonistic activity of actinomycete

strains

Out of 39 strains examined on antagonistic activity, 25% significantly active against MDR 7, MDR 13 and MDR 14, except MDR 6. The strains that exhibited significant activity were also tested against the crude extract of ethyl acetate. This cell antagonist test serves as an initial step to quickly select actinomycete isolates which are potential to produce secondary metabolites.

Genetic diversity of actinomycete isolates

Investigation results of genetic diversity of actinomycete isolates are presented in Figure 1, which showed that NBJ 24 isolate did not share the genetic diversity among isolates. Isolates that have same genetic diversity are NBJ 3 with NBJ 25 and NPC 9 , NBJ 27 with NPC 8, NPC 10 with NPC 11, NBJ 7 with NPC 12.

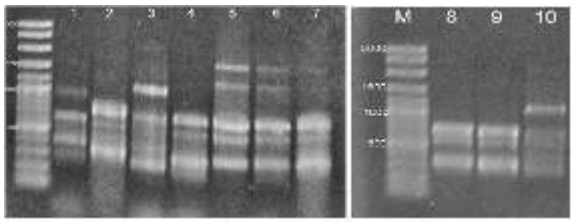

Figure 1. Genetic diversity of actinomycetes isolated from nudibranch were analyzed by rep-PCR with BOXA1R primer. M : Marker 100 bp DNA Ladder; 1: NBJ 24; 2: NBJ 3; 3: NBJ 7; 4: NBJ 25; 5: NBJ 27; 6: NPC 8; 7: NPC 9; 8: NPC 10; 9: NPC 11; 10: NPC 12.

The similarity in DNA fingerprints indicates that BOX repetitive sequences of the bacteria were located at the same position in chromosome. It, therefore, could be predicted to have a close relationships. Genersch and Otten (2003) explained that the DNA fingerprint by rep-PCR were useful for classification of bacteria subtype or strain. The result of genetic variability analysis with this rep-PCR indicates that only 1 isolate (NBJ 24) was species-specific, while the others were not species-specific group.

Antibacterial activity of isolates

Antibacterial activity of the selected actinomycetes against MDR6, MDR7, MDR13, dan MDR 14 is shown in Table 1. Three ethyl acetate extracts of the active isolates were shown to be potent antibacterial activity against MDR 7, MDR 13 and MDR 14, except MDR 6. Three active isolates are NBJ 24, NPC 8 and NPC 12. However, since the reagent used was ethyl acetate, other possibly active compounds that belong to non-polar or high polar could be missed.

Table 1Antibacterial activity of the selected actinomycetes against MDR6, MDR7, MDR13, dan MDR 14

Ethyl acetate extract	Diameter (mm)			
	MDR 6	MDR 7	MDR 13	MDR 14
NBJ 24	-	9,1	12,0	11,2
NPC 8	-	18,8	11,5	11,8
NPC 12	-	14,8	12,8	15,3

The emergence of multidrug resistant strains of *Escherichia coli* has complicated treatment decision and may lead to treatment failures. *E. coli*, the most common number of the family Enterobacteriaceae implicated in urinary tract infections, has showed increasingly resistant to antibiotics and caused some therapeutic problems (Moniri *et al*., 2003). It was revealed that none of the active isolates was capable of inhibiting the growth of MDR strain *E. coli* used in this study.

Morphology of actinomycetes colony

Strain NBJ 24 and NPC 12 do not produce aerial mycelium, but produce sporangia. Strain NPC 8 produces aerial mycelium and surface colony with brown color. Colony morphologies of the selected marine actinomycetes are represented in Figure 2.

Detection of PKS-I and NRPS genes

The presence of Polyketide Synthase-I (PKS-I) and NonRibosomal Peptide Synthetase (NRPS) genes were detected by

degenerated primers to understand their correlation with antibacterial activity of Actinomycetes isolates. Among the 3 isolates, NRPS gene fragment was found only in 1 isolate (data not shown). Non-ribosomal peptide synthetases (NRPS) and type I polyketide synthases (PKS-I) are biosynthetic systems involved in the synthesis of a large number of important biologically active compounds. Furthermore, not only PKS-I or NRPS gene is involved in the biosynthesis of bioactive secondary metabolites (Fingking and Marahiel, 2004).

The phylogenetic relationship

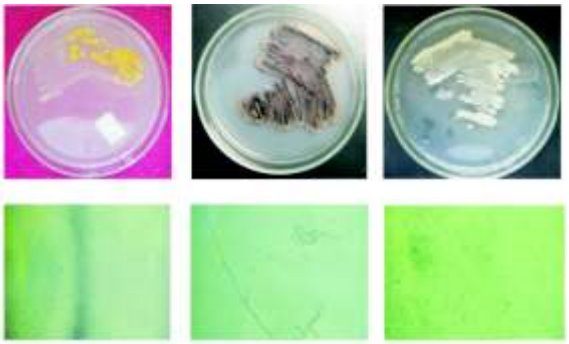

Figure 2. Colony morphologies of selected marine actinomycetes .A: NBJ 24; B: NPC 8:NPC 12

Molecular taxonomy of marine-derived actinomycetes

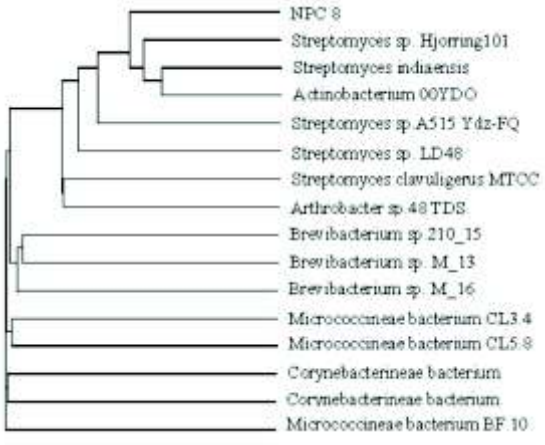

Figure 3. Phylogenetic relationship determined from almost complete 16S rDNA sequences of select NPC 8 isolate

determined from almost complete 16S rDNA sequences of the selected NPC 8 isolate is represented in Figure 3. A comparison of the nearly complete 16S rRNA gene sequences (averaging 1459 nucleotides) of the strains NPC 8 here against sequences in the GenBank database revealed homologies of greater than 96% to members of the *Streptomyces* sp.

According to Omura *et al.* (2001), Streptomyces is a genus of Gram positive bacteria that grows in soil, marshes and coastal marne habitats and forms filamentous mycelium-like eukaryote fungi. Morphological differentiation in Streptomyces involves the formation of a lawn of aerial hyphae on the colony surface that stands up into the air and differentiates into chains of spores.This process, unique among Gram-positive bacteria, requires the specialized coordination of metabolism and is more complex than other Gram-positive bacteria. The most interesting property of Streptomyces is its ability to produce secondary metabolites including antibiotics and bioactive compound.

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