Combination Methods for Screening Marine Actinomycetes Producing Potential Compounds as Anticancer

Yuyun Farida.[1], Jaka Widada[2*], and Edy Meiyanto[3]

1. Study Program of Biotechnology, Graduate School of Gadjah Mada University, Yogyakarta, Indonesia
2. Department of Microbiology, Faculty of Agriculture, Gadjah Mada University, Yogyakarta, Indonesia
3. Faculty of Pharmacy, Gadjah Mada University[3]

Abstract

Marine actinomycetes is a robust source of secondary metabolites including anticancer compounds . The objective of this research was to select marine actinomycetes producing potential compounds as anticancer used combination methods that consist of amplification PKS I (polyketide synthases type I) and NRPS (non ribosomal peptide synthetases) genes, analysis the diversity of secondary metabolites and genetic. Selected isolates were used for cytotoxicity assay. PKS I and NRPS genes were amplified using sets of degenerate primers. K1F and M6R were used for amplify ketosynthase and methyl-malonyl-CoA transferase modules of PKS I gene which targeted sequences 1200-1400 bp. A3F and A7R were used for amplify adenilation domains of NRPS gene which targeted sequences 700-800 bp. The diversity of secondary metabolites was analized by TLC and densitometry of ethyl acetate extracts. Genetic diversity was analized by repetitive DNA fingerprinting using BOXA1R primers. The cytotoxicity of secondary metabolites on T47D and MCF7 breast cell lines cancer was measured by MTT assay method.

Fifty two marine actinomycetes isolates were screened using combination methods. Ten isolates were detected encoding both PKS I and NRPS genes, whereas 11 isolates were detected encoding the NRPS gene. The screening by analysis of secondary metabolites and genetic diversity methods were obtained 6 selected isolates for cytotoxicity assay, which consist of 3 isolates encoding both PKS I and NRPS genes and 3 isolates encoding NRPS gene. Isolate 1 had high cytotoxicity with the IC_{50} on T47D cell was 19 µg/ml and the IC_{50} on MCF7 cell was 7 µg/ml. This findings suggests that combination methods were effective and efficient way to select marine actinomycetes producing potential compounds as anticancer.

Keywords : marine actinomycetes, PKS I, NRPS, secondary metabolites, *rep*-PCR, anticancer, cytotoxicity, T47D, MCF7.

Introduction

The actinomycetes or *Actinobacteria* are a large group of filamentous, gram positif bacteria, that produce many bioactive secondary metabolites and have a significant role in the recycling of organic matter (Atlas, 1997). More than 45% important bioactive compounds were produced by this bacterial group (Berdy, 2005). Mincer *et al.* (2002) revealed that select groups of marine actinomycetes from genus *Salinispora* have proven to be particularly rich source of new bioactive compounds including anticancer. More than 80% *Salinispora* strain were able to inhibit human tumor proliferative.

The oceans cover 70% of the earth's surface and harbor most of the planet's biodiversity. The microbiological component of this diversity remains relatively unexplored. Marine actinomycetes which have high potential anticancer compounds could be explored from this environment (Bull *et al.*, 2000).

Screening methods of new drug discov-

*corresponding author : Jaka Widada, Department of Microbiology, Faculty of Agriculture, Gadjah Mada University, Jl.Flora Bulaksumur Yogyakarta, Indonesia, 55281, email : jwidada@gmail.com

ery were needed for developing resource of important bioactive compounds. The exploration of new anticancer compounds are relatively expensive. The objectives of this research was to obtain efficient and effective screening methods on the discovery of marine actinomycetes producing potential compounds as anticancer.

A broad range of potential bioactive compounds polyketide and peptida compounds produced by microorganisms, among others by actinomycetes are synthesized by type I polyketida synthases (PKS I) and nonribosomal peptida synthetases (NRPS) (Dewick, 2002). All of the isolates were screened for genes encoding PKS I and NRPS. The detection of gene sequences involved in the synthesis of secondary metabolites would allow to focus on screening on the most metabolically talented groups. Ayuso-Sacido and Genilloud (2004) designed primers targeted to specifically amplify PKS I and NRPS genes. All actinomycetes isolates encoded PKS I and NRPS genes on this screening might be have secondary metabolites and genetics diversity. The isolates which have similarity profiles in secondary metabolites and genetics were chosen as representative isolates.

This research was to select marine actinomycetes producing potential compounds as anticancer used combination methods. The amplification PKS I and NRPS genes were continued by analysis the diversity of secondary metabolites (Cannel, 1998) and genetics (Sadowsky, 1996). Selected isolalates were used for citotoxycity assay on breast cancer cell lines T47D and MCF7.

Materials and Methods
Isolation of marine actinomycetes
Isolation of marine actinomycetes used solid starch nitrate medium (Waksman, 1961) following Ghanem (2000). Composition is given in g/L : starch, 20; KNO_3, 1; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5;

$FeSO_4.7H_2O$, 0.01; bacteriological agar, 20. All media were prepared by using seawater and contained 75 µg ml^{-1} of filter-sterililized cycloheximide as antifungal agent. Samples (seaweed, sponge, seawater) were collected from Krakal Beach, Gunung Kidul Yogyakarta. One gram samples (seaweed and sponges) diluting in 9 ml NaCl 0.85% and treated by heating in a water bath at 70°C for 60 minutes to reduce the number of unicellular bacteria in favor of actinomycetes.

Actinomycetes were recognized by their characteristic tough leathery colonies, branched vegetative mycelia, and, when present, aerial mycelia and spore formation. Actinomycetes colonies were incubated at room temperature (25-30°C).

Genomic DNA extraction
Genomic DNA extraction following the methods of Pospiech *and* Neumann (1995) was modified by Song *et al.* (2004). Mycelia (5 ml) grown in a starch nitrate broth shake culture 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 ìl (10 mg/ml) and incubated at 37°C for 1 hour (h). Then 50 ìl 10% SDS was added and incubated at 65°C with occasional inversion for 1 h. About of 50 ìl 5 M NaCl was added and incubated at 65°C for 1 h. Chloroform 400 ìl 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 trasnferred 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 precipitated with 0.1 vol 3 M

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

Profile of secondary metabolites

Secondary metabolites profile was performed on selected isolates which encoding PKS I and NRPS genes by PCR. Loopful selected actinomycetes on slant agar were inoculated into 5 ml soluble starch nitrate broth. Then it was incubated in a rotary shaker at 200-250 rpm for 5 days at room temperature. Then the culture was inoculated into 100 ml starch nitrate broth in 500 ml Erlenmeyer flask. The flask was incubated on shaker at room temperature until 11 days. The culture was transferred into 50 ml sterile conical tubes and centrifuged at 3000 rpm for 15 minutes. Futhermore, the supernatant was used as a source of secondary metabolites.

Extraction of secondary 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 minutes. The top layer is 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 sulfate and then filtrate was evaporated until dry.

TLC was performed by using the Silica gel GF$_{254}$ (E-Merck) with solvent system Butanol – Acetic Acid – Water (BAW, 4:5:1, v/v) dan Chloroform – Methanol – Water (CMW, 90:10:1, v/v). Spots were visulized by densitometry of UV light 366 nm and 254 nm to determine quantitatively the different substances. Data were subjected from Rf values of densiometry were analyzed by using Unweight Pair Group with Mathematical Average (UPGMA) method.

Repetitive DNA fingerprinting (rep-PCR)

The analysis of repetitive DNA fingerprinting was performed on selected isolates encoding PKS I and NRPS genes following the method of Sadowsky (Sadowsky *et al.*, 1996). The PCR primer derived from the repetitive sequences (5′CTACGGCAAGGCGACGCTGACGCTGACG-3′) was used to amplify the DNA samples. The PCR mixture contained 4 µl H$_2$O, 1 µl DNA genome of actinomycetes as template (50 ng/µl), 1 µl BOX A1R primer (16,5 pmol), and 6 µl Mega Mix Royal (MMR). The following PCR temperature profile was performed: hot start of 5 min at 95ºC, 30 cycles of 1 min at 94ºC, 1.5 min at 53ºC, 1 min at 68°C, and a final extension step at 68ºC for 10 min. The PCR product was visualized by 8% PAGE. Data were subjected from electrophoresis of PAGE were analyzed by using Unweight Pair Group with Mathematical Average (UPGMA) method.

Cytotoxicity assay (MTT assay)

Ethyl acetate extracts secondary metabolites from selected isolates by combination methods were used for cytotoxicity assay on T47D and MCF7 breast cell lines cancer. The cytotoxicity was measured by MTT *(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability test* (Mosmann, 1983). The IC$_{50}$ was determined by probit analysis using SPSS 11.5.

Results and Discussion

Fifty two marine actinomycetes isolates were screened using combination methods. Twenty one isolates detected using this methods, consist of ten isolates were detected encoding both PKS I and NRPS genes, whereas 11 isolates were detected encoding the NRPS gene. PKS I and NRPS genes were amplified using sets of degenerate primers. K1F and M6R were used for amplify ketosynthase and methyl-malonyl-CoA transferase modules of PKS I gene which targeted sequences 1200-1400 bp.

A3F and A7R were used for amplify adenilation domains of NRPS gene which targeted sequences 700-800 bp. The visualization of PKS I and NRPS genes of the 21 detected isolates in this methods is represented in Figure 1.
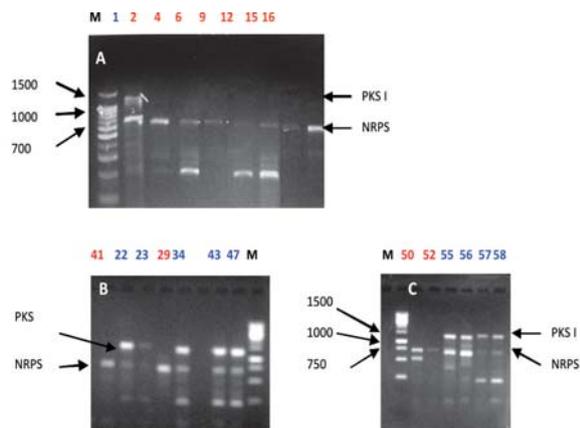

Figure 1. Visualization of PKS I and NRPS genes of 21 detected isolates by electrophoresis in 1% (w/v) agarose gels stained with ethydium bromide. 1, 22, 23, 34, 43, 47, 55, 56, 57, and 58 are isolates encoding PKS I and NRPS genes, meanwhile 2, 4, 6, 9, 12, 15, 16, 41, 29, 50, and 52 are isolates encoding NRPS gene. The size range of amplified fragments of PKS I (1200 – 1400 bp), and NRPS (700 – 800 bp). (A) used marker 100 bp DNA Ladder (B) & (C) used marker 1 kb DNA Ladder

PKS I and NRPS are biosynthetic systems involved in the synthesis of a large number of important biologically active compounds produced by microorganisms, among others by actinomycetes. The detection of gene sequences involved in the synthesis of secondary metabolites to evaluate the biosynthetic potential. KS I sequences detected in 10 of 52 isolates (19%) and always associated with NRPS sequences. NRPS sequences detected in 21 of 52 isolates (40%), 21% (11 isolates) were detected without associated with PKS I sequences. Twenty one detected isolated used this method would be selected using the next screening specifically the analysis of secondary metabolites and repetitive DNA fingerprinting.

Profiles data of ethyl acetate extract secondary metabolites by densitometry of UV light 366 and 254 nm using CMW and BAW (unpublished) as solvent system were subjected from Rf values and analyzed by UPGMA method using NTSYS program. Dendrogram of analysis secondary metabolites using CMW as solvent of marine actinomyces encoding both PKS I and NRPS genes of UV light 366 nm is represented in Figure 2.
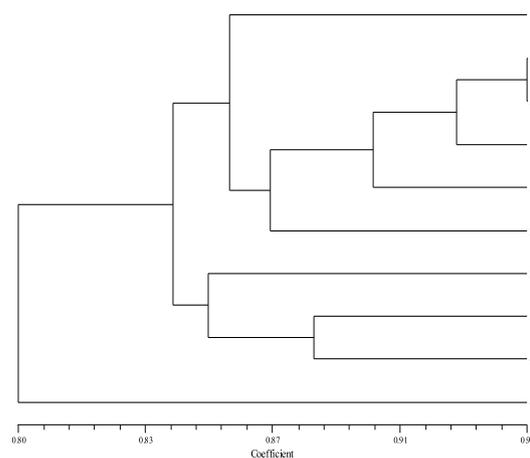

Figure 2 . Dendrogram of densitometry analysis of ethyl acetat extract secondary metabolites of marine actinomycetes encoding both PKS I and NRPS genes with CMW solvent on TLC, UV light 366 nm. Data was analized by UPGMA method using NTSYS program. This result divided into 3 groups. Group 1 consist of isolates 1, 55, 58, 56, 57, and 23. Group 2 consist of isolates 22, 34, and 43. Group 3 consist of isolate 47.

Figure 2 showed grouping result according to similarity coefficient in dendrogram, divided into 3 groups. This result would perform next on the Table 2. According to this data, isolate 55 and 58 have the highest similarity coefficient value (0.94). It was showed that they have most similarity of secondary metabolites, meanwhile isolate 47 has the lowest similarity coefficient value (0.80), it was showed that isolate 47 most different among others.

Dendrogram of analysis secondary metabolites using CMW as solvent of marine

actinomycetes encoding both PKS I and
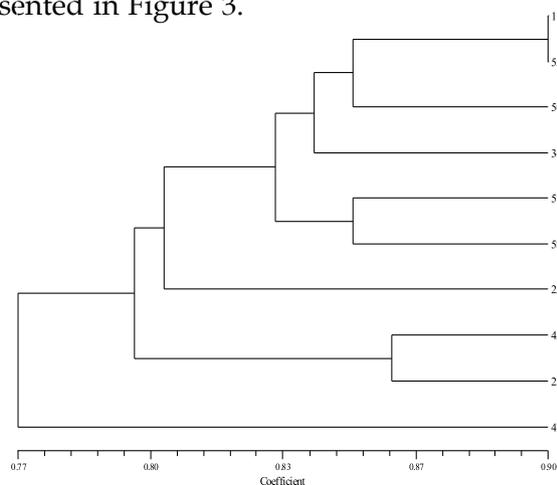NRPS genes of UV light 254 nm is repre-
sented in Figure 3.

Figure 3 . Dendrogram of densitometry analysis of
ethyl acetat extract secondary metabolites of marine
actinomycetes encoding both PKS I and NRPS genes
with CMW solvent on TLC, UV light 254 nm. Data
was analized by UPGMA method using NTSYS pro-
gram. This result divided into 4 groups. Group 1 con-
sist of isolates 1, 55, 56, 34, 57, and 58. Group 2 consist
of isolate 22. Group consist of isolates 43 and 23. Group
4 consist of isolate 47.

Figure 3 showed grouping result accord-
ing to similarity coefficient in dendrogram,
divided into 4 groups. This result would
perform next on the Table 2.   According to
this data, isolate 1 and 55 have the highest
similarity coefficient value (0.90). It was
showed that they have most similarity of
secondary metabolites, meanwhile isolate
47 has the lowest similarity coefficient value
(0.77), it was showed that isolate 47 most
different among others.

Dendrogram of analysis secondary me-
tabolites using CMW as solvent of marine
actinomycetes encoding NRPS gene of UV
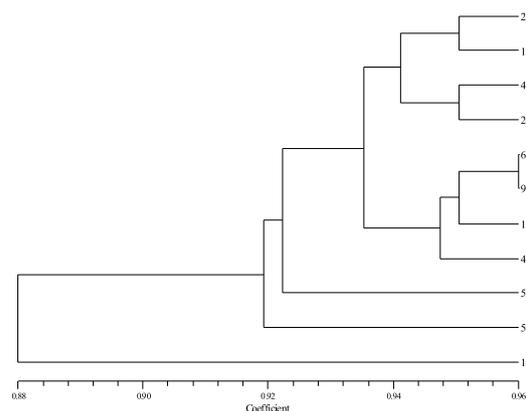light 366 nm is represented in Figure 4.

Figure 4 . Dendrogram of densitometry analysis of
ethyl acetat extract secondary metabolites of marine
actinomycetes encoding NRPS gene with CMW sol-
vent on TLC, UV light 366 nm. Data was analized by
UPGMA method using NTSYS program. This result
divided into 3 groups. Group 1 consist of isolates 2,
12, 4, 29, 6, 9, 15, 41, and 52. Group 2 consist of isolate
50. Group 3 consist of isolates 3 and 16.

Figure 4 showed grouping result accord-
ing to similarity coefficient in dendrogram,
divided into 3 groups. This result would
perform next on the Table 2.   According to
this data, isolate 6 and 9 have the highest
similarity coefficient value (0.96). It was
showed that they have most similarity of
secondary metabolites, meanwhile isolate
16 has the lowest similarity coefficient value
(0.88), it was showed that isolate 16 most
different among others.

Dendrogram of analysis secondary me-
tabolites using CMW as solvent of marine
actinomycetes encoding NRPS gene of UV
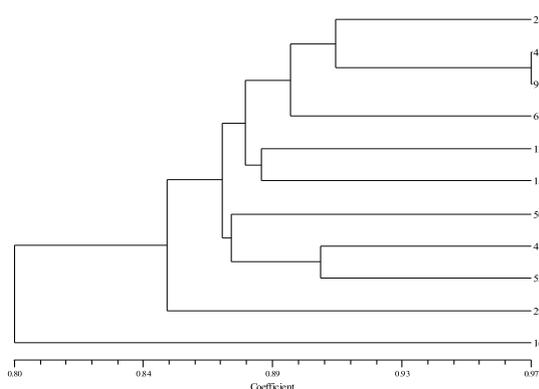light 254 nm is represented in Figure 5.

Figure 5 . Dendrogram of densitometry analysis of ethyl acetat extract secondary metabolites of marine actinomycetes encoding NRPS gene with CMW solvent on TLC, UV light 254 nm. Data was analized by UPGMA method using NTSYS program. This result divided into 4 groups. Group 1 consist of isolates 2, 4, 9, 6, 12, and 15. Group 2 consist of isolates 50, 41, and 52. Group 3 consist of isolate 29. Group 4 consist of isolate 16.

Figure 5 showed grouping result according to similarity coefficient in dendrogram, divided into 4 groups. This result would perform next on the Table 2. According to this data, isolate 4 and 9 have the highest similarity coefficient value (0.97). It was showed that they have most similarity of secondary metabolites, meanwhile isolate 16 has the lowest similarity coefficient value (0.80), it was showed that isolate 16 most different among others.

Repetitive DNA fingerprinting or *rep-*PCR was used to determine the diversity of marine actinomycetes. Dendrogram of analysis repetitive DNA fingerprinting 21 isolates used BOXA1R primer, visualized by PAGE 8% with silver staining (unpublished) is represented in Figure 6. The analysis data according to repetitive DNA fragments, analyzed by UPGMA method using NTSYS program.
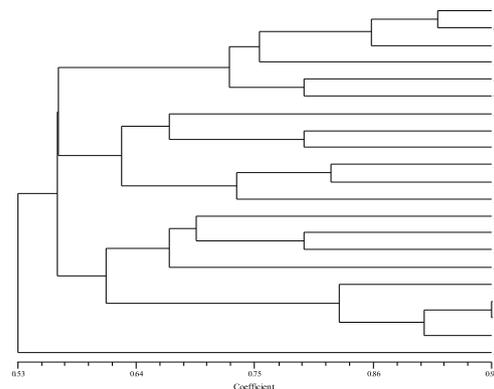

Figure 6 . Dendrogram of repetitive DNA fingerprinting used BOXA1R primer of 21 selected marine actinomycetes isolates using PCR PKS I and NRPS genes method, visualized by PAGE 8% with silver staining. Data was analized by UPGMA method using NTSYS program. This result divided into 6 groups. Group 1 consist of isolates 1, 5, 2, and 52. Group 2 consist of isolates 4 and 9. Group 3 consist of isolate 43, 58, 50, 55, 56, and 57. Group 4 consist of isolate 22, 23, 29, and 41. Group 5 consist of isolates 34, 12, 15, and 16. Group 6 consist of isolate 47.

Dendrogram on the Figure 6 showed the dividing into 6 groups of repetitive DNA fingerprinting 21 isolates according to similarity coefficient. This grouping was used as a base for selected 6 isolates for cytotoxicity assay.

Dendrogram of repetitive DNA fingerprinting of marine actinomycetes encoding both PKS I and NRPS genes is represented in Figure 7.
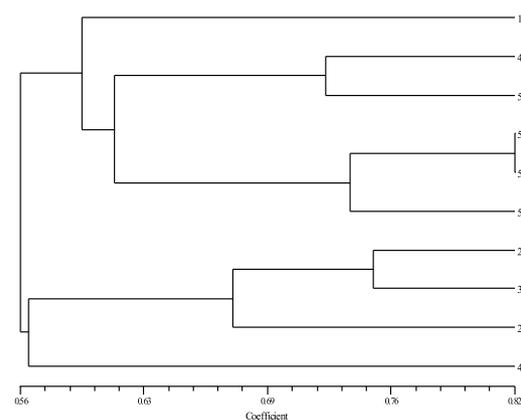
Figure 7 . Dendrogram of repetitive DNA fingerprinting used BOXA1R primer of marine actinomycetes encoding both PKS I and NRPS visualized by PAGE 8% with silver staining. Data was analized by UPGMA method using NTSYS program. Symbol (*) showed grouping result according to similarity coefficient. This result divided into 3 groups. Group 1 consist of isolates 1, 43, 58, 55, 56, and 57. Group 2 consist of isolates 22, 34, and 23. Group 3 consist of isolate 47.

Figure 7 showed a grouping result according to similarity coefficient in dendrogram, it is divided into 3 groups. This result would perform next on the Table 2. According to this data, isolate 55 and 56 have the highest similarity coefficient value (0.82). It was showed that they have most similarity of secondary metabolites, meanwhile isolate 47 has the lowest similarity coefficient value (0.57), it was showed that isolate 16 most different among others.

Dendrogram of repetitive DNA fingerprinting of marine actinomycetes encoding NRPS gene is represented in Figure 8.
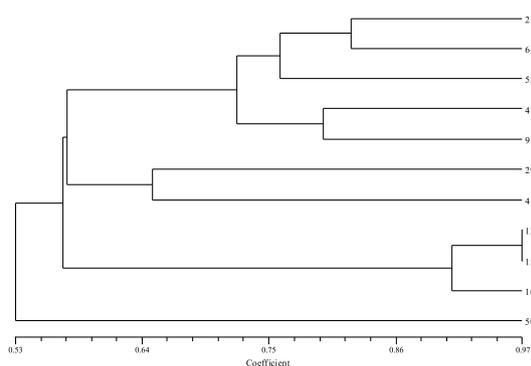

Figure 8 . Dendrogram of repetitive DNA fingerprinting used BOXA1R primer of marine actinomycetes encoding NRPS visualized by PAGE 8% with silver staining. Data was analized by UPGMA method using NTSYS program. This result divided into 3 groups. Group 1 consist of isolates 2, 6, 52, 4, 9, 29, and 41. Group 2 consist of isolates 12, 15, and 16. Group 3 consist of isolate 50.

Figure 8 showed a grouping result according to similarity coefficient in dendrogram, it is divided into 3 groups. This re-sult would perform next on the Table 2. According to this data, isolate 12 and 15 have the highest similarity coefficient value (0.97). It was showed that they have most similarity of secondary metabolites, meanwhile isolate 50 has the lowest similarity coefficient value (0.53), it was showed that isolate 16 most different among others.

All of the result of data analysis that are performed in dendrogram Figure 2, 3, 4, 5, 6, 7, and 8, are represented in Table 1. This data was made in order to ease the selection of 6 isolates for cytotoxicity assay.

Tabel 1. The Result of Data Analysis of Secondary Metabolites and Genetics Diversity Based on Dendrogram

Isolates encoding both PKS I and NRPS Genes			Isolates Encoding NRPS Gene		
Densitometry		Rep-PCR	Densitometry		rep-PCR
254 nm	366 nm		254 nm	366 nm	
1	1	1	2	2	2
55	55	43	4	12	6
56	58	58	9	4	52
34	56	55	6	29	4
57	57	56	12	6	9
58	23	57	15	9	29
				15	41
				41	
				52	
*	*	*	*	*	*
22	22	22	16	16	12
	34	34			15
	43	23			16
*	*	*	*	*	*
47	47	47	50	50	50
			41		
			52		
*	*	*	*	*	*
43			29		
23					
*			*		

■ The candidates of selected isolates encoding both PKS I and NRPS genes

■ The candidates of selected isolates encoding NRPS gene

The colourful code of isolates showed that those isolates always perform in all dendrograms grouping in the same line to each dendrograms.

The candidates of selected isolates encoding both PKS I and NRPS genes are isolates 1, 55, 56, 57, 58, in line 1. Line 2 is isolate 22. Line 3 is isolate 47. The candidates of selected isolates encoding NRPS gene are isolates 2, 4, 9, in line 1. Line 2 is isolate 16. line 3 is isolate 50. The isolates in line 1 need to be chosen to get one isolate represented line 1. It deliberated the result of *rep*-PCR 21 isolates and the amount of Rf value in densitometry using solvent system BAW (unpublished). Isolate 1 is selected as the isolate represented actinomycetes encoding both PKS I and NRPS genes and isolate 9 represented actinomycetes encoding NRPS gene. So, the result of the combination methods for screening marine actinomycetes obtained 6 isolates for cytotoxicity assay method. Isolates 1, 22, and 47 represented the actinomycetes encoding both PKS I and NRPS genes. Isolates 9, 16, and 50 represented the actinomycetes encoding NRPS gene.

Cytotoxicity assay measured by MTT cell viability test on T47D and MCF7 breast cancer cell lines. T47D cell was treated with ethyl acetate extract secondary metabolites of marine actinomycetes. The serial concentration of the treatment were 6,25; 12,5; 25; 50; 100 µg/ml with total amount of cell was 5.10^3 cells/ml and incubated 24 hours. The result of probit analysis of this treatment are represented in Figure 9. All of the treatment showed the reducing of cell viability significantly based on ANOVA ($p<0.05$).
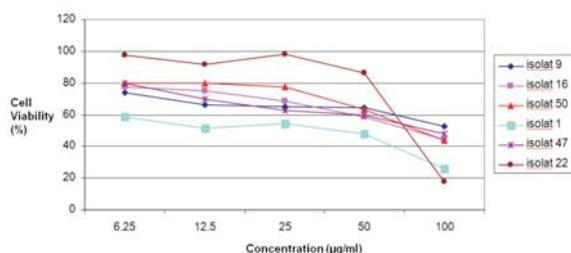

Figure 9. The effect of ethyl acetate extract secondary metabolites of marine actinomycetes on T47D cell's viability. Every point represented the averages of triplicate. The IC_{50} (in µg/ml): isolate 9 (224), isolate 16 (83), isolate 50 (102), isolate 1 (19), Isolate 47 (90), Isolate 22 (71).

Isolate 1 had the lowest IC_{50} (19 µg/ml), and the highest IC_{50} was isolate 9 (224 µg/ml). Cytotoxicity assay of 6 isolates on T47D cell obtained 4 isolates had IC_{50} less than 100 µg/ml (isolates 1, 22, 16 and 47). Meanwhile 2 isolat (isolates 9 and 50) had IC_{50} more than 100 µg/ml, both isolates represented actinomycetes encoding NRPS gene. All of the isolates represented actinomycetes encoding both PKS I and NRPS had low IC_{50}. MCF7 cell was treated with ethyl acetate extract of secondary metabolites marine actinomycetes. The serial concentration of the treatment were 12,5; 25; 50; 100; 200µg/ml with total amount of cell was 5.10^3 cells/ml and incubated 48 hours. This treatment particularly for isolates 9, 16, 22, 47, and 50. Special for isolate 1 used serial concentration 1,75; 2,5; 5; 10; 20; 40 µg/ml. The result of probit analysis of this treatment is represented in Figure 10 and 11. All of the treatment showed the reducing of cell viability significantly based on ANOVA ($p<0.05$). Probit analysis of cytotoxicity secondary metabolites isolate 9, 16, 22, 50, 47 are represented in Figure 10.
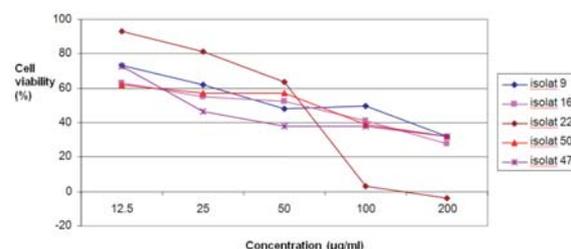

Figure 10. The effect of ethyl acetate extract secondary metabolites of marine actinomycetes on MCF7 cell's viability. Every point represented the averages of triplicate. IC_{50} (in µg/ml): isolate 9 (62), isolate 16 (42), isolate 22 (46), isolate 50 (47), Isolate 47 (34).

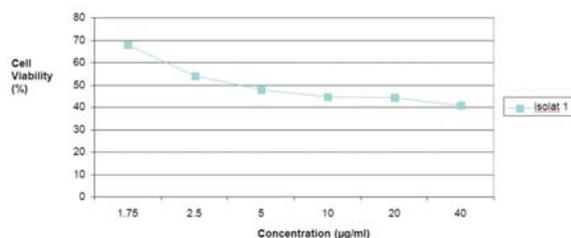Probit analysis of cytotoxicity secondary metabolites isolate 1 is represented in Figure 11.

Figure 11. The effect of ethyl acetate extract of secondary metabolites of marine actinomycetes on MCF7 cell's viability. Every point represented the averages of triplicate. IC$_{50}$ (in μg/ml): isolat 1

Isolate 1 had the lowest IC$_{50}$ (7 μg/ml), and the highest IC$_{50}$ was isolate 9 (62 μg/ml). Cytotoxicity assay of 6 isolates on MCF7 cell obtained that all of isolates had IC$_{50}$ less than 100 μg/ml.

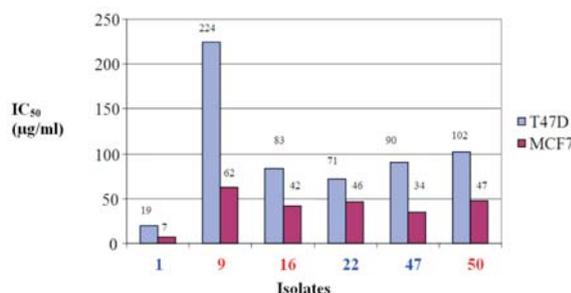All of the result of cytotoxicity is represented in Figure 12.

Figure 12. IC$_{50}$ of cytotoxicity assay on T47D and MCF7. Isolates 1, 22, and 47 are marine actinomycetes encoding both PKS I dan NRPS genes, meanwhile isolates 9, 16, and 50 are marine actinomycetes encoding NRPS gene. The number upon the bar on the graphic showed the IC$_{50}$ each isolates on each cancer cells.

The combination methods for screening marine actinomycetes producing potential compounds as anticancer in this study obtained the selected isolates of marine actinomycetes which had high cytotoxocity on T47D and MCF7. Isolate 1 had highest cytotoxicity with the IC$_{50}$ on T47D cell was 19 μg/ml and the IC$_{50}$ on MCF7 cell was 7 μg/ml. This findings suggests that combination methods were effective and efficient way to select marine actinomycetes producing potential compounds as anticancer.

References

Atlas, R. M, 1997, Principles of Microbiology, *Wm. C. Brown Publishers*, Dubuque, Iowa.

Berdy, J., 2005, Bioactive microbial metabolites: a personal view. J. Antibiot. 58:1-26.

Bull, A. T., Ward, A. C., Goodfellow, M., 2000, Search and Discovery Strategies for Biotechnology : the Paradigm Shift, *Microbiology and Molecular Biology Reviews*, Sept. 2000, p. 573 – 606, Vol. 64, No. 3.

Cannel, Richard J. P., 1998, Natural Products Isolation. *Humana Press. Inc.*, Totowa, New Jersey.

Dewick, P. M., 2002, Medicinal Natural Product, A Biosynthetic Approach. 2nd ed. *John Wiley & Sons Ltd*. England.

Ghanem, Nevine B., Soraya A. Sabry, Zeinab M. El-Sherif, and Gehan A. Abu El-Ela. Isolation and enumeration of marine actinomycetes from seawater and sediments in Alexandria. *J. Gen. Appl. Microbiol.*, 46, 105-111.

Jaekyeong Song, Seong-Chan Lee, Jun-Won Kang, Hyung-Jin Baek, and Joo-Won Suh, 2004, Phylogenetic analysis of Streptomyces spp. Isolated from potato scab lesions in Korea on the basis of 16S rRNA gene and 16S-23S rDNA internally transcribed spacer Sequences. *International Journal of Systematic and Evolutionary Microbiology*, 54, 203-209.

Jensen, P.R., Williams, P.G., Oh, Dong-Chan., Zeigler, L., Fenical, W., 2007, Species-Specific Secondary Metabolite Production in Marine Actinomycetes of the Genus *Salinispora*. *Applied and Environmental Microbiology*, Feb. 2007, p. 1146 – 1152, Vol. 73, No. 4.

Moore, B.S., Trischman, J.A., Seng, D., Kho, D., Jensen, P.R., Fenical, W., 1998,

Salinamides, Antiinflammatory Depsipeptides from Marine Streptomycete., *J. Org. Chem.* 1999, 64, 1145 – 1150.

Mosmann, T., 1983, Rapid Colorimetric Assay for Cellular Growth and Survival: Aplication to Proliferation and Citotoxicity Assays, *J. Immunol Methods*, 65(1-2):55-63.

Sadowsky, M. J., L. L. Kinkel, J.H. Bowers and J.L. Schottel., 1996, Use of repetitive intergenic DNA sequences to classify pathogenic and disease-suppresive *Streptomyces* strain, *Appl. Environ. Microbiol.* 62:3489-3493.

Song, J., S. Lee, J. Kang, H. Baek and J. Suh, 2004, Phylogenetic analysis of Streptomyces spp. Isolated from potato scab lesions in Korea on the basis of 16S rRNA gene and 16S-23S rDNA internally transcribed spacer Sequences. *International Journal of Systematic and Evolutionary Microbiology.* 54: 203-209.