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DEEP SEA *Bacillus flexus* AND THE EVALUATION OF ANTI-MYCOBACTERIAL ACTIVITY

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ABSTRACT

Pathogenic bacteria, especially *Mycobacterium*, happens to be one of the cause of disease in society. The fact that this bacterium is resistant to several commercial antibiotics, meanwhile the development of new effective antibacterial compound to overcome this problem is needed. The search of antibacterial compound in this research was done with the source from deep sea. The aims of the study was to find the new source of antimycobacterium producers as well as to characterize the potential active compound. The selected deep-sea microorganism was cultured aerobically under room condition (29°C, 1 ATM). The cultivation under room condition was done using 10x 1.5 L M2 medium broth. Antibacterial compound was extracted using ethyl acetate, and separated using HPLC. Preliminary active compound identification was done using GC-MS. The antibacterial activity of each fraction was examined. The result from GC-MS showed that the suggested active compound was 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester which showed moderate activity against *Mycobacterium smegmatis*. Molecular characterization of potential strain showed 99% similarity with *Bacillus flexus*. Furthermore, in this study proved that this deep sea *B. flexus* could grow without adjusting to the extreme condition such aslow temperature, high pressure and low lighting.

KEYWORDS: Active compound, *Bacillus flexus*, deep sea microorganism, anti-Mycobacterium.

INTRODUCTIONS

Pathogenic bacteria is one of the cause of disease that happened in the society, for instance diarrhea and food poisoning. Nowadays, the most infectious case in Indonesia was tuberculosis. This disease was caused by *Mycobacterium* infection, that classified to the top 10 deathly diseases. World Health organization (2014) reported that among 9 million infections cases, 1,5 million of them were died. To overcome this problem, various antibiotics have been used widely but the fact that resistant case was indicated for some patient. Developing the new antibiotic was needed to find the most effective to the existing tuberculosis drug (Ventola 2015).

In the last 50 years, marine organisms has been the producer of more than 20.000 natural products. However, the technology to develop has led to a breakthrough where 22.000 secondary metabolites were isolated from marine microorganisms and hovering a significant percentage those metabolites have biological activities (Blunt et al. 2002).The potential of deep sea marine microorganisms were still limited to explore.

The major part of biosphere is represented by ocean, covering about 97% of the Earth's water, and it is situated in the deep sea (depth greater than 3000 meters), accounting for 75% of the ocean's volume (Tapilatu 2016). Moreover, Indonesian Eastern marine areas make up more than 80 percent of this region's total area and located in the coral triangle area, which primarily the spot of the world biodiversity (Tapilatu 2016). By considering its vast coverage, it is obvious that it has a great potential concerning the biodiversity of marine bacteria.

Deep sea environment is lack of nutrition, temperature, light, oxygen and salinity. Its constant darkness and hydrostatic pressure that increases every 10 meters make the environment extreme, thus believed to be the hostile environment for most of the marine microorganisms (Radjasa 2004). However, those extreme conditions trigger the deep-sea microorganisms to develop their unique physiology ability, not only to survive, but also affect their ability to produce secondary metabolites that cannot be found in terrestrial microorganisms (Fenical 1993; Skropeta & Wei 2014).

Throughout the years, ecological molecular studies of sea microorganisms have isolated and identified various of compounds from deep sea microorganisms and reported that its secondary metabolites has an antibacterial activity (Stach & Bull 2005; Chen et al. 2016). The types of deep sea microorganisms are characterized by its pressure-loving (barophilic) and low temperature-loving (psychrophilic) (Munn 2004). Nevertheless, in consonance to Pettit (2011), pH, temperature, and extreme pressure are not necessary in terms of culturing deep sea microorganisms, because of deep sea environment it self has several of physical and chemical gradient environment resulting in diverse population of deep sea microorganisms. Moreover, some researches have proved that deep sea microorganisms can be cultured under laboratory condition and able to produce bioactive compounds (Nakagawa et al. 2005; Nunoura, et al. 2008). Furthermore, developing country like China also carried the deep sea microorganism research. One of their finding is the active antibacterial and antitumor compounds such as pseudonocardians A-C, grincamycins B-F, and abyssomicins J-L. (Pan et al. 2015).

In this research about 80 extracts of deep sea microorganisms strain collected from East Indonesia Island have been screened for biologically active compounds. The potent anti-bacterial extract will continue to separate the anti-mycobacterial compound. This research was conducted to identify anti-mycobacterium target from deep-sea sediment microorganisms and to find out the potential species of the anti-mycobacterium substances producer.

MATERIAL AND METHODS

Materials

Media Used for cultivating the selected bacteria was M2 Medium that contains 0.5 g peptone (Himedia), 0.1 gr yeast extract (Himedia), and 16 g bacteriological agar (Himedia) per litre tropical seawater. Nutrient Agar medium contained 5 g/L peptic digest of animal tissue, 5g/L sodium chloride, 1.5 g/L beef extract, 1.5g/L yeast extract were used for anti-mycobacterial assay. Wild strain *Mycobacterium smegmatis* was purchased from Microbiology Laboratory of The University of Indonesia.

Organic solvent used for HPLC was methanol HPLC grade (Merck). Ethyl acetat p.a. Merck was used to dissolve the potential fractions.

Instruments used for analysis were High Performance Liquid Chromatography (HPLC) Hitachi-Chromaster, Gas Chromatography-Mass Spectroscopy (GC-MS) (Aglient Technologies 7890 Gas Chromatography with Auto Sampeler and 5975 Mass Selective Detector and Chemstation data system), Nuclear Magnetic Resonance (NMR)(Bruker Avance III 500 Console magnex 11.75T).

Methods

Bacterial Isolations: Eight selected bacterial strains were used in this research. Those bacteria were isolated from the deep sea sediment of Sumba Island, East Indonesia in November 2016. M2 medium was used for culturing all the selected isolates. Bacterial strains were transferred into solid medium, which has the same compositions as the maintenance medium. For antibacterial screening purposes, all of the isolated strains were culture in 10 mL M2-broth during 72 hours at room temperature using shaker incubator. After harvested, all of bacterial broth were extracted using ethyl acetate. Extracts were applied for antibacterial assay, and the most potential strain was recommended for further analysis.

Bacterial cultivation: The selected strain was propagated in the semi-large scale cultivation in the 15 L M2 medium medium broth. Cultivation was carried on the rotary shaker incubator for 72 hours at 29°C with the rotation speed of 150 rpm. After cultivation, the bacterial broth was extracted using ethyl acetate. The extract was concentrated using vacuum rotary evaporator at 40° C.

Antibacterial assay: Antibacterial assay was conducted using agar diffusion method against *M. smegmatis* (Balouiri, Sadiki, Ibnsouda, 2016). Approximately 15 µg of bacterial extracts and fractions were applied into paper disc (diameter 6 mm), then laid on agar surface that contained of pathogenic bacterial culture. After one day of incubation at 30° C, the diameter of inhibition was determined. The same procedure was applied to the antibiotic rifampicin as a positive control.

Microorganisms Characterization: The characterization of microorganism from the selected strain was done using gram staining and 16S rRNA sequencing method. The sequencing was done at Genetic Science Laboratory in West Jakarta. The construction of phylogeny tree was built by analysing the sequence using NCBI BLAST data base and MEGA7 software.

Isolation of antibacterial compound: Ethyl acetate extract of selected bacteria was evaporated and dried. The extract was prepared to inject into Gas Chromatography – Mass Spectrometry instrument. The separation compounds was done using High Performance Liquid Chromatography (HPLC) with UV detector with C18, ID 250x10 mm Agilent column chromatography. Solvent applied in this system was isocratic system using 70:30 methanol - water. All of fractions were applied to the antimicrobial assay. The profile of potential antibacterial fraction was checked using Thin Layer Chromatography method with hexane- ethyl acetate solvent method.

Structural Determination of Compound: Structural determination of active compound done using GC-MS and ^{1}H-NMR. Mestre-Lab Nova application was used to

compare the predicted ^{1}H-NMR of the most potential compound resulted from GC-MS analysis and ^{1}H-NMR.

RESULT AND DISCUSSIONS

Screening of antibacterial

Screening for antibacterial activities using 15 µL samples were presented in Table 1. The results show that all extracts isolated from deep sea were effective against *M. smegmatis*. However, among all the extracts, strain STA54/ 200m/ 10^{-3}.2 was the strongest antibacterial with 12.7 mm diameter inhibition against *S. aureus*, 12.7 mm against *B. subtilis*, and 17.9 mm against *M. smegmatis*. Based on these results, marine deep sea bacterium STA54/ 200m/ 10^{-3}.2 was selected for further studies in the isolation of antibacterial compound and this study was continued by focusing on antibacterial against *Mycobacterium*.

Table 1: Antibacterial Activity of Marine Bacteria Extracts.

Sample	Inhibition Zone (mm)		
	Escherichia coli	*Staphylococcus aureus*	*Mycobacterium smegmatis*
STA 47/ 5m/ 10^{-2}.2	8.15	9.10	11.80
STA 47/ 100m/ 10^{-3}.6	-	-	-
STA 47/ 1000m/ 10^{-3}.4	9.20	12.30	13.60
STA 50/ 25m/ $10^{=1}$.2	8.80	11.40	13.90
STA 53/ 3500m/ 10^{-4}.1	-	9.20	18.30
STA 54/ 200m/ 10^{-3}.2	12.6	12.7	17.90
STA 54/ 200m/ 10^{-1}.3	9.70	9.55	10.20
STA 58/ 200m/ 10^{-1}.3	8.10	11.20	13.10

M. smegmatis was used in this research as a model to target an active compound to prevent tuberculosis is based on the fact that this bacterium has 6 out of 11 *M. tuberculosis* histidine kinase and 9 out of 13 genes of *M. smegmatis* are homologous with *M. tuberculosis*. Moreover, *M. smegmatis* is considered a rapid growth bacteria that can be used in biosafety level 1 or 2 and it expresses the same gene like *M. tuberculosis*, hence it helps to target an anti-mycobacterium compound (Tyagi, 2002).

Microorganism Characterization

Results of microorganism characterization by gram staining was presented in Figure 1. The figures show that the microorganism is a gram positive bacterium and it has a rod-shape (basil). It is then confirmed by molecular identification conducted by Genetic Science Laboratory that the strain based on BLAST Homology using NCBI database and the construction of phylogenetic tree using MEGA7 are *Bacillus flexus* strain KM16 (Figure 2). Previous study between 2013 and 2017 indicated that *B. flexus* has been isolated from many sources and cultured in various conditions were reported to have numbers of biological activities.

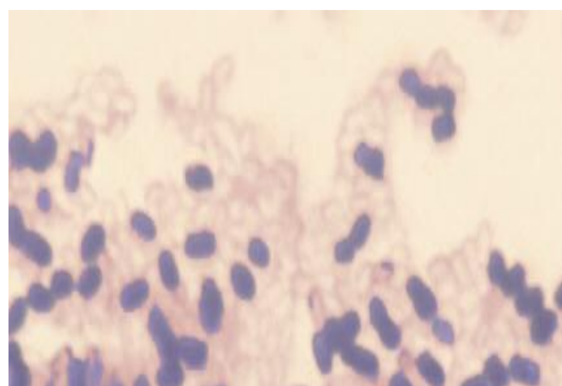

Figure 1: Profile of gram staining bacteria result.

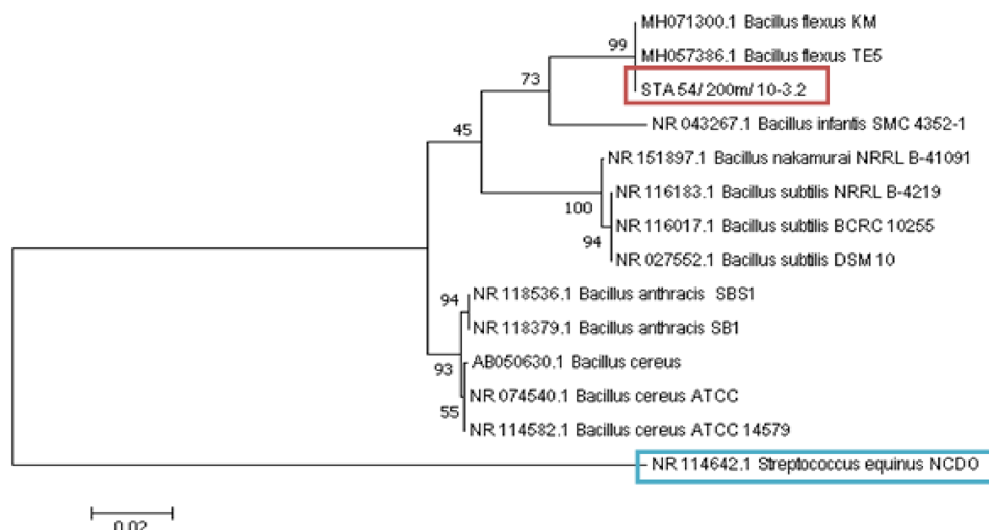

Figure 2: The phylogeny tree of selected strain STA 54/200m/10-3.2.

Streptococcus equinus was used in phylogenetic tree as an out group to support the result that the tree is reliable and to prove that the strain is really homologous to *B. flexus*.

Bacillus flexus is a gram positive bacterium that can grow well both in aerobic and facultative anaerobe environment, with the range of pH 4.5-9.5 and temperature 17-37°C (Turnbull, 1996). Although most studies on *B. flexus* were on bioremediation and biodegradation, there are some reports on antibacterial potential of *B. flexus*. This bacterium was reported has an antibacterial activity against *Pseudomonas* and *Shigella* (Ramasubburayan *et al*., 2014) and pathogenic bacteria such as *Staphylococcus*, *B. subtilis*, and *E. coli* (Fitri *et al*., 2017). Nonetheless, there is no previous report that this bacterium has an antibacterial activity toward *Mycobacterium*, hence this might be potential in targeting an antibacterial compound against *Mycobacterium*.

Isolation of Antibacterial Compound

Approximately 0.502 g extract of STA 54/200/10^{-3}.2. was separated using Reverse Phase-HPLC solvent system. The HPLC fractions and antibacterial assay result could be seen in the table 2.

Table 2: Result of anti-*M. smegmatis* activity test of separated fractions.

Sample fraction /control (50 µg)	Weight (mg)	Rtime (minute)	Diameter inhibitions against *M.smegmatis*)
F1	2 .0	4.6	17.4 mm
F2	2.3	5.8	19.1 mm
F3	1.0	6.1	13.1 mm
F4	0.9	6.9	12.4 mm
F5	0.9	9.3	13.6 mm
F6	0.2	8.1	17.8 mm
F7	0.4	8.9	13.3 mm
F8	0.2	9.7	13.4 mm
F9	0.3	13.7	15.9 mm
F10	1.0	15.2	14.8 mm
F11	0.2	17.8	16.9 mm
F12	0.5	22.1	12.9 mm
F13	0.6	26.2	13.1 mm
F14	0.1	30.6	11.4 mm
F15	27.1	33.1	17.5 mm
F16	0.4	35.3	12.2 mm
F17	0.6	39.1	17.2 mm (partial)
F18	0.0.5	41.2	13.4 mm
F19	1.3	44.1	8.9 mm
F20	0.8	50.5	11.7 mm
F21	0.5	53.8	11.2 mm
Metanol	-		11.1 mm
Rifampicin (2.5µg)	-		11.25 mm

All the fractions were applied into antimicrobial assay. Four fractions (F1, F2, F6, and F15) were active against *Mycobacterium smegmatis*. The diameter inhibition of those fractions against *M. smegmatis* were 17.4 mm, 19.1 mm, 17.8 mm, and 17.5 mm respectively. Further analysis was done to predict the active compounds. Detailed anti-mycobacterium assayed of HPLC fractions could be shown in TABEL 2

GC-MS & ^{1}H analysis

The preliminary study of secondary metabolites contained in the bacterial extract using GC-MS. The GC-MS spectrum indicated that there were at least 20 peaks, that was indicating 20 compound as described in Figure 3.

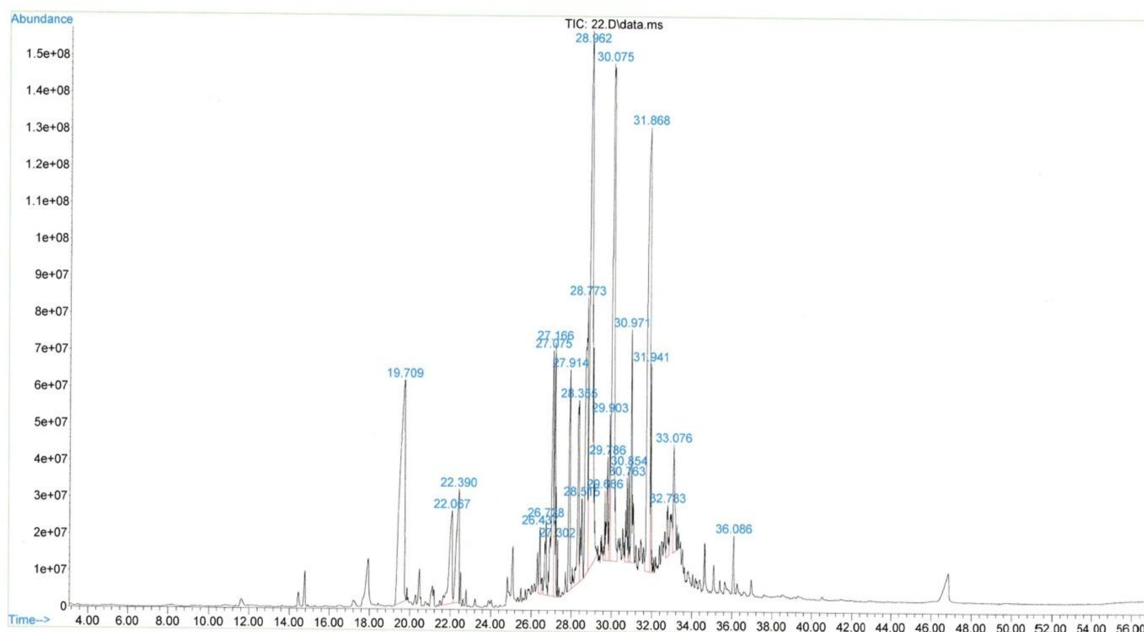

Figure 3: GC-MS Spectrum of STA54/200m/ 10^{-3}.2 Extract.

Based on the data, the first peak was appeared at the retention time (Rt) of 19.712 minutes and ended in Rt 33.075 minutes. From all the components that were detected, there are 3 compound with highest peak, which were eluted in Rt.29.965 mins, 28.868 mins, and 31.868 mins, with the proportion of 16.99%, 15.46%, and 13.59% in the extract. Among the three compounds, 2 of them (Hexadecanoic and Dodecanoic Acid) were abundant fatty acid derived compounds that mostly found in marine extract spectrum. The very interesting compound that appeared at retention time 31.868 mins selected to be further analysed. Based on GC-MS library, this compound is considered to be 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester. This compound in previous study had been reported as antibacterial properties (Ezhilan & Neelamegam, 2011). Figure 4 is the mass spectra of the compound.

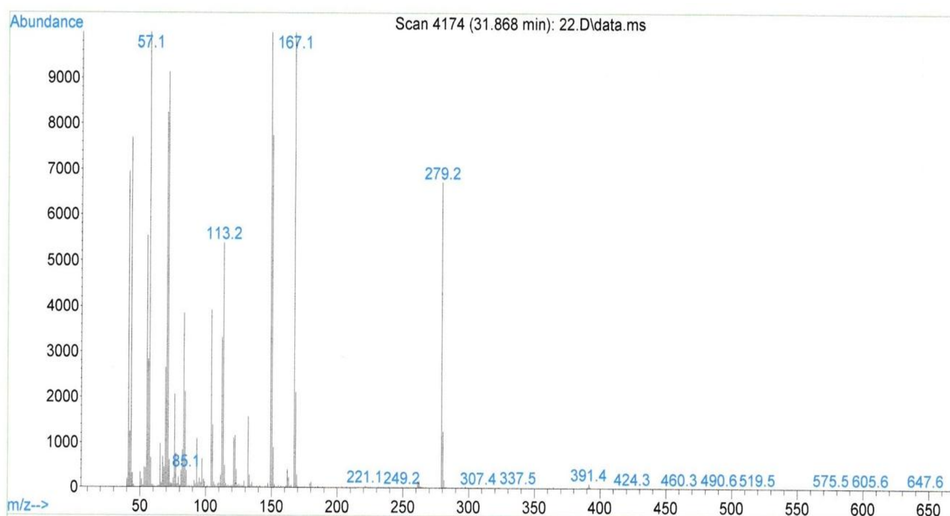

Figure 4: Mass Spectra of fraction at retention time 31.868 minute.

As it is illustrated by the Figure 4, the base peak were m/z 57.1 and 167.1, while the parent peak was m/z 279.2. Additionally, 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester has molecular mass of 278.35 g/ mol, thus making [M+1] : 279.2 is the peak of molecular ion. This compound is known to have other name, which is 2-(((2-ethylhexyl) oxy) carbonyl) benzoic acid, indicating it was a benzoic acid-derived compound.

Considering the 1H NMR spectrum of F1 fraction resulted the chemical shift at:

Table 3: Proton NMR chemical shift of fraction F1.

Code	Chemical Shift (ppm)	Multiplicity	Functional Group
A	7.982	s, 1H	Aromatic (-CH Aromatic)
B	7.260	d, J = 8.0 Hz, 1H	Aromatic (-CH Aromatic)
C	5.601	d, J = 19.8 Hz, 2H	Alkene (-O-CH)
D	5.300	s, 4H	Alkene (-O-CH)
E	4.028	s, 1H	Ether/ Alcohol/ Ester
F	3.829	s, 2H	Ether/ Alcohol/ Ester (-O-CH_3)
G	3.763	s, 2H	Ether/ Alcohol/ Ester/(-O-CH_3)
H	3.699	s, 2H	Ether/ Alcohol/ Ester/
I	2.576	s, 1H	Alkyne
J	2.355	t, J = 7.5 Hz, 2H	Allylic/Benzylic/ Ketone =C=CH, CH-C-O
K	2.265	d, J = 8.0 Hz, 1H	Allylic/Benzylic/ Ketone =C=CH, Ph-CH, CH-C-O
L	2.101	s, 1H	Allylic/Benzylic/ Ketone =C=CH, Ph-CH, CH-C-O
M	1.431	s, 2H	Alkane CH-CR_3
N	1.301	s, 2H	Alkane CH-CR_3
O	1.256	s, 4H	Alkane CH-CR_3
P	0.907	m, 5H	Methyl CH_3
Q	0.816	s, 1H	Methyl CH_3

The proton NMR signal of fraction F1 showed that there is carbonyl proton at 7.9 ppm, benzylic ketone at chemical shift 2.1-2.3 ppm (code J-L) in table, indicating the benzoic group. The proton NMR supporting there was a benzoic acid derived compound.

CONCLUSION

This study reported that isolate strain STA 54/100m/10^{-3}. Isolated from deep sea sediment of Sumba Island was *Bacillus flexus* strain KM16 that contained antibacterial metabolite, especially toward *Mycobacterium smegmatis*. The chemical structural analysis indicated that FH1 obtained from separation of the extract had a similarity with compound detected in GC-MS analysis, which is 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester. This study was the first that reported this compound has an antibacterial activity toward *M. smegmatis* and produced by marine *B. flexus*.

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CONFLICT OF INTEREST

No conflict of interest regarding this publication.

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