

Antibacterial Activity and GC-MS Based Metabolite Profiles of Indonesian Marine *Bacillus*

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ABSTRACT

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Investigating Indonesian marine bacteria producing active compounds is key to finding a cultivable source of marine drugs. Screening the potential strain as well as profiling the active compounds are important steps to identifying the targeted substances. Several steps done in this study were to isolate some *Bacillus* strains from several marine environment areas, evaluate the antibacterial activity using the agar diffusion method, and characterize the secondary metabolite using GC-MS spectroscopy. Several active antimicrobial compounds derived from marine microorganisms were identified using GC-MS such as pyrrolo [1,2-a] pyrazine-1,4-dione, octatriacontyl pentafluoropropionate. We found that some marine bacillus showed antimicrobial activity, such as *B. flexus*, *B. tequilensis*, *B. subtilis*, and *Bacillus* sp. Profiling of metabolites on GC-MS showed the presence of several bioactive compounds in the ethyl acetate extract, which were identified to be nitrogen compounds such as pyrrolo[1,2-a]pyrazine-1,4-dione, phthalates compounds (butyl isohexyl ester and 1,2 benzendicarboxilate bis (2-ethylhexyl) ester), and dibutyl phthalate. Some phenolic compounds also were found, such as tris (2,4-di-ter-butylfenil) fosfat, phenol, 2,4-bis (1,1-dimethyl ethyl), and phenol 3,5-bis (1,1-dimethyl ethyl). Finally, fatty acid derivatives such as n-hexadecanoic acid, cis-vaccenic acid, 7-hexadecene, farnesol isomer A, and stigmastan-3,5-diene were also identified in several marine bacillus.

Keywords: Marine Bacillus, antimicrobial, GC-MS, bioactive compounds

INTRODUCTION

Since drug development requiring several potential higher marine organisms such as sponge, tunicate, and other marine invertebrates faces a deadlock in supply, marine microorganisms have become the priority target when investigating new marine drug candidates. The ocean is the richest source of microbial diversity that distinguishes natural products (Hughes & Fenical, 2010). Learning about terrestrial microorganisms has led to some vital antibiotic innovations, such as penicillin, streptomycin, and vancomycin, among other commercial antibiotics. Similarly, studies have since claimed that marine microorganisms also produce several active compounds.

The reported common marine microorganisms that produced active compounds were *Marinospora*, *Brevibacillus*, *Pseudomonas*, *Nocardia*, *Streptomyces*, *Aspergillus*, *Penicillium*, and *Alternaria*. (Debbab et al., 2011). Among the diversity of marine microorganisms, marine

bacillus was the promising genera that produce secondary metabolisms, such as lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipoamides, and isocoumarins (Hamdace et al., 2011, Barruzi et al., 2011). They are often found in many ocean habitats and can live in many conditions such as high pressure, temperature, salinity, and pH (Rampelotto, 2010). *Bacillus* strains need rich nutrition and suitable environmental conditions for optimum growth (Mondol et al., 2013). In the marine origins, marine bacillus also produced the unique compounds that showed active pharmacologically.

The potency of marine bacillus for producing various secondary metabolites that are active against antimicrobial, anticancer, and anti-algal activities was reported by Mondol et al., 2013. Several cyclic lipopeptide isolated from marine *Bacillus pumilus* has activity against pathogenic bacteria, such as surfactin analogs, glycocholic acid, amicoumacins A and B, and lipoamides A-C

(Berrue et al., 2009). The Marine bacillus has been shown to produce basiliskamide A, basiliskamide B, tupuseleiamide A, tupusleiamide B, and Bogorol A, potentially active against MRSA /Methicillin-resistant *Staphylococcus aureus* and VRE/ Vancomycin-resistant Enterococci (Barsby et al., 2001). Basiliskamide A and B were vigorously active against *C. albicans* and *A. fumigatus*. Further research to find fruitful results were continuously carried on. In this study, we explored the potential marine bacillus isolated from various Indonesian marine environments and biotas. This study aimed to evaluate the potency of tropical marine bacillus for producing active secondary metabolites as well as to characterize the group of active compounds and compare it to the other antimicrobial metabolite derived from the bacillus genus.

MATERIALS AND METHODS

General Experimental Procedures

The strains of marine bacillus were isolated from marine sediment and sponges collected from several areas in Indonesia such as Sulawesi Sea, Seribu Island, and Sumba. Media used for isolating the strains were modified marine broth (M1, M2, and M3), containing original seawater (from Ancol sea water line), peptone, yeast extract, and agar. All of the media for the first isolation was added with the antifungal agent (nystatin). Media used for antibacterial assays were nutrient agar and Mueller Hinton Agar (MHA). Ampicillin and rifampicin were used as the positive control.

The solvent used for extracting active substances were ethyl acetate, acetone, and methanol. The instrument used for analyzing compounds was GC-MS.



Figure 1. Sampling Location: 1 Makassar Strait (1°24.162'1.41022" N, 119°31.345'118.91080" E /150 m), 2. Seribu Island (5°51'09.3"S 106°36'58.7"E), 3. Sumba (10°05'27.6"S 119°38'52.6"E)

Isolation of the strains from the marine environment.

The method for bacterial isolation was referred to the Peter Schupp (pers. com). About 1 g of marine sediment or sponge was washed using seawater sterile and added about 5 mL solution contained 20% glycerol and marine broth medium. During transportation, the solution was frozen using ice dried. Arrived in the laboratory, the solution was diluted until 10^{-4} using seawater sterile and spread on agar media such as M1, M2, and M3 medium. One liter of M1 medium contained 16 g agar, 10 g amylum, 4 g yeast extract, 2 g peptone, 1 L seawater, and 50 mg nystatin. Each liter, M2 medium contained 16 g agar, 3.75 g marine broth, and 1 L demineralized water. M2 (10% Marine Broth) medium consisted of 0.5 g peptone, 0.1 g yeast extract, 0.01 g ferric citrate, 1.945 g sodium chloride, 0.88 g magnesium chloride 0.32 g sodium sulphate, 0.18 g calcium chloride, 55 mg potassium chloride, 16 mg sodium bicarbonate, 8 mg potassium bromide, 3.4 mg strontium chloride, 2.2 mg boric acid, 0.4 mg sodium silicate, 0.16 mg ammonium nitrate, 0.8 mg disodium phosphate, 0.24 mg sodium fluoride and dissolved in 1 L distilled water. M3 medium was not enriched in nutrition and contained 16 g agar, 1 L seawater, and 50 mg nystatin only. After 2 weeks of incubation at room temperature, the colony was picked and isolated.

Molecular characterization

DNA isolation was done using PCR colony method (Packeiser et al., 2013). A single cell was picked up from the solid surface medium and suspended in 50 μ L water (nuclease-free). The cell lysis process was done using a vortex for 10 seconds and incubated at 98°C for 5 min. The lysate was spun down to separate cell debris and supernatant. The supernatant was used for the DNA template for PCR amplification.

The amplification of 16S rDNA fragments was done using the reagent GoTaq (Promega) with primers as 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Zhang et al., 2009; Palaniappan et al., 2010). The PCR condition was set with the initial denaturation at 95°C for 1 min, denaturation at 95°C for 15 s, annealing at 52°C for 15 s, and extension time at 68°C for 45 s. The PCR process was carried out in as many as 35 cycles. PCR products were then purified using PEG precipitation method (Hiraishi et al., 1995) and continued for the sequencing cycle. The result of the sequencing cycle

was purified again using the *ethanol purification method*. The order of nitrogenous bases was analyzed using an *automated DNA sequencer* (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The sequencing data was processed using Bioedit program, homology sequence for 16SrDNA was browsed for similarity using an online database (Eztaxon server www.ezbiocloud.net) (Kim & Jun, 2012).

Bacterial Culture

The two L batch culture of Bacillus strains was prepared using the media for isolating strains (M1/M2 or M3) broth. The bacterial broth was incubated in a shaker incubator for 72 h, 28°C at 150 rpm. After harvested, the broth was centrifuged for separating pellet and supernatant.

Extraction of bacillus fermentation broth.

The pellet resulting from Bacillus broth was extracted using a polar organic solvent (acetone), and the supernatant was extracted using a semipolar solvent (ethyl acetate). Extracts were checked for biological activity against various pathogenic bacteria and remaining for further analysis. The potential extracts were applied for the spectroscopic method for preliminary profiling of known potential compounds.

Evaluation of antimicrobial activity

Biologically activity screening was done for antibacterial and anti-mycobacterial activity. The bacteria used for screening were *Staphylococcus aureus*, *Bacillus subtilis*, *Vibrio eltor*, *Escherichia coli*, and *Mycobacterium smegmatis*. The antibacterial activity was conducted using Kirby-Bauer methods, while the mycobacterium smegmatis assayed was combined with the MURR method.

The bacteria that were used for assays (bioindicator) were swabbed onto Petri dishes containing Mueller-Hinton Agar (MHA) with 0.9 % NaCl solution. 6 mm diffusion paper disks were prepared alongside ampicillin and rifampicin as the antibiotic controls. Methanol was also used as solvent control since it was used to dilute the dried extracts. Filter paper discs were impregnated with 100 micrograms of extract and antibiotic controls, then transferred to the diffusion disks containing the microorganism's test. Each sample and control were duplicates. The agar plates were incubated at 37°C for 24 h. Inhibition zones were measured using Vernier calipers.

Anti-*Mycobacterium smegmatis* assay (MURR Method)

An aliquot of Rifampin stock solution (20 g/mL) and extract (10 mg/mL) was dissolved with sterile DMSO for stock and stored at 4°C. Aliquots (10 µL) of Rifampin stock solution were diluted with MGIT medium (480 µL) and DMSO (10 µL) to obtain 0.4 g/mL and 200 g/mL of Rifampin. The assay was carried out using a sterile 96-well micro-plate, about 100 µL of MGIT medium containing *M smegmatis* was dropped in each well. The extracts and Rifampin against a suspension of *M. smegmatis* with the cell density of 1.0×10^6 cells/mL. Microtitre plates (MTP) were incubated (37°C, 5% CO₂) for 24 to 168 h) in a humid environment (>95%). After incubation, 50 L of resazurin solution in 5% Tween 80 (60 g/mL) was added to each well, and MTP was incubated (37°C; 5% CO₂) in a humid environment for 24 h. MTP was covered with sterile microplate sealing film before growth was measured with a microplate fluorometer. Mycobacterial growth was measured as relative fluorescence units (RFU) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm at 37°C. For the possibility of autofluorescence (correction), blanks were prepared for each treatment with MGIT medium (100 L) and included throughout the assay. The percentage inhibition of mycobacterial growth in each test sample and positive control was calculated using an equation.

Gas chromatography analysis

GC-MS was used to profile the substances that contained inactive extracts or fractions. GC-MS analysis was done using Gas Chromatography-Mass Spectroscopy (GC-MS) Agilent Technologies 7890 Gas Chromatography with autosampler and 5975 Mass Selective Detector and Chemstation data system operated with a HP Ultra 2, capillary column length (m) 30×0,25 (mm) LD×0,25 (µm) film thickness. The oven temperature program was adjusted between 70-270°C with a temperature increase rate of 10°C /min. The helium was used for carrier gas with the pressure set as 12 kPa, a total rate of 30 mL/min, and a split ratio of 1:50.50.50.50.00. The Agilent internal library system was used for the analysis of the compounds.

Table I. Isolated active bacillus strains and characterization

Strain code	Media isolation (16S-rDNA)	Molecular identification	% similarity	Source/depth
STA.7.MB.10-1.3	M2	<i>Bacillus sp. Bac123R 16S ribosomal RNA gene, partial sequence</i>	95	Surface water column /Sulawesi/150 m
STA25.Msed.2.10 ⁻¹ -1.3	M2	<i>Bacillus kochii</i>	100	Deep-sea sediment /Sulawesi/3700 m
STA3.Msed.10 ⁻¹ -1.2	M2	<i>Bacillus safensis</i>	99	Deep-sea sediment (Sulawesi /1500 m)
M1.Sp1.dil.3b.3	M1	<i>Bacillus subtilis subsp. inaquosorum KCTC 13429(T)</i>	99,78	sponge (Theonella, Seribu island 5 m)
M2.Sp3.dil.Ie	M2	<i>Bacillus aerophilus 28K(T)</i>	100%	Litisthis Sponge (Seribu Island 5 m)
M1.SP3.121015.101.a	M1	<i>Bacillus tequilensis</i>	100%	Litisthis Sponge (Seribu island 5 m)
STA 54/ 200m/ 10-3.2	M2	<i>Bacillus flexus</i>		Deepsea sediment (Sumba 6000 m)
STA4.Sed.090816.143 0M.F.M1.1 0 ⁻¹ .1	M1	<i>Bacillus subtilis</i>	99%	Deep-sea sediment (1430 m, Sumba)

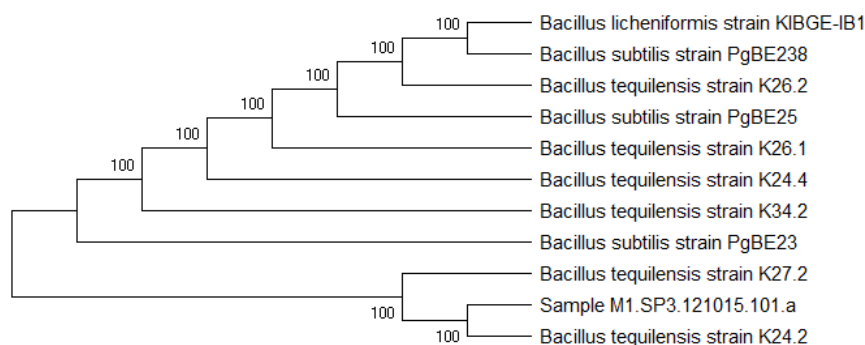


Figure 2. The phylogeny tree of strain M1.SP3. to the related isolated marine bacillus.

RESULTS AND DISCUSSION

Bacterial isolation and molecular characterization

Isolation of marine *Bacillus* using various types of media was intended to get the new species of *Bacillus*. In this study, only the active antimicrobial strain did for the further molecular characterizations.

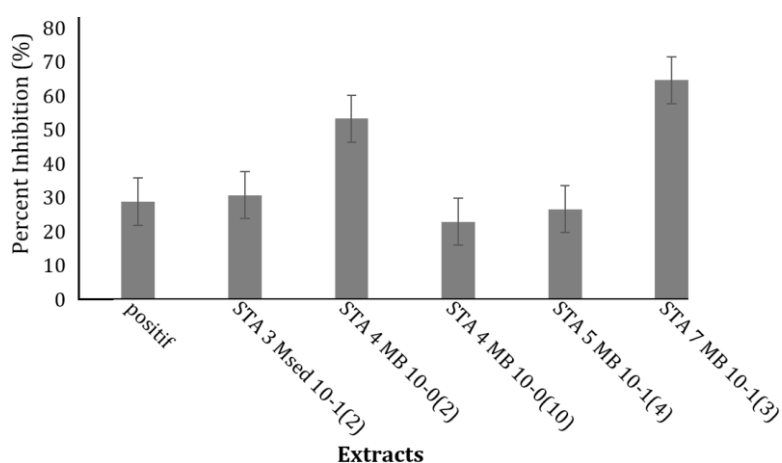
All of the marine *Bacillus* was isolated using the M2 medium, which contained 10% Marine Broth medium (Table I). There was 81 colony isolated from this medium. The other study used several media for isolating the marine *Bacillus* was related to the purpose of the research, such as Locus Bean Gum medium contained CMC and Xylane were the medium used to isolate marine *Bacillus* sp to generate enzymes such as manannase, xylannase, and cellulose (Yopi et al., 2017). Bennetht's broth medium was the medium

for isolating the *Bacillus* to generate fatty acid bioactive compounds (Mondol et al., 2013). In this study, the minimum nutrition was used to get the potential bacteria.

From the phylogenetic tree formed (Figure 2), *Bacillus subtilis* and *Bacillus tequilensis* are closely related. Research conducted by Kianngam et al. (2014), identified *B. tequilensis* and *B. subtilis* using biochemical methods and 16S rRNA sequencing analysis showed that *B. subtilis* has 99% similarity to *B. tequilensis*. The phylogeny tree also shows the relationship between *B. subtilis* and *B. licheniformis*. *B. licheniformis* has a close relationship with *B. subtilis* based on sequencing analysis of 16S rRNA and 16S-23S internal transcribed spacer. It is estimated that 80% of *B. licheniformis* genome sequences contain orthologous genes from *B. subtilis* (Rey et al., 2004).

Table II. Antibacterial activity of marine *Bacillus* strains

Extract of the bacteria	Diameter inhibition against (mm)				
	<i>V. eltor</i>	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>
<i>Bacillus</i> sp. STA.7.MB.10 ¹ .3	11.8	0	0	10.65	nd
<i>Bacillus kochii</i> STA25.Msed.2.	13.5	15.5	12	8.2	nd
<i>Bacillus safensis</i> STA3.Msed.10 ⁻¹ .2	14.1	7.6	13.65	15.8	0
<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> M1.Sp1.dil.3b.3	14.5	11.85	15.15	9.75	nd
<i>Bacillus aerophilus</i> M2.Sp3.	15.1	6.65	12.6	8.5	nd
<i>Bacillus tequilensis</i> M1.SP3.	8.05	6.65	15.25	6.9	nd
<i>Bacillus flexus</i> STA 54	nd	nd	12.7	12.6	18.3
<i>Bacillus subtilis</i> STA4.Sed.090816	nd	nd	nd	9.4	11.85
Rifampicin	38.37	nd	29.65	14.9	17
Ampicillin	34.05	47.2	16.9	19.65	19.15

Figure 3. The screening test of anti-*Mycobacterium smegmatis* from the isolated strains

Based on the phylogeny tree, sample M1.SP3 has a close relationship with *B. tequilensis* strain K24.2 indicated by the same branch. The presence of two isolates in the same branch indicates the similarity of species.

Antibacterial assay

Almost all of the isolated marine bacillus showed weak, moderate, and strong activity against several bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Vibrio eltor*, and *Mycobacterium smegmatis*. By using Kirby Bauer test, almost all bacillus strains showed moderate activity against several antibacterial bioindicators (Table II). The strongest activity showed by strain *Bacillus flexus* against *M. smegmatis*. The evaluation of anti-mycobacterial using MURR method exhibited that *Bacillus* sp strain STA.7.MB.10-1.3 was very strong activity against *M. smegmatis*, with the inhibition 64.63 % (Figure 3).

Bacillus sp. strain STA.7.MB.10-1.3 very strong inhibits *Mycobacterium smegmatis* growth (Figure 3), indeed strongest than positive control rifampicin. Also, *Bacillus safensis* strain STA3.Msed.10-1(2) showed a little bit higher than positive control with 30% inhibition.

Based on the antibacterial activity data, the chosen strains are to be analyzed for active secondary metabolite components using GC-MS. Preliminary identification of active compounds derived from the most potential *Bacillus* strains using GC-MS (Table III). However, further analysis will be done after detailed chemical separation to find the potential pure compounds.

The medium used for isolated potential *Bacillus* strains that are active against human pathogenic bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, and *Vibrio eltor* was less nutrition with 10% Marine Broth medium. Among the used medium in this study, medium M2 was the richest mineral ingredient, with a small carbon source.

Table III. Compounds identified in marine bacillus strains

Strain	Compounds identified using GC MS	Yield(%)
<i>Bacillus subtilis</i> subsp.	Methyl chloride	4.67
<i>inaquosorum</i> M1.Sp1.	Methane, chloro	3.98
	1-(6-methyl-2-piperidyl)propan-2-o	5.24
	1h-pyrazol,3-ethoxy	3.03
	1-octene	4.32
	4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	5.42
	n-hexadecanoic acid	19.65
	Cis-vaccenic acid	9.8
	Oleic acid	8.43
	Stigmastan-3,5-diene	6.34
	Farnesol isomer A	7.89
<i>Bacillus</i> sp	Phenol 3,5-bis (1,1 -dimethylethyl)	1.10
STA.7.MB.10-1.3	Pirrol[1,2-a]pyrazine- 1,4-dione, hexahydro-3-(phenylmethyl)	1.90
	1,2-benzenedicarboxylic acid, dioctyl ester	82.20
	7,8,17,18-Tetrahydro-35-methoxy-1,3,21,23-tetramethyl-16H,31H-5,9,15,19-dimethano-10, 14-metheno-26,30-nitrilo-6H,25H-	2.59
	dibenzo(b,s)(1,21,4,8,14,18)dioxatetraazacyclooctacosine	
<i>Bacillus safensis</i>	Benzene 1,2,4-trimethyl	3.10
STA3.Msed.10-1.2	Phenol, 2,4-bis (1,1-dimethylethyl)	39.10
	Octatriacontyl pentafluoropropionate	1.84
	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18,20,20-icosamethylcyclo decasiloxane	2.27
<i>Bacillus flexus</i> STA 54.	Phenol, 2,4-bis(1,1-dimethylethyl)	11.20
	Phthalic acid, butyl isohexyl ester	3.09
	1-Hydroxy-1-(4-Butylphenyl)-2-Butanone	3.33
	7-Hexadecene, (Z)	1.46
	Dibutyl Phthalate	6.50
	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	13.59
	Ethanol - 2 - (Tetradecyloxy)	1.65
	1- Tricosene	1.85
<i>Bacillus tequilensis</i>	1,2 benzendicarboxilate bis (2-ethylhexil) ester	67.74
M1.SP3.	Tris (2,4-di-ter-butyl phenyl) phosphate	32.26
	Bis-(2-ethylhexyl) phthalate	14.18

The induction to produce secondary metabolites in microbe is rarely done by reducing the nutrition, inducing a pathogenic strain, or other physical conditions. In this study, several *Bacillus* strains that were isolated using minimum nutrition M1 produced active substances that inhibit moderate until strong against pathogenic bacteria that have already been mentioned. A study on the Metagenomic of *Bacillus* genus reported that more than 8% of them produced antibiotics (Chen et al., 2007). A study of the biosynthesis gene cluster revealed that several *Bacillus* strains produced antibiotics such as carotenoid (*Bacillus akibai*, *Bacillus pseudofirmus*, *Bacillus wakoensis*, *Bacillus*

cellulosilyticus, *Bacillus horikoshii*, *Bacillus aurantiacus*, *Bacillus daliensis*, and *Bacillus bogoriensisoccore*), and tetra hydroypyrimidine ectoine (widespread in *Bacillus* strains) (Khurana et al., 2020).

The preliminary study of compounds contained in active extract indicated several compounds. The important active compounds present in the potential extract such as Pirrolo[1,2-a]pyrazine- 1,4-dione, hexahydro-3-(phenylmethyl), previously have been produced by marine bacteria *Bacillus* sp, which was isolated from sponges showed active against marine pathogens *Vibrio alginolyticus*, *Vibrio*

vulnificus, *Vibrio parahaemolyticus*, *Aeromonas salmonicida*, *Flavobacterium* sp., *Edwardsiella* sp., *Proteus mirabilis* and *Citrobacter brackii* (Mohan et al., 2016). Octatriacontyl pentafluoropropionate was previously reported to be found in seaweed *Sargassum fusiforme* and confirmed as antibacterial and antiMDR activity. Besides, those GC-MS analysis data, the previous work reported that *Bacillus kochii* strain STA25.Msed.2.10^{-1.3} was containing Bengamide E (Wibowo et al., 2017). Bengamide E in a previous study was also found in the sponge *Pachastrissa* sp. (Fernandez et al., 1999), *Jaspis coriacea*, and myxobacteria *Myxococcus virescens* (Johnson et al., 2012). Bengamide E compound was also reported active against anti-tumor, cytotoxic, antibacterial, & immunomodulation (Johnson et al., 2012). The most potential antimycobacterium producer *Bacillus* sp STA.7.MB.10-1.3 contained 7,8,17,18-Tetrahydro-35-methoxy-1,3,21,23-tetramethyl-16H,31H-5,9,15,19-dimethano-10, 14-metheno-26,30-nitrilo-6H,25H-dibenzo(b,s) (1,21,4,8,14,18) dioxatetraazacyclooctacosine. This compound was previously reported to exist in marine cyanobacteria *Geitlerinema* sp. CNP 1019 extract showed cytotoxic against brine shrimp larvae, HT-29 (colon), HeLa (cervix), MCF7 (breast), KB (oral), and Hop2 (lung) cancer cell lines (Maruthanayagam et al., 2013).

Based on the GC-MS analysis of ethyl acetate extract of *Bacillus tequilensis* M1.SP3.121015.101 and *Bacillus flexus* STA 54/200m/ 10^{-3.2}, there were phthalate compound groups such as phthalic acid, butyl isohexyl ester, Bis-(2-ethylhexyl) phthalate. Phthalate compounds are reported able to inhibit the growth of several bacteria such as *S. epidermidis*, *S. aureus*, *S. pneumoniae*, *E. coli*, *M. luteus*, *K. pneumonia*, *S. flexneri*, *V. cholerae*, and *P. aeruginosa* (Viswanathan and Shobi, 2018). Phthalates also could interfere with the function of cells as DNA methyltransferase. DNA methyltransferase plays a role in the process of methyl group substitution in specific areas of DNA. The DNA methylation process is used as a signal for the regulation of gene expression and DNA replication. Inhibition of methyltransferase causes genes could not to be expressed and the DNA replication process to be disrupted (Surat, 2019). In addition, phthalates can inhibit the action of methyl-CpG binding protein (Valinluck et al., 2004).

Another compound such as tris (2,4-di-ter-butylphenyl) phosphate which belongs to the phenol group has biological functions as an antimicrobial. The phenolic compound can inhibit

the activity of the fungus *Aspergillus niger*, *Fusarium oxysporum*, and *Penicillium chrysogenum* (Varsha et al., 2015). In addition, it also can inhibit the activity of *E. coli*, *S. aureus*, and *P. aeruginosa* (Abdullah et al., 2011). Phenolic compounds can damage the cytoplasmic membrane; increase its permeability and cause the release of intracellular fluids such as proteins, nucleic acids, and inorganic ions (Amborabe et al., 2002). Phenolics are lipophilic, making it easier for them to pass through microbial cell membranes and cause a lowering of the cytosolic pH resulting in protein denaturation and disrupting cell activity (Campos et al., 2016). Besides, phenol can cause damage to cell wall structures as well. The previous study reported that marine bacillus commonly produces antimicrobial substances such as bacteriocins and other peptide groups (Moubayed et al., 2022).

This study confirmed that *Bacillus* sp strain STA.7.MB.10-1.3 is very strong against *Mycobacterium smegmatis*. The molecular identification strain STA.7.MB.10-1.3 has a 95% similarity with the *Bacillus* sp. The phylogeny three of this strain showed that this strain suggested a new species, but generating a comprehensive conclusion needs a further experiment.

CONCLUSION

In this study, several marine *Bacillus* strains such as *B. flexus*, *B. tequilensis*, *B. subtilis*, *Bacillus aerophilus* showed activity against pathogenic bacteria. *B. tequilensis* active against *Staphylococcus aureus* with diameter inhibition 15.25 mm, *B. flexus* active against *Mycobacterium smegmatis* with diameter inhibition 18.3 mm, *B. subtilis* active against *Mycobacterium smegmatis*, and *Bacillus aerophilus* active against *Vibrio eltor* with diameter inhibition 15.1 mm. They play an important role in producing active compounds. Several active compounds isolated from that marine bacillus were nitrogen compounds such as bengamide E, pyrrolo[1,2- a]pyrazine-1,4-dione, phthalates compounds (phthalic acid, butyl isohexyl ester, and bis-(2-ethylhexyl) phthalate). Some phenolic compounds also were found, such as tris (2,4-di-ter-butylphenyl) fosfat, phenol, 2,4-bis (1,1-dimethylethyl), phenol 3,5-bis (1,1-dimethylethyl) and 7,8,17,18-tetrahydro-35-methoxy-1,3,21,23-tetramethyl-16H,31H-5,9,15,19-dimethano-10,14-metheno-26,30-nitrilo-6H,25H- dibenzo(b,s) (1,21,4,8,14,18)dioxatetraazacyclooctacosine. Finally fatty acid derivatives such as n-hexadecanoic acid, cis-vaccenic acid, 7-hexadecene, farnesol isomer A, stigmastan-3,5-diene were also

identified in several marine *Bacillus*. Those findings were quite different from the most antimicrobial compounds derived from the *Bacillus* genus. The common antimicrobial compounds derived from the *Bacillus* genus were from ribosomally synthesized peptides such as bacteriocins and small molecules of peptides synthesized enzymatically or nonribosomal pathways.

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