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Isolation and Antibacterial Screening of Marine Heterotrophic Bacteria from Makassar Strait, Indonesia

(Pengasingan dan Penyaringan Antibakteria Bakteria Heterotrofik Marin dari Selat Makassar, Indonesia)

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ABSTRACT

The biological potential of microorganisms in Indonesian waters has yet to be widely reported despite their high diversity. This research aimed to isolate bacteria from seawater and seabed sediments in Makassar Strait and then screen their potential antibacterial abilities. Subsequently, we tried to determine the bioactive compound content of the bacterial extracts. We purified 15 bacterial isolates and based on a 16S rRNA analysis, we found that these bacteria represented three phyla, namely Actinobacteria, Firmicutes, and Proteobacteria. All the isolates were found to have antibacterial capabilities, while 2 isolates showed positive results for inhibiting the growth of the pathogens tested. Molecular analysis studies provided information that the two isolates which were *Bacillus sp*., namely *B. stratosphericus* TR4 and *B. thuringiensis* TR13, were detected to have non-ribosomal peptide synthetase (NRPS) genes, which are thought to contribute to their antibacterial potential. Compounds from the imidazole group, quinolone ester derivatives, steroid derivatives, and acetamide derivatives were found in the extracts of both strains. Further research needs to be conducted to better understand each compound's antibacterial mechanisms and possible interesting bioactive properties.

Keywords: Antibacterial; LC-MS; marine bacteria; NRPS

ABSTRAK

Potensi biologi mikroorganisma di perairan Indonesia masih belum dilaporkan secara meluas walaupun mempunyai kepelbagaian yang tinggi. Penyelidikan ini bertujuan untuk memencilkan bakteria daripada air laut dan sedimen dasar laut di Selat Makassar seterusnya menyaring potensi antibakteria bakteria tersebut. Selepas itu, kami cuba menentukan kandungan sebatian bioaktif ekstrak bakteria. Kami telah memencilkan 15 pencilan bakteria dan berdasarkan analisis rRNA 16S, kami mendapati bahawa bakteria ini mewakili tiga filum, iaitu Actinobacteria, Firmicutes dan Proteobacteria. Kesemua pencilan didapati mempunyai keupayaan antibakteria, manakala 2 pencilan menunjukkan keputusan positif untuk menghalang pertumbuhan patogen yang diuji. Kajian analisis molekul memberikan maklumat bahawa 2 pencilan iaitu *Bacillus* sp., *B. stratosphericus* TR4 dan *B. thuringiensis* TR13 dikesan mempunyai gen bukan ribosomal peptida sintetase (NRPS) yang dianggap menyumbang kepada potensi antibakteria. Sebatian daripada kumpulan imidazol, sebatian ester kuinolon, sebatian steroid dan sebatian asetamida ditemui dalam ekstrak kedua-dua strain. Kajian lanjut perlu dijalankan untuk lebih memahami mekanisme antibakteria setiap sebatian dan kemungkinan sifat bioaktif yang menarik.

Kata kunci: Antibakteria; bakteria marin; LC-MS; NRPS

INTRODUCTION

Over the past 30 to 40 years, the world has focused on marine species to discover new natural resources. More than 12,000 compounds have been created by a group of animals, plants, microorganisms, and hundreds more are discovered each year in marine (Donia & Hamann 2003). The majority of these compounds have so far been found in marine invertebrates, especially sponges (Lei & Zhou 2002). In addition, bacteria isolated from sediments, seawater, and marine organisms show their ability to produce widely applied bioactive compounds (Piel 2009). This is because marine ecosystems provide different habitats, including pressure, high salinity, low temperatures, and fluctuating oxygen concentrations

(Bull, Ward & Goodfellow 2000). All these factors exert pressure on marine microorganisms to evolve in ways that differentiate them from terrestrial microorganisms, and these pressures are likely to impact the genetic diversity and metabolism of marine microbes (Lam 2006; Manivasagan et al. 2013). Therefore, marine microorganisms, including bacteria, can respond to environmental changes and adapt quickly to ensure survival in this dynamic habitat by producing unique products known as secondary metabolites.

The utilisation of these distinctive molecules in research has facilitated the discovery of novel bioactive compounds exhibiting antibacterial, antifungal, anticancer, anti-quorum sensing, and antiviral properties and apply them to current clinical challenges (Carroll et al. 2021; Gulder & Moore 2010; Saurav et al. 2017; Valliappan, Sun & Li 2014). Secondary metabolites are synthesised as a result of stress stimuli, and some of these compounds have demonstrated beneficial properties in the fields of biotechnology and pharmaceuticals (Engel, Jensen & Fenical 2002; Zhang et al. 2005). In the future, if thoroughly screened and dedicated research is performed, marine bacteria possess the potential to serve as a valuable source of antimicrobial agents, thereby addressing the challenge of drug-resistant infections in the forthcoming century (Hancock 2007). This requires researchers to continue exploring the potential microbes from the sea to provide a source of new medicines in the future.

Makassar Strait is one of the waters in Indonesia which plays a vital role because it is the main gateway to the Indonesian Throughflow (ITF), which significantly influences the ecosystem in the region, including the microorganisms. However, information regarding the diversity and potential of microorganisms in this area still needs to be improved. Here, we present research results from our efforts to systematically isolate and identify marine bacteria based on molecular genetic data and characterise the antibacterial activity and bioactive compounds of extracts of potential isolates.

MATERIALS AND METHODS

SAMPLES

Samples were obtained during the TRIUMPH (TRansport Indonesian seas, Upwelling, and Mixing Physics) expedition which was carried out in December 2019. Seawater and seabed sediment samples were taken at Bontang station with coordinates 0° 13.5308' North 117° 47.6941' East. Seawater samples were obtained using a carousel water sampler type SBE 32 at a depth of 150 m (thermocline zone) and 600 m, while seabed sediment at a depth of 636 m, taken at a depth of 0 - 2 cm below the seabed (bsf) using a correr. On board, 5 L of seawater was filtered using a 0.22 µm Whatman membrane filter. All samples were stored in a sterile falcon and stored at -20 °C until testing in the laboratory.

ISOLATION OF MARINE BACTERIA

The isolation of marine bacteria was carried out using the spread plate method. The seawater filter sample was cut into small sizes, which was then mixed with sterile distilled water in the first dilution tube and vortexed. One gram of sediment sample was weighed and vortexed in 9 mL of distilled water and vortexed for 10 min. Dilutions were carried out to 10^{-5} times and 100 µL from the last three dilution treatments were spread on Marine Agar (MA) and Actinomycetes Isolation Agar (AIA). Then, the petri dish was incubated in an incubator at 25 °C for 48 h. The growing colonies were selected based on morphological differences and purified on MA and AIA medium. The pure isolate was stored on glycerol at -20 °C.

EXTRACTION AND ANALYSIS OF MARINE BACTERIA DNA

Extraction of marine bacterial DNA was carried out using the Presto™ Mini gDNA Bacteria Kit (Genaid). The amplification of the 16S rRNA gene was performed using the primers, 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′TACGGYTACCTTGTTACGACTT-3′). The DNA amplification program involved pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min 30 s, and extension at 68 °C for 1 min. It was then followed by a final extension of 68 °C for 5 min. The PCR process was repeated for 30 cycles (de Fretes et al. 2021). Visualisation of PCR products was carried out using safe green DNA staining and electrophoresis using 1% agarose gel at 100 volts for 30 min. The PCR products were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (1st Base Pte. Ltd, Singapore). The 16S rRNA gene sequences were then used as queries in the BLAST analysis on the NCBI website. Phylogenetic tree construction was performed using MEGA X with Neighbor-joining tree and bootstrap method with replications of 1000.

AVAILABILITY OF NUCLEOTIDE SEQUENCES

The 16S rRNA gene sequences of the isolates examined in this investigation were submitted to GenBank and assigned the accession numbers ON338678, ON338679, ON338680, ON338682, ON338683, ON338684, ON338686, ON340568, ON340602, ON340603, ON340604, ON340605, ON340607, ON340608, and ON340610.

SCREENING THE ANTIBACTERIAL POTENTIALS OF THE MARINE BACTERIA

The ability of marine bacteria as antibacterial agents was carried out using the colony-picking method (Hettiarachchi et al. 2017). The test pathogens, *Escherichia coli* and *Staphylococcus aureus*, were inoculated into 5 mL of Mueller Hinton Broth and incubated at 35 °C for 18 h. Then, 100 µL of the test bacteria (10^8 CFU/mL - McFarland 0.5) were spread on Mueller Hinton Agar medium on a petri dish. After that, the marine bacterial isolates were inoculated into a petri dish using a sterile toothpick. Petri dishes were incubated at 35 °C for 24 h and the zone of inhibition of the test bacteria was observed.

DETECTION OF THE NON-RIBOSOMAL PEPTIDE SYNTHETASE (NRPS) GENE

The primers used were A2gamF (5′-AAG GCN GGC GSB GCS TAY STG CC-3′) and A3gamR (5′-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3′). DNA amplification was carried out with the following cycle that involved predenaturation at 96 °C for 3 min, then denaturation (96 °C for 30 s), annealing (58 °C for 40 s), and extension (68 °C for 2 min 30 s). After that, it was followed by a final extension of 72 °C for 10 min. The PCR process was repeated for 40 cycles (Radjasa et al. 2007). The PCR product that has been amplified will be visualised using safe green DNA staining and electrophoresis using 1% agarose gel for 30 min at 100 volts.

EXTRACTION FOR BIOACTIVE COMPOUNDS

The TR4 and TR13 strains were combined in a culture and mixed with 1-butanol in a ratio of 5:1. The mixture was then incubated for 1 h at 37 °C, with continuous shaking at a speed of 200 rpm per min. The centrifugation process was conducted for a duration of 10 min at a relative centrifugal force of 8000 times the acceleration due to gravity. The resultant organic layer was gathered and subjected to evaporation with a rotary evaporator (Baharudin et al. 2021). The present study employed tandem mass spectrometry with liquid chromatography (LC-MS/MS) for the analysis under specific experimental conditions. High-performance liquid chromatography (HPLC) separation was conducted using an ultra-performance liquid chromatography (UPLC) BEH C18 column with dimensions of 50 mm × 2.1 mm and a particle size of 1.7 µm. The mobile phase was composed of two solvents namely solvent A, which was deionised water, and solvent B, which was a mixture of CH_3CN and water containing 1% formic acid. The experimental parameters employed for mass spectrometry analysis included the ionisation parameters which were in the positive ion mode, the capillary voltage was set to 3000 V, while the cone voltage was set to 30 V. The mass analyser was utilised to scan a range of m/z values spanning from 100 to 1200 atomic mass units (amu).

RESULTS AND DISCUSSION

MARINE BACTERIA

The marine environment is rich in ecological biodiversity and can be regarded as an untapped resource for prospecting novel bioactive compounds. Bacterial strains derived from seawater, sediments, and marine species have demonstrated the capacity to synthesise a wide range of novel medicinal chemicals, exhibiting various uses (Piel 2009). The results of the isolation of marine bacteria that have been collected from seawater filter and sediment samples showed that there were 15 isolates with different colony morphology, of which 7 isolates were found in MA medium, namely TR1, TR3, TR7, TR10, TR12, TR14, and TR15, while 8 isolates were found in AIA medium, namely TR2, TR4, TR5, TR6, TR8, TR9, TR11, and TR13. Five isolates, TR1 to TR5 were isolated from seawater samples at a depth of 150 m, and TR6 and TR7 were from a depth of 600 m. Meanwhile, TR10 to TR15 came from seabed sediment. Sequences of 15 marine bacterial isolates were compared with reference strains in GenBank. The results of the BLAST analysis showed that marine bacteria obtained from seawater and seabed sediment samples came from the phylum Firmicutes (*Bacillus, Rossellomorea, Staphylococcus*), Actinobacteria (*Rhodococcus, Kocuria, Kytococcus*), and Proteobacteria (*Halomonas*). The phylogenetic tree of marine bacterial isolates based on the 16S rRNA gene sequence is shown in Figure 1.

The interesting finding was that the isolates obtained belonged to three different phyla, namely Actinobacteria, Firmicutes, and Proteobacteria, showing the abundance of marine bacterial species that have been found. However, research results showed that culturing marine bacteria in the laboratory is very limited by various factors and the differences with the native conditions in the sea. Zhang et al. (2021) explained that these factors included stress, such as incubation pressure and temperature, nutrient requirements, and low growth rates. Based on empirical evidence, it has been determined that a mere 2% of the bacterial population on our planet may be readily cultivated using culture-dependent techniques (Wade 2002).

This study obtained 7 genera of marine bacteria, which have been reported previously. One of the important genera of the Firmicutes group is *Bacillus*, where species of this genus are often isolated from marine sediment samples and other marine habitats. This species has high-temperature tolerance and can grow quickly in liquid culture (Ntougias & Russell 2000; Rossi et al. 2002). Various species of the genus *Rhodococcus* have been recovered from various habitats such as soil, freshwater, marine sediments, and herbivore droppings. *Rhodococcus* has potentials in industry because it has diverse metabolic activities, including the degradation of aliphatic and aromatic hydrocarbons. However, some bacteria are also known as animal and plant pathogens (Ramaprasad et al. 2018). Stackebrandt et al. (1995) reported that *Kytococcus sedentarius* is a species of *Kytococcus*,

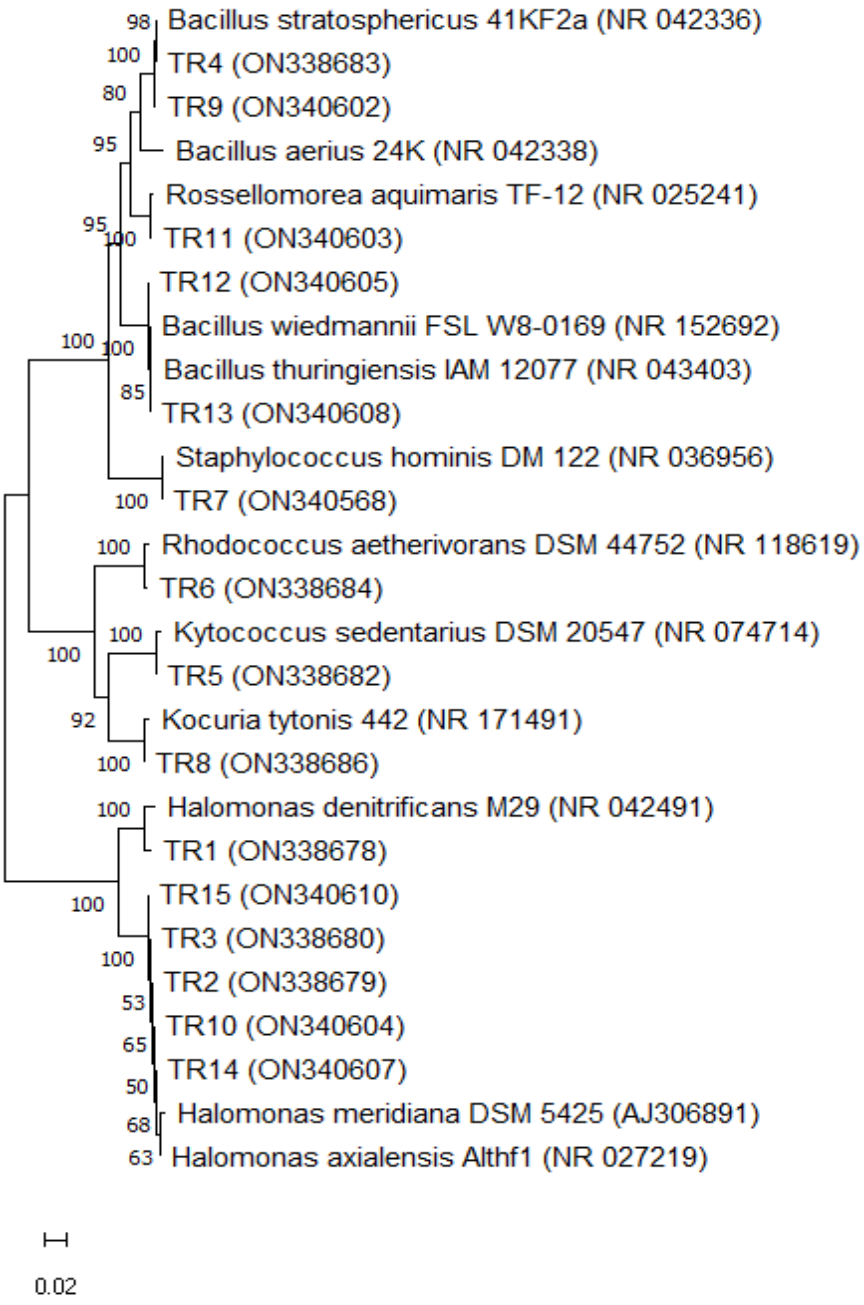


FIGURE 1. A phylogenetic tree of 15 isolates marine bacteria obtained from Makassar Strait

previously known as *Micrococcus sedentarius*, which was first isolated from the marine environment, marine sediments, and was also found on human skin. Likewise, members of the genus *Kocuria* have been reported to be isolated in extreme habitats such as desert soil, salt water, seawater, air, marine sediments, and fermented seafood (Park et al. 2010). The *Halomonas* genus consists of halophilic heterotrophic bacteria and is the largest genus in the Halomonadaceae family. It is found in various environments with varying salinity, such as soil, estuaries, lakes, deep seas, and seafood and marine invertebrates (Jiang et al. 2014). Research by Sanz-Sáez et al. (2020) showed that *Halomonas* is one of the heterotrophic bacteria found in the marine culture collection project (MARINHET).

ANTIBACTERIAL ASSAY

The results showed that most of the marine bacterial isolates (*Halomononas* sp. TR1, *Halomononas* sp. TR3,

TABLE 1. Antibacterial assay of the isolates obtained against *E. coli* and *S. aureus*

Bacterial isolates	Test bacteria	
	E. coli	*S. aureus*
TR1	-	+
TR2	-	-
TR3	-	+
TR4	+	+
TR5	-	+
TR6	-	+
TR7	-	+
TR8	+	-
TR9	-	+
TR10	-	+
TR11	-	+
TR12	-	+
TR13	+	+
TR14	+	-
TR15	+	-

+/-, indicates an inhibition zone formed around the colony

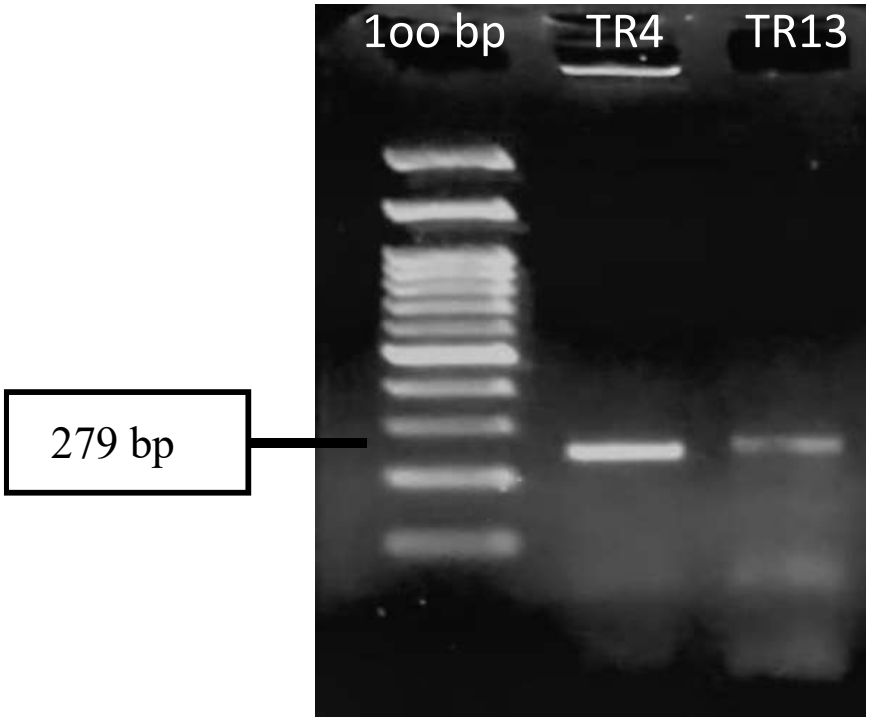
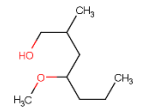
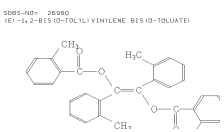
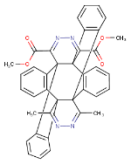
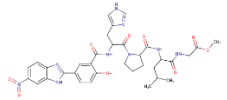
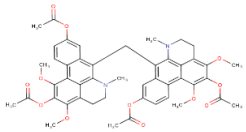
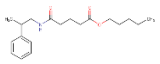
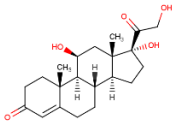


FIGURE 2. *Visualisation* of NPRS gene fragment from B. stratosphericus TR4 and B. thuringiensis TR13

TABLE 2. Identification of secondary metabolites in butanol extract of *B. stratosphericus* TR4 and *B. thuringiensis* TR13

No	Rt (min)	Compound name	Class	Calculated mass	Observed mass	Major fragment ion	Molecular formula	Compound structure
		B. stratosphericus TR4						
1	0.42	4-Methoxy-2-methyl-1-heptanol	Alkyl alcohol	159.8762	160.1925	60.2290, 70.2851, 88.2741, 132.1243, 160.2971	$C_9H_{20}O_2$	
2	3.81	(E)-1,2-bis(o-tolyl)vinylene bis(o-toluate)	Carboxylic acid derivatives	476.1330	476.2698	83.1522, 122.8793, 172.1662, 453.5678, 476.5000	$C_{32}H_{28}O_4$	
3	4.38	1,4-bis(Methoxycarbonyl)-9,12-dimethyl-2,3,10,11-tetraaza-4a,8b : 12a,16b-bis(orthobenzeno) tetraphenylene	Orthobenzeno derivates	604.1055	604.2110	83.2771, 141.3215, 122.8168, 283.8686, 453.5053, 588.5408, 604.6631	$C_{38}H_{28}N_4O_4$	
4	4.80	2-Hydroxy-5-(6-nitro-1H-benzo[d] imidazol-2-yl)-benzoyl His-Pro-Leu-Gly Dev	Imidazole derivatives	717.1797	717.2870	60.2290, 83.2771, 340.5365, 679.7145, 701.7740, 717.7097	$C_{34}H_{39}N_9O_9$	
5	5.11	4H-Dibenzo[de,g]quinoline-2,9-diol, 7,7'-methylenebis[5,6-dihydro-1,3-dimethoxy-6-methyl-, tetraacetate (ester)	Ester quinoline derivatives	830.0754	830.3050	60.1040, 83.2147, 122.6919, 172.2911, 397.2072, 792.8885, 814.8238, 830.8221	$C_{47}H_{46}N_2O_{12}$	
6	8.76	Glutaric acid, monoamide, N-(2-phenylpropyl)-, pentyl ester	Glutaric acid	319.1923	319.2871	60.1040, 122.7543, 230.5146, 274.4970, 319.4810	$C_{19}H_{29}NO_3$	
7	8.95	4-Pregnen-11Î²,17,21-triol-3,20-dione • 11,17,21-Trihydroxypregn-4-ene-3,20-dione, (11.beta.)	Steroid derivatives	362.0572	362.3620	60.1665, 83.2147, 244.5088, 290.4911, 334.6010, 362.4672	$C_{21}H_{30}O_5$	

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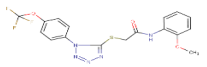
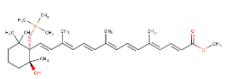
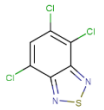
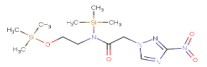
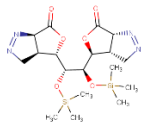
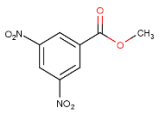
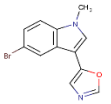
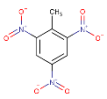
8	9.59	N-(2-methoxyphenyl)-2-[1-[4-(trifluoromethoxy)phenyl]tetrazol-5-yl]sulfanyl-acetamide	Acetamide derivative	425.0769	425.3490	60.2290, 83.2771, 203.4015, 288.4918, 425.3873	$C_{17}H_{14}F_3N_5O_3S$	
9	17.04	Methyl ester of (+)-(5S,6S)-6-Hydroxy-5-trimethylsilyloxy-5,6-dihydro-10'-apo-.beta.-carotin-10'-oic acid	Methyl ester	512.7029	512.7359	60.1665, 131.7494, 239.5190, 313.4830, 353.3451, 512.8048	$C_{31}H_{48}O_4Si$	
10	18.39	2,1,3-Benzothiadiazole, 4,5,7-trichloro- • 4,5,7-Trichloro-2,1,3-benzothiadiazole • 4,5,7-Trichlorobenzthiadiazole-2,1,3	Benzothiadiazole	239.2965	239.4501	60.1665, 80.2771, 131.4996, 239.5109	$C_6HCl_3N_2S$	
11	19.08	N-(2-Hydroxyethyl)-2-(3-nitro-1H-1,2,4-triazol-1-yl)acetamide, 2TMS derivative	Acetamide derivative	359.1857	359.4920	83.2147, 131.6245, 267.5622, 341.5987, 359.5306	$C_{12}H_{25}N_5O_4Si_2$	
12	20.11	4,4'-[1'',2''-bis[(Trimethylsilyl)oxy] ethane-1'',2''-diyl] - bis[3a,4,6,6a-tetrahydro-3H-furo[3,4-c]pyrazol-6-one]	Pyrazole derivatives	454.0103	454.1788	61.1659, 149.2402, 279.4951, 303.5208, 413.5153, 454.6301	$C_{18}H_{30}N_4O_6Si2$	
13	21.11	3,5-dinitrobenzoic acid, methyl ester • Benzoic Acid, 3,5-Dinitro-, Methyl Ester • Methyl 3,5-dinitro benzoate	Benzoic acid derivatives	226.0154	226.1378	60.1040, 131.6870, 132.4366, 143.1809, 185.2224, 226.1415	$C_8H_6N_2O_6$	
14	22.16	5-(5-bromanyl-1-methyl-indol-3-yl)-1,3-oxazole	Oxazole derivatives	275.9898	277.1200	60.2290, 132.1243, 155.2372, 183.1609, 227.1210	$C_{12}H_9BrN_2O$	
		B. thuringiensis TR13						
1	0.39	2,4,6-trinitrotoluene	Toluene derivatives	227.0178	227.1324	60.0416, 70.1602, 86.2753, 88.0868, 227.1411	$C_7H_5N_3O_6$	

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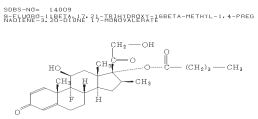
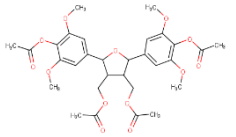
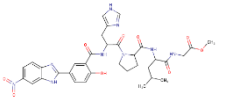
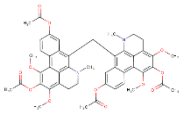
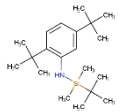
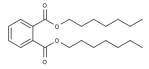
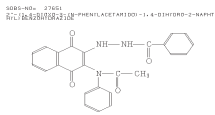
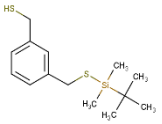
2	3.81	9-fluoro-11beta,17,21-trihydroxy-16beta-methyl-1,4-pregnadiene-3,20-dione 17-monovalerate	Steroid derivatives	476.5419	476.6152	60.1040, 83.2147, 122.7543, 172.2287, 453.5678, 475.6252, 476.6250	$C_{27}H_{37}FO_6$	
3	4.37	4-[5-(4-Hydroxy-3,5-dimethoxyphenyl)-3,4-bis(hydroxymethyl)oxolan-2-yl]-2,6-dimethoxyphenol, tetraacetate	Dimethoxyphenol tetraacetate	604.3798	604.5107	60.2290, 83.2771, 114.3215, 284.0560, 566.6696, 588.6033, 589.7280, 604.6005	$C_{30}H_{36}O_{13}$	
4	4.80	2-Hydroxy-5-(6-nitro-1H-benzo[d] imidazol-2-yl)-benzoyl His-Pro-Leu-Gly Dev	Imidazole derivatives	717.2870	717.7074	60.1665, 83.2771, 331.6020, 340.5365, 679.7770, 701.7740, 717.7097	$C_{34}H_{39}N_9O_9$	
5	5.09	4H-Dibenzo[de,g]quinoline-2,9-diol, 7,7'-methylenebis[5,6-dihydro-1,3-dimethoxy-6-methyl-, tetraacetate (ester)	Ester quinoline derivatives	830.3050	830.8872	60.2290, 83.2147, 397.0198, 792.8885, 814.8862, 815.8236, 830.8821	$C_{47}H_{46}N_2O_{12}$	
6	8.74	2,5-Di-tert-butylaniline, N-(tert-butyldimethylsilyl)	Tert-butyldimethylsilyl	319.3920	319.4778	60.1040, 230.4522, 274.4970, 318.6062, 319.6059	$C_{20}H_{37}NSi$	
7	8.95	diheptyl phthalate	Dipentyl phthalate	362.1714	362.2457	60.1040, 290.5536,334.5385, 362.5297	$C_{22}H_{34}O_4$	
8	9.47	2'-(1,4-dioxo-3-(N-phenylacetamido)-1,4-dihydro-2-naphthyl)benzohydrazide	Benzohydrazide	425.1920	425.2778	83.2771, 122.7543, 288.4919, 425.3873	$C_{25}H_{19}N_3O_4$	
9	13.22	1,3-Benzenedimethanethiol, S-(tert-butyldimethylsilyl)- • 1,3-Benzenedimethanethiol, TBDMS derivative	tert-Butyldimethylsilyl (tBDMS) Derivatives	283.9498	284.1771	60.1040, 83.2271, 122.7543, 284.5558	$C_{14}H_{24}S_2Si$	

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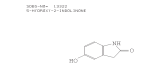
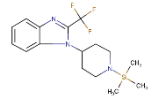
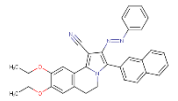
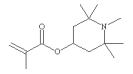
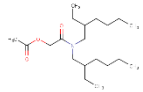
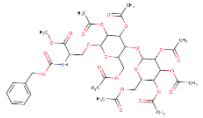
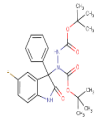
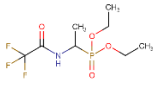
10	13.69	5-hydroxy-2-indolinone	Indolinone derivatives	149.0178	149.0476	60.1040, 83.2771, 149.1153	$C_8H_7NO_2$	
11	14.99	1-(4-Piperidinyl)-2-(trifluoromethyl)-1H-benzimidazole, TMS • 1-(4-Piperidyl)-2-(trifluoromethyl)benzimidazole, N-trimethylsilyl	Imidazole derivatives	341.1880	341.4507	83.1522, 131.7494, 341.5362	$C_{16}H_{22}F_3N_3Si$	
12	17.05	8,9-Diethoxy-5,6-dihydro-3-(naphthalen-2”-yl)-2-(phenyldiazenyl)pyrrolo[2,1-a] isoquinoline-1-carbonitrile	Quinoline derivatives	512.1260	512.2212	60.2290, 83.2771, 131.6870, 239.4484, 313.5455, 512.6173	$C_{33}H_{28}N_4O_2$	
13	18.42	1,2,2,6,6-pentamethyl-4-piperidyl methacrylate	methacrylate derivatives	239.1528	239.2250	60.1040, 83.2147, 131.7494, 239.3859	$C_{14}H_{25}NO_2$	
14	19.12	Acetoxyacetamide, N,N-bis(2-ethylhexyl)	Acetamide derivative	341.2216	341.4794	83.2771, 131.7494, 276.4997, 341.5362	$C_{20}H_{39}NO_3$	
15	20.14	Alanine, N-carboxy-3-[(4-O-.alpha.-d-glucopyranosyl-.beta.-d-glucopyranosyl)oxy]-, N-benzyl methyl ester, heptaacetate (ester)	Alanine	871.5778	871.7663	61.0410, 149.2402, 167.2311, 413.5153, 454.5676, 871.8807	$C_{38}H_{49}NO_{22}$	
16	21.13	Di-tert-butyl 1-(5-fluoro-2-oxo-3-phenylindolin-3-yl)hydrazine-1,2-dicarboxylate	Dicarboxylate	457.1990	457.2012	131.7494, 152.2387, 226.2040, 441.5707, 457.5044	$C_{24}H_{28}FN_3O_5$	
17	22.29	N-(1-diethoxyphosphorylethyl)-2,2,2-trifluoro-acetamide	Acetamide derivative	277.0790	277.0690	60.2290, 114.0117, 155.2372, 160.1721, 183.1609, 277.1835	$C_8H_{15}F_3NO_4P$	

Bacillus sp. TR4, *Kytococcus* sp. TR5, *Rhodococcus* sp. TR6, *Staphylococcus* sp. TR7, *Bacillus* sp. TR9, *Halomonas* sp. TR10, *Rossellomorea* sp. TR11, *Bacillus* sp. TR12, *Bacillus* sp. TR13) were able to inhibit the growth of *S. aureus* and only 5 isolates (*Bacillus* sp. TR4, *Staphylococcus* sp. TR7, *Bacillus* sp. TR13, *Halomononas* sp. TR14, *Halomononas* sp. TR15) showed positive results for *E. coli* (Table 1).

The non-ribosomal peptide synthetase (NRPS) gene was amplified using primers, A2gamF and A3gamR, which had a specific DNA length of the PKS and NRPS sequences of 279 base pairs (Piel 2002). The results showed that the NRPS gene was detected in two isolates, namely *B. stratosphericus* TR4 and *B. thuringiensis* TR13 (Figure 2).

BIOACTIVE COMPOUNDS FROM MARINE BACTERIA

Based on the results of the LC-MS/MS analysis in Table 2, *B. stratosphericus* TR4 and *B. thuringiensis* TR13 have 14 and 17 compounds, respectively.

Many bioactive compounds were identified from marine bacteria, such as antibacterial, antiviral, antifungal, anti-quorum sensing, and anticancer substances (Carroll et al. 2021; Saurav et al. 2017; Valliappan, Sun & Li 2014). Therefore, exploring the marine environment for antimicrobial agents plays a significant role in drug development and biomedical research. In this study, 2 isolates namely *B. stratosphericus* TR4 and *B. thuringiensis* TR13 showed positive results for inhibiting the growth of the pathogens tested. It has been reported before that *Bacillus* sp. found from marine environment produce antimicrobial peptide and antibiotic (Abriouel et al. 2011; Stein 2005). Strains belonging to the *B. thuringiensis* group have been known to produce most of the secondary metabolites, including antimicrobial compounds, especially bacteriocins (Salazar-Marroquin et al. 2016). Meanwhile, *B. stratosphericus* is known as a marine bacterial strain that is capable of producing antibiotic compounds. Recent research by Wang et al. (2020) demonstrated that these bacteria can produce micrococcin P3, a novel thiopeptide antibiotic capable of targeting Gram-positive bacteria. In *Bacillus* sp., NRPS is involved in primary metabolite biosynthesis (Ayuso-sacido & Genilloud 2005). Polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS) are two examples of complex enzymatic machinery that have been shown to be responsible for the synthesis of marine bacterial bioactive secondary metabolites.

The synthesis of secondary metabolites by marine bacteria presents promising opportunities for the discovery and development of innovative natural chemicals. Moreover, marine bacteria are emerging as a promising reservoir for the discovery and advancement of innovative medicinal medicines. Marine microorganisms, when subjected to comprehensive screening and thorough examination, have the potential to supply us with antimicrobial agents essential for treating drug-resistant infections during the course of the next century (Srinivasan et al. 2021). Research has shown that marine bacteria are responsible for the production of around 23,000 bioactive secondary metabolites. The biological activities of secondary metabolites produced by marine bacteria, among many marine microorganisms, include a wide range of functions, including possible antibacterial properties (Stincone & Brandelli 2020). Compounds from the group of imidazole derivatives, ester quinolone derivatives, steroid derivatives, and acetamide derivatives were found in the extracts of both strains. Aromatic heterocycles, particularly the imidazole ring, have been employed as a structural framework in the past few years to synthesize a variety of bioactive chemicals with anticancer, antiviral, antifungal, antibacterial, antidiabetic, and other activities (Chen et al. 2018; Duan et al. 2013; Li et al. 2013; Rani, Sharma & Singh 2013). Imidazole derivatives have also been isolated from marine bacteria such as *Bacillus* sp. (Yan et al. 2022) and *Pelomonas puraquae* (He et al. 2014). In addition, compounds from steroid derivatives have been reported to have antibacterial abilities against various pathogens such as *S. aureus* (Vollaro et al. 2020). Research by Chi et al. (2021) showed that steroid compounds isolated from marine fungi have antibacterial activity which can inhibit the growth of pathogenic bacteria *Vibrio anguillarum*, *Edwardsiella tarda*, and *Micrococcus luteus*. In future, we will purify the bioactive compounds to obtain a more comprehensive report on the biological potency of each compound.

CONCLUSION

Our findings showed that 15 bacterial isolates from the phylum Actinobacteria, Firmicutes, and Proteobacteria were successfully isolated, and 2 isolates had the potential to be antibacterial, namely *B. stratosphericus* TR4 and *B. thuringiensis* TR13. This was supported by the presence of NRPS genes and antibacterial bioactive compounds found in both isolates. Several compounds, such as imidazole and steroid derivatives, need to be purified and further screened for their antibacterial activity. Further comprehensive research on the potential and activity of bioactive compounds from bacteria is required for the manufacture of new drugs from the sea.

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