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Characterization of marine bacteria isolates GaN 2 and RaN 2 from Papuma Coastal, Jember

Syubbanul Wathon[1], Kartika Senjarini[1*], Rike Oktarianti[1], Fitrianti Milhatul Hasanah[1], Melinda Dwi Putri Oktavia[1], Endah Puspitasari[2], Antje Labes[3]

[1]Biology Department, Faculty of Mathematics and Natural Sciences, University of Jember,
Jl. Kalimantan 37 Kampus Tegalboto, Jember, East Java, 68121, Indonesia
[2]Faculty of Pharmacy, University of Jember
Jl. Kalimantan I No.2, Sumbersari, Jember, East Java, 68121, Indonesia
[3]Faculty of Energy and Biotechnology, Flensburg University of Applied Sciences,
Kanzleistraße 91–93, 24943 Flensburg, Germany
*corresponding author: senjarini@unej.ac.id

ABSTRACT

The exploration of secondary metabolites for various human needs has now extended to aquatic environments. The fluctuating and dynamic nature of aquatic environments can stimulate marine bacteria to adapt by producing secondary metabolites. The diversity of marine bacteria has become increasingly promising as a target for obtaining various potential secondary metabolites. This study aims to isolate potential marine bacteria that produce secondary metabolites from the Papuma Beach area in Jember. Marine bacterial characterization can be carried out through morphological and molecular approaches. In this study, bacterial isolates were collected from marine algae at aquatic sites. Bacteria were isolated from the algae using the spread plate and streak plate methods on nutrient agar dissolved in artificial seawater as the medium until single colonies were obtained. Morphological characterization of the single colonies was conducted by observing colony shape and color, as well as performing Gram staining. Molecular characterization of selected single colonies was performed by amplifying the 16S rDNA as a bacterial molecular marker. The results of this study showed that marine bacteria that grew well and remained stable on artificial media were successfully isolated from green and red algae samples, labeled GaN 2 and RaN 2, respectively. The GaN 2 bacterial isolate was identified as Fictibacillus barbaricus strain SQ5-4, while the RaN 2 bacterial isolate was identified as *Bacillus altitudinis* strain 41KF2bT.26. According to previous studies, *Fictibacillus barbaricus* is known to have potential as a bioremediation agent, while *Bacillus altitudinis* has potential applications as an antitumor, antimicrobial, and antioxidant-producing agent.

Keywords: Characterization, marine bacteria, morphology, secondary metabolite, 16S rDNA

INTRODUCTION

Coastal areas are part of the marine ecosystem and harbor many organisms (Lambert et al., 2019). The exploration of marine organism diversity is continually being carried out, with microbial diversity being one of the key focuses. Marine bacteria are a group of marine microorganisms believed to have abundant biodiversity. The fluctuating conditions of coastal ecosystems cause various types of marine bacteria to adapt by adjusting to environmental changes. Marine bacteria adapt these changing environmental conditions by producing metabolic compounds (Stincone & Brandelli, 2020).

Marine bacteria play a crucial role in maintaining the biogeochemical balance of marine ecosystems (Tapilatu, 2016). They exhibit several characteristics, such as the ability to thrive in environments with fluctuating temperatures, high salinity levels, and relatively high pressure (Stincone & Brandelli, 2020). These factors enable marine bacteria to develop complex metabolic capabilities, making them potential producers of unique bioactive compounds (Maldonado-Ruiz et al., 2024). Various metabolic compounds produced by marine bacteria have the potential to be utilized for human needs. The primary and secondary metabolites of marine bacteria are

being studied for their potential applications in the medical and pharmaceutical fields (Maldonado-Ruiz et al., 2024).

Marine bacteria can be isolated from various components of the aquatic habitat, such as seawater, sediment, and associations with other living organisms (Nweze et al., 2020). Several previous studies have explored the potential of marine bacteria to produce metabolite compounds with antimicrobial properties. Marine bacteria are known to produce antimicrobial compounds belonging to several phyla, including Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria, Planctomycetes, and Cyanobacteria (Stincone & Brandelli, 2020). Other groups of marine bacteria from the phylum of *Streptomyces*, *Pseudomonas*, *Pseudoalteromonas*, *Bacillus*, *Vibrio*, and *Cytophaga* produce various metabolite compounds with antibiotic properties (Srinivasan et al., 2021).

More specifically, the metabolite compounds produced by marine bacteria are utilized as drug compounds. Several new drugs that have been discovered from marine bacteria. Secondary metabolites produced by marine bacteria have led to the development of various products, such as anti-inflammatory, anticancer, and antibiotics. One example of a metabolite compound produced by marine bacteria is macrolactin-A. This compound can inhibit murine melanoma B16-F10 cancer cells, mammalian herpes simplex virus, and protect T lymphocyte cells from human immunodeficiency virus replication (Dongare et al., 2020). Marine bacteria have also been found to produce protease inhibitor compounds (Paul et al., 2021).

Considering the potential of marine bacteria as producers of various metabolite compounds important to the medical field, the exploration of marine bacteria agents isolated from Indonesia is essential. The exploration of new marine bacterial requires characterization to determine their potential (Kyule et al., 2022). Bacterial characterization is conducted based on morphological and molecular characteristics (Zhou et al., 2022). Morphological characterization is performed both macroscopically and microscopically, while molecular characterization utilizes 16S-rDNA as bacterial molecular markers (Church et al., 2020). Bacterial characterization is crucial to specify local isolates with that can produce essential metabolite compounds.

Papuma coastal area, located in Jember Regency, is a peninsula connected to the ocean and has the potential to serve as a new site for obtaining local marine bacterial isolates. This study focuses on the isolation of marine bacterial strains with the potential to produce secondary metabolites from Papuma Beach, Jember—a coastal area that remains largely underexplored. The aim is to uncover novel microbial candidates that may contribute to the discovery of bioactive compounds for pharmaceutical or industrial applications. This study will highlight the importance of exploring under investigated marine environments as reservoirs of potentially valuable microbial resources and support the continued bioprospecting of coastal ecosystems like Papuma Beach.

METHOD

Sample collection

Sample collection was conducted at Papuma coastal area in Jember, at the coordinates 8°25'60"S, 113°32'59.8"E (Figure 1). The collected samples included green algae and red algae.

Figure 1. Map of marine bacteria sampling locations

The sampling process began with the measurement of abiotic factors, including water salinity, pH, and temperature, as secondary data and as reference conditions for the growth medium. Green algae were collected using a sterile cotton swab by rubbing it on the surface of rocks submerged in seawater and placed into a sterile tube. Red algae samples were collected using tweezers and placed into a sterile tube.

Marine bacteria isolation

The isolation of marine bacteria began by taking 5 grams of green and red algae samples from Papuma Beach, which were then soaked in 10 mL of artificial seawater (containing 33 PSU artificial sea salt (Sigma Aldrich, USA) diluted into aquadest)). The suspension was subsequently diluted using a serial dilution method (10^1 to 10^5) to ensure that the isolated colonies are not too dense. 100 µl samples are cultured on nutrient agar (containing meat extract, peptone, agar and dissolved in artificial seawater as the medium (Sigma Aldrich, USA)), which has been adjusted to match the environmental conditions at the time of sampling. The spread plate technique is applied for culturing the bacteria. Incubation was carried out at a temperature of 25℃ and observed after two days. The grown colonies are observed and counted, followed by a purification process to obtain single colonies. Purification is carried out using the streak plate method based on morphotype differences. The purification process is repeated at least three times to obtain completely pure marine bacterial colonies.

Bacterial characterization

a. Morphological characterization

The morphology of single bacterial colonies is observed using two methods: macroscopic and microscopic analysis. Macroscopic identification involves observing the number of colonies, colony shape, color, edges, and elevation. Morphological characterization is conducted based on Bergey's Manual Book, while microscopic observation is performed using the Gram staining method to determine the Gram reaction and cell shape. The Gram staining procedure follows the method described by (Amankwah et al., 2022), with slight modifications.

b. Molecular characterization

Genomic DNA isolation was performed using the heat-shock method. A single colony from the agar medium was grown in liquid media and incubated on a shaker at room temperature for approximately 24 hours. Genomic DNA was then isolated using the Freeze and Thaw method, based on the heat shock principle, with modifications from (Wangka et al., 2020). The obtained DNA was visualized through electrophoresis at 100 V, 500 mA, for 40 minutes. The quality of the amplified DNA was assessed through electrophoresis at 100 V, 500 mA, for 45 minutes. Bacterial isolates with visible DNA bands were subsequently amplified using 16S rDNA-encoding DNA with primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3') (Fukuda et al., 2016). Table 1 shows the PCR mix composition in this study. Table 2 presents the PCR condition and temperature gradient used in this study.

Table 1. PCR mix composition

Components	Volume (µl)
PCR master mix	25
DNA template	4
Primer 27F	2,5
Primer 1492R	2,5
ddH_2O	16
Total volume	50

Table 2. Temperature gradient in PCR condition

PCR Condition	Tempe-rature	Time (minutes)	Cycle
Initial denaturation	94℃	5	1 x
Denaturation	94 ℃	1	33 x
Annealing	53 ℃	1	
Extension	72 ℃	1	
Final extension	72 ℃	10	1 x

The amplified DNA was subsequently purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega, USA). The purified

product was then sequenced. DNA sequencing was carried out by sending the purified PCR product to 1st Base. The raw sequencing data were then edited using the BioEdit. The edited consensus sequence was compared with previously documented 16S rDNA bacterial sequences in the GenBank database. The DNA sequences were further analyzed for their phylogenetic relationships through phylogenetic tree analysis and genetic distance analysis using the MEGA 11. The constructed phylogenetic tree is subsequently analyzed using the Kimura-2 parameter genetic distance method, with a bootstrap value of 1000 repetitions. The Neighbor-Joining (NJ) phylogenetic tree, based on Kimura's 2-parameter model, typically illustrates the topological relationships among taxa (Prasetya et al., 2011).

RESULTS AND DISCUSSION

The marine bacteria isolation began with the collection of several samples from the Papuma coastal area in Jember. The collected samples included green and red algae from the sampling location. Marine bacteria often form symbiotic relationships with various types of marine algae, as these organisms provide essential nutrients for bacterial growth (Nweze et al., 2020). The algae sample collection was accompanied by the measurement of abiotic factors at the sampling location. The measurements showed seawater salinity at 33 PSU, a temperature of 25.4 ℃, and a pH of 7.9. These abiotic factor measurements serve as reference parameters for bacterial growth media to support optimal bacterial growth. The nutrient content, pH, and temperature of the media significantly influence the success of bacterial cultivation in artificial laboratory conditions (Rodrigues & de Carvalho, 2022).

Bacterial isolation was performed using the spread plate method with several dilution series on NA media, using artificial seawater as the solvent, adjusted to match the salinity of the sampling location (33 PSU). This medium was used to provide an optimal environment for bacterial growth. In this study, the spread plate results from the 10^{-6} dilution series were selected, as bacterial colony growth in this series was not too dense, allowing for easy colony separation. Bacterial isolates from green algae on NA media were labeled as GaN (a), while those from red algae on NA media were labeled as RaN (b). The visualization of marine bacterial isolation from each algae sample is shown in Figure 2. Colonies with different morphologies were then purified to obtain single colonies. The colony purification was conducted using repeated quadrant streaking to ensure that the obtained bacterial colonies were pure single colonies.

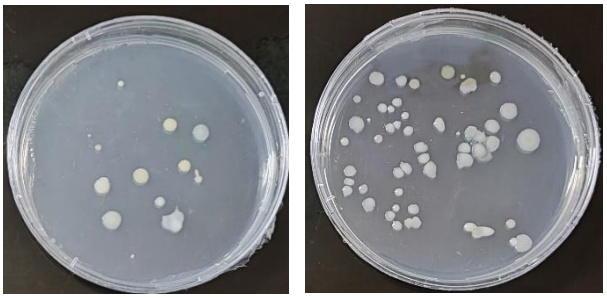


Figure 2. Marine bacteria isolated from samples: (a) green algae, (b) red alga.

The purification of the bacterial isolate showed a total of 8 bacterial colonies with distinct morphologie obtained from all samples. The purification of green algae samples resulted in 4 isolates, labeled GaN 1, GaN 2, GaN 3, and GaN 4. Similarly, the purification of red algae samples resulted in 4 isolates, labeled RaN 1, RaN 2, RaN 3, and RaN 4. Some bacterial isolates could not be successfully cultured in the laboratory and exhibited a decline in growth capability over time. Among the successfully cultured bacterial isolates, some displayed distinctive morphological characteristics, including the bacterial isolate from the green algae sample labeled GaN 2 (a) and the bacterial isolate from the red algae sample labeled RaN 2 (b), as shown in Figure 3. These bacteria isolates were selected due to their good and stable growth in laboratory conditions. The GaN 2 and

RaN 2 isolates also exhibited distinct morphologies compared to other colonies.

The selected single colonies from the green algae sample labeled GaN 2 and the red algae sample labeled RaN 2 were further examined for their colony morphology using a stereo microscope. The colonies were observed after 2 days of incubation. The morphological characteristics of the GaN 2 and RaN 2 bacterial isolates are presented in Table 3. The GaN 2 bacterial isolate has a round colony shape with irregular edges, a yellowish-white color, and a flat elevation. Meanwhile, the RaN 2 bacterial isolate has an irregular colony shape with entire edges, a white color, and a raised elevation. Both GaN 2 and RaN 2 bacteria belong to the Gram-positive rod-shaped bacterial group, confirmed by their purple staining when observed under a microscope. Gram staining is used for the characterization of bacteria because it provides a quick, reliable, and widely used method to classify bacteria into two major groups, which are Gram-positive and Gram-negative). This classification based is on the structural differences in bacterial cell walls. Gram-positive bacteria have thick peptidoglycan cell walls that retain a crystal violet stain, giving them a purple appearance. In contrast, Gram-negative bacteria have thinner peptidoglycan layers and retain only the final counterstain, safranin, which gives the cells a red appearance (Paray et al., 2023).

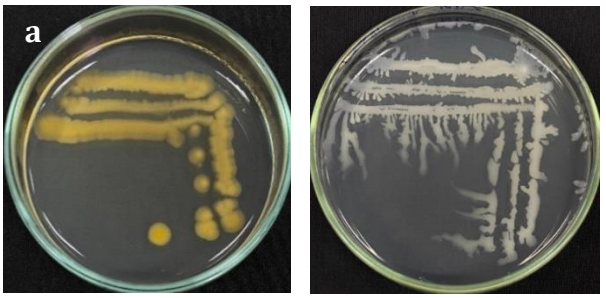
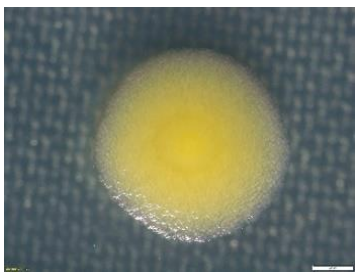
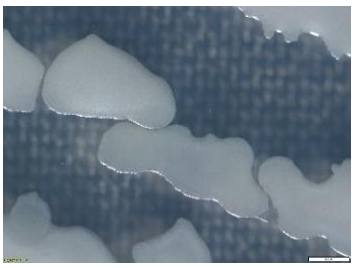
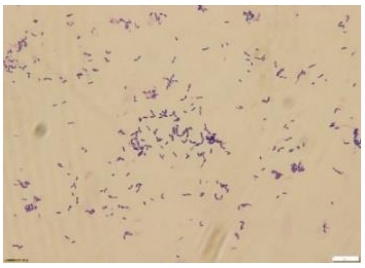
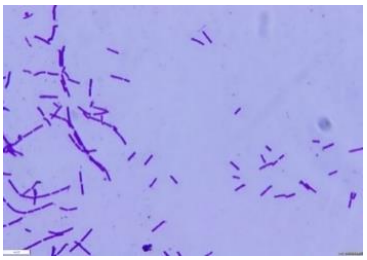
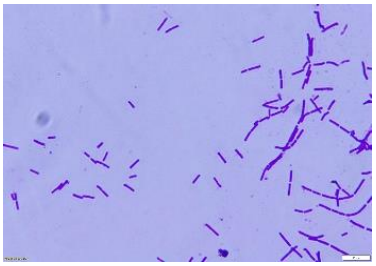
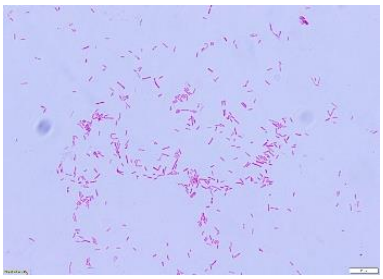


Figure 3. Colony morphology of marine bacterial isolates: (a) GaN 2 (a) and (b) RaN 2

Morphological characters are not sufficient to identify bacteria species. Many bacteria have similar shapes. Morphological traits provide information of genetic or biochemical differences between species. Environmental conditions can influence the expression of bacterial morphology. Molecular characterization is required for bacterial identification (Akihary & Kolondam, 2020).

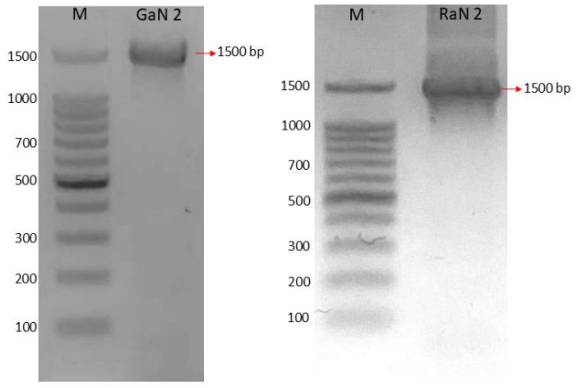
Table 3. Morphological characteristics of marine bacterial isolates GaN 2 and RaN 2

Isolate	Morphological Colony				
	Colony	Shape	Colour	Edge	Elevation
GaN 2	 16x	round	Whitish yellow	irregular	flat
RaN 2	 16x	irregular	white	entire	raised

Isolate	Microscopic Morphology		
	Gram Staining	Gram Type	Cell Shape
GaN 2	 1000x	Gram-positive	Basil
RaN 2	 1000x	Gram-positive	Basil
Bacillus cereus (K+)	 1000x	Gram-positive	Basil
Escherichia coli (K-)	 1000x	Gram-negative	Basil

The molecular characterization of bacterial isolates begins with DNA isolation. Once the DNA is successfully isolated, it is amplified. The PCR is conducted using 16S rDNA, which are widely used as marker genes in bacterial identification (Akihary & Kolondam, 2020). Figure 4 shows the visualization of the PCR product that the amplified DNA is approximately 1500 base pairs in length.

The GaN 2 and RaN 2 isolates have sequence lengths that match the expected length of the 16S rDNA. The 16S rDNA typically



(a) (b)

Figure 4. Visualization of the 16S-rDNA amplification from marine bacterial isolates, (a) GaN 2 and (b) RaN 2

has a length of approximately 1500 bp (Fukuda et al., 2016). Therefore, it can be concluded that the amplified product from the PCR analysis corresponds to the 16S rDNA of these marine bacteria.

The PCR product is then purified to remove any other components than the 16S rDNA sequence. Purified PCR product was subsequently sequenced. The chromatograms generated from the sequencing were then edited using the BioEdit. The result of this editing process was a consensus sequence. The consensus sequence length of the GaN 2 isolate, after editing, was approximately 1404 bp, while the RaN 2 isolate had a consensus sequence length of approximately 1416 bp. The obtained consensus sequences were then aligned with sequences in the GenBank database using the BLAST tool on NCBI. Regarding to the alignment analysis, the top five sequences in GenBank were selected based on max score, total score, query cover, e-value, and also percent identity. The sequence alignment analysis obtained through BLAST on NCBI database are presented in Table 4.

Table 4. Collection of marine bacteria sequences from NCBI database

Isolate	Scientific name	Max score	Total Score	Query Cover	E Value	Per. Ident	Accession Number	Country
GaN 2	*Fictibacillus barbaricus* strain SQ5-4	2591	2591	100%	0	100	MN235852.1	China
	Fictibacillus arsenicus strain FJAT-46930	2591	2591	100%	0	100	MG651541.1	China
	Fictibacillus phosphorivorans strain V6456	2591	2591	100%	0	100	PP962901.1	China
	Fictibacillus phosphorivorans strain V6190	2591	2591	100%	0	100	PP962858.1	China
	Fictibacillus phosphorivorans strain V6484	2586	2586	100%	0	100	PP257341.1	China
RaN 2	*Bacillus altitudinis* strain 41KF2bT.26	2615	2615	100%	0	100	MN543858.1	China
	Bacillus pumilus strain EB202	2615	2615	100%	0	100	MH142609.1	South Korea
	Bacillus altitudinis strain 1910ICU267	2615	2615	100%	0	100	MT225779.1	China
	Bacillus sp. (in: Bacteria) strain ATXP13	2615	2615	100%	0	100	MT035879.1	Egypt
	Bacillus aerius strain TS2-12A	2615	2615	100%	0	100	MN098858.1	Mexico

Key parameters to consider in the sequence alignment results using BLAST include max score, total score, query cover, e-value, and percent identity (Gaffar & Sumarlin, 2021). Query coverage represents the percentage of sequence length homology between the BLAST result and the sequences in the GenBank database. The E (Expect)-value is a statistical value that indicates the probability of the alignment occurring by chance between the query sequence and the database sequences in GenBank. Percent identity represents the level of similarity between the BLAST result sequence and the sequences in the GenBank database (Fitrian & Madduppa, 2020). The BLAST analysis for both bacterial isolates showed identical max and total scores, indicating that all nucleotide segments of the aligned sequences matched the nucleotide segments in GenBank. The e-value obtained from the BLAST analysis was 0. A lower e-value (closer to zero) indicates a higher degree of sequence similarity, and vice versa. An e-value of 0 confirms that the sequences of GaN 2 and RaN 2 are identical to sequences in GenBank (Amatullah et al., 2023).

The percent identity value for the GaN 2 bacterial isolate showed 100% sequence

homology with 100% query coverage to the genus *Fictibacillus*. The BLAST results indicated 100% similarity to the bacterial species *Fictibacillus barbaricus* strain SQ5-4 (MN235852.1), *Fictibacillus arsenicus* strain FJAT-46930 (MG651541.1), *Fictibacillus phosphorivorans* strain V6456 (PP962901.1), *Fictibacillus phosphorivorans* strain V6190 (PP962858.1), and *Fictibacillus phosphorivorans* strain V6484 (PP257341.1). The RaN 2 bacterial isolate showed a percent identity of 100% with 100% query coverage to the genus Bacillus. The BLAST results showed 100% similarity to *Bacillus altitudinis* strain 41KF2bT.26 (MN543858.1), *Bacillus altitudinis* strain 1910ICU267 (MH142609.1), *Bacillus altitudinis* strain 1910ICU267 (MT225779.1), *Bacillus* sp. (in: Bacteria) strain ATXP13 (MT035879.1), and *Bacillus aerius strain* TS2-12A (MN098858.1).

The homology values obtained indicate that the GaN 2 sequence shares a very high similarity with *Fictibacillus barbaricus, Fictibacillus arsenicus*, and *F. phosphorivorans*, while the RaN 2 sequence closely resembles *Bacillus altitudinis*, *Bacillus* sp., and *Bacillus aerius*. A percent identity value greater than 97% can be categorized within the same species. A percent identity value between 93% and 97% can be categorized within the same genus but different species (Amatullah et al., 2023). The obtained BLAST analysis was further used to reconstruct a phylogenetic tree of the marine bacteria.

Phylogenetic trees are essential tools in microbial taxonomy and evolutionary biology, providing a visual representation of the evolutionary relationships among organisms based on molecular sequence data. In microbiology, the 16S rRNA gene is widely used for phylogenetic analysis due to its presence in all prokaryotes, its functionally conserved regions, and its hypervariable regions that allow discrimination at the genus and species levels (Hassler et al., 2022). In the present study, a phylogenetic tree was constructed using 16S rRNA gene sequences from marine bacterial

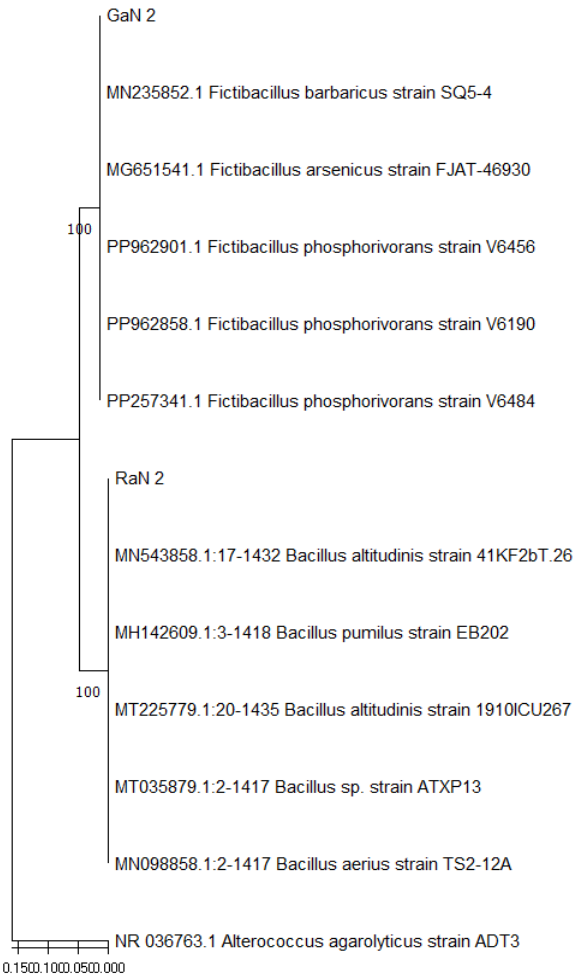


Figure 5. Phylogenetic tree of GaN 2 and RaN 2 isolates based on 16S rDNA sequences

isolates.. The tree topology reflects the degree of evolutionary relatedness among these organisms, with branch lengths indicating genetic distance and nodes representing hypothetical common ancestors. The closer the branching points (nodes) of two sequences, the more recent their common ancestor, and therefore the more closely related they are. The bootstrap values shown on the branches (e.g., 100) are statistical measures of confidence in the branching order, typically obtained by resampling the dataset multiple times (commonly 1,000 replicates). A bootstrap value of 100% suggests a very high confidence in the grouping of sequences at that node (Keklik, 2023).

The outgroup (in this case, *Alterococcus*

agarolyticus) serves as a reference point to root the tree. Rooting allows for the inference of the direction of evolutionary change, distinguishing ancestral from derived traits among the ingroup taxa. The clustering of sequences into distinct clades, such as the *Fictibacillus* and *Bacillus* groups observed here, indicates shared evolutionary histories and helps in the taxonomic identification and classification of unknown isolates. Such classification is critical not only for understanding microbial diversity but also for predicting physiological traits, ecological functions, and potential applications in biotechnology (Miller et al., 2018). Phylogenetic analysis thus complements phenotypic and biochemical methods, enabling a more robust and scientifically sound identification of environmental bacterial isolates.

The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replications by using the MEGA 11. The neighbor-joining method in phylogenetic analysis is based on genetic distance values, measuring the branch/node distances in the phylogenetic tree structure, which exhibit varying evolutionary rates (Djoemharsjah et al., 2023). The phylogenetic tree was constructed using *Alterococcus agarolyticus* as the outgroup. *Alterococcus agarolyticus* was chosen as the outgroup in this analysis because it belongs to a different taxonomic level than the genus of *Fictibaccius* and *Bacillus*. The bootstrap values in the phylogenetic tree indicate the level of accuracy. Bootstrap values approaching 100%, or at least above 70%, indicate a high level of accuracy (Murtafi'ah & Aeni, 2023).

The construction of phylogenetic tree is presented in Figure 5. Evolutionary relationships in the phylogenetic tree are depicted by the branch lengths, where shorter branch lengths indicate smaller evolutionary changes and thus a closer relationship (Murtafi'ah & Aeni, 2023). According to the phylogenetic tree, the GaN 2 isolate has a very close relationship with *Fictibacillus barbaricus* strain SQ5-4 (MN235852.1), while the RaN 2 isolate is closely related to *Bacillus altitudinis* strain 41KF2bT.26. This suggests that the GaN 2 isolate is molecularly identified as *Fictibacillus barbaricus* strain SQ5-4, and the RaN 2 isolate is identified as *Bacillus altitudinis* strain 41KF2bT.26.

Fictibacillus barbaricus was the first reported species of the genus *Fictibacillus*. Initially, this species was classified as *Bacillus barbaricus* in 2003. (Glaeser et al., 2013), as cited in (Li et al., 2024), reported that *B. barbaricus* was reclassified as *Fictibacillus barbaricus*. The genus *Fictibacillus* forms round colonies with a yellowish-white or cream color and a shiny appearance, with irregular elevation and margins. Bacterial cells of this genus are rod-shaped, often forming chains, and belong to the Gram-positive. The GaN 2 isolate exhibits morphology consistent with *Fictibacillus barbaricus*. This genus is also known to produce spherical or elliptical endospores.

According to Li et al. (2024), species of the genus *Fictibacillus* are primarily isolated from marine environments. Studies have reported that 12 strains of *Fictibacillus* isolated from marine sediments have the potential to produce biosurfactant compounds that play an important role in aquatic environments. Research by Pandey et al. (2021) revealed that one species, *Fictibacillus phosphorivorans*, has the potential to produce hydrocarbonoclastic and biosurfactant compounds, which function as bioremediation agents. Another study reported that species of the *Fictibacillus* genus isolated from marine sponges can produce antimicrobial compounds (Anteneh et al., 2021). Several studies have shown that these bacteria can produce biosurfactants—surface-active compounds produced by microorganisms such as bacteria. These compounds can degrade hydrocarbons (Couto et al., 2015) and effectively enhance the dispersion of hydrophobic compounds, making them useful for addressing oil-contaminated environments (Uzoigwe et al., 2015). According to Couto et al. (2015),

F. barbaricus is capable of producing biosurfactants, specifically bioemulsifiers, with an emulsification index of 63%.

Based on the phylogenetic tree, the RaN 2 isolate is identified as *Bacillus altitudinis* strain 41KF2bT.26. The genus *Bacillus* belongs to the phylum Firmicutes, characterized by Gram-positive bacteria capable of forming spores, having a rod shape, and being facultatively aerobic or anaerobic. The metabolic diversity and spore-forming ability of *Bacillus* species enable them to thrive in various natural environments, including diverse soil conditions, freshwater and marine environments, plants, and animals (Fu et al., 2021). One species in the genus *Bacillus*, *Bacillus altitudinis*, was first isolated from a terrestrial environment, specifically from air samples collected at an altitude of 41,000 meters in India (Liu et al., 2013). Evolutionary adaptation has allowed the distribution of *Bacillus altitudinis* to extend into aquatic environments, including both freshwater and marine ecosystems (Fu et al., 2021). Most *Bacillus altitudinis* strains found in marine environments have been isolated from seawater, and sediments, and also associated with several marine organisms (Fu et al., 2021). *Bacillus altitudinis* isolated from marine environments have been found to produce several bioactive compounds with potential roles as antitumor, antimicrobial, and antioxidant agents (Mohamed et al., 2018).

The similarity score can also be obtained from the genetic distance (pairwise alignment). This is because genetic distance and similarity are two sides of the same coin, representing complementary ways to measure the relationship between two sequences or organisms. Genetic distance is commonly used in phylogenetic analysis to construct evolutionary trees. Genetic distance quantifies the difference between two sequences, such as DNA, RNA, or protein sequences (Tamura et al., 2021). The similarity score can be derived from genetic distances when interpreting relationships. The similarity score measures the degree of similarity between two sequences, expressed as a percentage or score (Kumar et al., 2024). It is often calculated as the proportion of sites where the organism sequences differ. The more identical or conserved sites between two organism sequences, the higher of the similarity score.

The genetic distance analysis is conducted to determine the genus and species of the GaN 2 and RaN 2 isolates based on the 16S rDNA sequences. A bacterial isolate can be classified within the same genus if its similarity value is greater than 97%, and within the same species if its similarity value exceeds 99% (Akihary & Kolondam, 2020). Based on the similarity scores, the GaN 2 isolate exhibits a very high similarity of 100% with the species *Fictibacillus barbaricus*, *Fictibacillus arsenicus*, and *Fictibacillus phosphorivorans*. Similarly, the RaN 2 isolate shows 100% similarity with the species *Bacillus altitudinis*, *Bacillus pumilus*, *Bacillus* sp., and *Bacillus aerius.* Table 5 shows the genetic distance analysis of GaN2 and RaN2 bacterial isolates.

CONCLUSION

The GaN 2 bacterial isolate, obtained from green algae samples in this study, exhibited morphological characteristics of round colonies with a yellowish-white color, irregular edges, and flat elevation. The RaN 2 bacterial isolate, obtained from red algae samples in this study, exhibited morphological characteristics of irregular colonies with white color, entire edges, and raised elevation. Both bacteria belong to the group of Gram-positive, rod-shaped bacteria. Molecular analysis based on the 16S-rRNA sequence identified the GaN 2 bacterial isolate as *Fictibacillus barbaricus* strain SQ5-4 (MN235852.1), while the RaN 2 isolate was identified as *Bacillus altitudinis* strain 41KF2bT.26 (MN543858.1), with 100% percent identity, 100% query coverage, and 0 E-value score.

Table 5. 16S rDNA sequence similarity of GaN 2 and RaN 2 isolates

No.	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	GaN 2																		
2	MN235852.1 *Fictibacillus barbaricus strain* SQ5-4	100%																	
3	MG651541.1 *Fictibacillus arsenicus strain* FJAT-46930	100%	100%																
4	PP962901.1 *Fictibacillus phosphorivorans strain* V6456	100%	100%	100%															
5	PP962858.1 *Fictibacillus phosphorivorans strain* V6190	100%	100%	100%	100%														
6	PP257341.1 *Fictibacillus phosphorivorans strain* V6484	100%	100%	100%	100%	100%													
7	RaN 2	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%												
8	MN543858.1:17-1432 *Bacillus altitudinis strain* 41KF2bT.26	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%											
9	MH142609.1:3-1418 *Bacillus pumilus strain* EB202	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%	100%										
10	MT225779.1:20-1435 *Bacillus altitudinis strain* 1910ICU267	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%	100%	100%									
11	MT035879.1:2-1417 *Bacillus* sp. *strain* ATXP13	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%	100%	100%	100%								
12	MN098858.1:2-*1417 Bacillus aerius strain* TS2-12A	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%	100%	100%	100%	100%							
13	MN865920.1 *Bacillus altitudinis strain* JBRI-MO-2019-0008	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%	100%	100%	100%	100%	100%						
14	MN421109.1 *Bacillus altitudinis strain* SK2-22	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%	100%	100%	100%	100%	100%	100%					
15	MH801101.1 *Bacillus* sp. *strain* XIXJ283	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%	100%	100%	100%	100%	100%	100%	100%				
16	MK453427.1 *Bacillus* sp. *strain* Alg-AMLN-14-2	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%	100%	100%	100%	100%	100%	100%	100%	100%			
17	MN252912.1 *Bacillus aerius strain* MN-1	91.71%	91.71%	91.71%	91.71%	91.71%	91.71%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		
18	NR 036763.1 *Alterococcus agarolyticus strain* ADT3	69.35%	69.35%	69.35%	69.35%	69.35%	69.35%	67.88%	67.88%	67.88%	67.88%	67.88%	67.88%	67.88%	67.88%	67.88%	67.88%	67.88%	

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