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Research Article

Identification of a New Compound (4-Fluoro-2-Trifluoromethyl Imidazole) Extracted from a New Halophilic *Bacillus aquimaris* Strain Persiangulf TA2 Isolated from the Northern Persian Gulf with Broad-Spectrum Antimicrobial Effect

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Background: The unique ecosystem of the Persian Gulf has made it a rich source of natural antimicrobial compounds produced by various microorganisms, especially bacteria, which can be used in the treatment of infectious diseases, especially those of drug-resistant microbes.

Objectives: This study aimed to identify antimicrobial compounds in the bacteria isolated from the northern region of the Persian Gulf in Abadan (Chavibdeh port), Iran, for the first time.

Materials and Methods: Sampling was performed in the fall on November 15, 2019, from 10 different stations (water and sediment samples). The secondary metabolites of all isolates were extracted, and their antimicrobial effects were investigated. 16S ribosomal ribonucleic acid sequencing was used for the identification of the strains that showed the best inhibition against selected pathogens, and growth conditions were optimized for them. A fermentation medium in a volume of 5000 mL was prepared to produce the antimicrobial compound by the superior strain. The extracted antimicrobial compounds were identified using the gas chromatography-mass spectrometry technique. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for the superior strain. The effects of salinity, pH, and temperature on the production of antimicrobial compounds were determined by measuring the inhibitory region (mm) of methicillin-resistant *Staphylococcus aureus* (MRSA).

Results: Four new strains with antimicrobial properties (i.e., *Halomonas* sp. strain Persiangulf TA1, *Bacillus aquimaris* strain Persiangulf TA2, *Salinicoccus roseus* strain Persiangulf TA4, and *Exiguobacterium profundum* strain Persiangulf TA9) were identified. The optimum growth temperatures were determined at 37-30, 37, and 40 °C for TA1 and TA2, TA4, and TA9 strains, respectively. The optimum pH values for the four strains were 7, 6-7, 7.5, and 6.5-7.5, respectively. The optimal salt concentrations for the four strains were 15%, 2.5-5%, 7.5%, and 5%, respectively. The ethyl acetate extract of strain Persiangulf TA2 showed extensive antimicrobial activity against human pathogens (75%) and MRSA. The most abundant compound identified in TA2 extract was the new compound 4-fluoro-2-trifluoromethyl imidazole. The MBC and MIC for the ethyl acetate extract of strain TA2 were 20 and 5 mg. mL^{-1} (*Staphylococcus aureus*), 40 and 20 mg. mL^{-1} (MRSA*, Escherichia coli*, and *Enterococcus faecalis*), 40 and 10 mg. mL^{-1} *Acinetobacter baumannii*), and 80 and 40 mg. mL^{-1} (*Staphylococcus epidermidis, Shigella* sp.*, Bacillus cereus*, and *Klebsiella pneumoniae*), respectively. The optimal conditions for antibiotic production by TA2 strain were 5% salt concentration, pH of 7, and temperature of 35 °C.

Conclusion: Newly detected natural compounds in TA2 strain due to superior antimicrobial activity even against MRSA strain can be clinically valuable in pharmacy and treatment.

Keywords: Antimicrobial; *Bacillus aquimaris*; Imidazole; MRSA; Persian Gulf; GC-MS

1. Background

Currently, much research has been focused on discovering new, long-acting antibiotics that are effective in the prevention and treatment of diseases (1). The widespread use of chemical antibiotics has led to the spread of infectious diseases caused by multidrug-resistant pathogens, and the death rate from these pathogenic microbes is increasing. In recent years, much attention has been paid to bioactive compounds, especially of marine origin. Among aquatic organisms, antimicrobial compounds derived from bacteria have had an amazing effect in controlling microbial infections (1-5).

A more serious threat than methicillin-resistant *Staphylococcus aureus* (MRSA) is the spread of Gram-negative infectious agents that have become resistant to all available antimicrobial compounds (6). Aquatic ecosystems have a very different ecological structure and, as a result, have unique organisms with the potential to produce secondary metabolites, compared to terrestrial ecosystems. To date, numerous bioactive compounds with different functions from aquatic organisms have been reported, some of which are antimicrobial compounds. They have been widely used in pharmacy and medicine to treat infectious diseases caused by microbial pathogens (7-12).

The production of different bioactive compounds, such as antibiotic compounds by marine organisms, especially bacteria, is due to unbalanced and variable physical and chemical conditions of their habitat and the variety of food sources in marine environments for microorganisms. Therefore, in these harsh conditions, only microorganisms are able to survive that adapt to these conditions and use unique strategies for survival which is one of these methods of producing secondary metabolites (13-15). Due to the geographical location of the Persian Gulf, it usually has a high temperature and a relatively high salinity. Therefore, these special ecological conditions have caused high biodiversity in this region (16-18). Among marine microorganisms, *Bacillus* has been recognized as an effective biological control agent and producer of a variety of secondary metabolites with different biological applications, including antimicrobial applications. Additionally, these strains probably have a great potential for controlling human, animal, and plant pathogens (5).

2. Objectives

The Persian Gulf is a source of new undiscovered compounds due to its wide biodiversity. This study was performed in the northern region of the Persian Gulf in Abadan (Chavibdeh port), Iran, to identify and purify antimicrobial compounds in isolated bacteria. To date, no studies have been performed on this area research in this geographical location. With the identification of new antimicrobial compounds in the Persian Gulf, its position in terms of various medical, biological, and industrial sciences will be improved.

3. Materials and Methods

3.1. Sampling Site and Collection

Sampling was performed in the fall on November 15, 2019, from the northern part of the Persian Gulf in Khuzestan province within the port of Chavibdeh in Abadan from 10 different stations (water and sediment samples) under completely sterile conditions. Water pH, salinity, and temperature were also measured. The water samples were collected using sterilized-Niskin bottles; subsequently, Sterile 500 ml bottles were used to collect the water samples, and a Van Veen grab was used to collect sediment samples. The sediment samples were collected from each station in a sterilized plastic bag after each sampling. All the samples were transferred to the laboratory at 4 °C.

3.2. Isolation of Bacteria

The water samples (in the amount of 70 µL) and sediment samples (after dilution) were shredded over Marine agar 2216 (HiMedia, India) plates and incubated. The plates were then incubated at different temperatures (25, 30, and 37 °C) for 5 days, and the grown colonies were purified (19, 20).

3.3. Screening for Antimicrobial Compound-Producing Bacteria

Bacterial strains were inoculated in 250 mL Marine broth (MB; HiMedia, India) medium. The media were then placed in a shaker incubator for 7 days. Its temperature was set at 30 °C and at a speed of 160 rpm. For obtaining crude bacterial extract after incubation, the medium was centrifuged at 4 °C for 20 minutes (10,000 rpm). Ethyl acetate (KBR) of equal volume was used to extract the bacterial secondary metabolite from the supernatant, and the solvent was removed at 37 °C. Extraction was performed twice from each strain (21-23). The disk diffusion method was used to investigate the effect of the antimicrobial activity of bacterial extracts on the tested pathogen strains. Half McFarland suspension was prepared from pathogenic bacteria grown

in the Müeller-Hinton broth medium after 24 hours. The metabolite extracted from the bacterium was first dried and then dissolved in metatol (at a concentration of 100 mg. mL^{-1}). The extract was added to a sterile filter paper disc (diameter: 6 mm) in a volume of 35 µL. The dried discs were then placed on lawn cultures and incubated for 24 hours at 37 °C. The zone of inhibition around the paper discs was measured in millimeters. Various antibiotics (Difco) were used as controls. This experiment was repeated twice for each strain, and its antimicrobial properties were confirmed (23, 24).

3.4. Identification of Antibiotic-Producing Bacteria

The morphological and biochemical properties of marine bacterial strains with antibacterial activity were determined (25). In addition, the identification of extracellular hydrolases, such as alpha-amylase, protease, and lipase, in strains with antimicrobial properties was investigated (26-28). 16S ribosomal ribonucleic acid (16S rRNA) sequencing was used for the molecular identification of bacterial strains that showed the best inhibition against the selected pathogens. Bacterial deoxyribonucleic acid samples were amplified using 16S rRNA primers, mainly forward (5́-TCACGGAGTTT-GATCCTG-3́) and reverse (5́-GCGGCTGCACGTAGTT-3́) (29). The sequences of all types of strains used for the analysis were retrieved from the NCBI GenBank database and https://lpsn.dsmz.de/. The sequences were aligned, and phylogenetic trees were constructed using MEGA software (version 7.0) and reconstructed using maximum-likelihood methods. The phylogenetic tree of the bacterial strain indicated an evolutionary relationship with the selected sequence.

3.5. Detection and Identification of Bioactive Antimicrobial Metabolites in Marine Bacterial Extract

Gas chromatography-mass spectrometry (GC-MS) (Agilent 7890 Gas Chromatograph-5975 Mass Spectrometer detector) was used to identify compounds in the secondary metabolites. The gas chromatograph was equipped with a capillary column (30 × 0.25 µm ID × 0.25 µm df) and attached to the mass spectrometer section containing an Elite-5MS (5%-phenyl methylpolysiloxane) (30). The quadrupole mass analyzer and MSD ChemStation software were used to examine the chromatograms and the obtained mass spectra. The National Institute of Standards and Technology database and the Wiley Online Library were used to examine the mass spectrum of GC-MS results.

Table 1. Antimicrobial activity of four selected strains on pathogenic microbes by disk diffusion method (results repeated three times).

Average of inhibition zone (mm) ± standard deviation				
Strain	**No.1**	**No. 2**	**No. 3**	**No. 4**
Staphylococcus aureus PTCC1112	10 ± 0.66	19± 0.33	-	-
Enterococcus faecalis PTCC 29272	-	-	-	-
Staphylococcus epidermidis - Clinical (AJUMS*)	13.83 ± 0.55	19 ± 0.72	10.33 ± 0.22	15.08 ± 0.44
Enterococcus faecalis - Clinical (AJUMS)	-	11.5 ± 0.33	-	-
Bacillus cereus - Clinical (AJUMS)	-	18.71 ± 0.68	11.23 ± 0.27	-
Acinetobacter baumannii 12156	-	8.23 ± 0.17	-	-
Pseudomonas *aeruginosa* ATCC27853	-	-	-	-
Escherichia coli - Clinical (AJUMS)	-	11.9 ± 0.26	-	-
Shigella sp. - Clinical (AJUMS)	13.38 ± 0.37	19.36 ± 0.24	-	-
Klebsiella pneumonia - Clinical (AJUMS)	11.63 ± 0.75	12.13 ± 0.31	-	-
Candidia albicans PTCC 1167	-	-	-	-
MRSA* -Clinical (AJUMS)	-	16.25 ± 0.25	-	-

* AJUMS: Ahvaz Jundishapur University of Medical Sciences

* MRSA: Methicillin resistant *Staphylococcus aureus*

3.6. Provision of Fermentation Media for Selected Strain with Better Antimicrobial Activity

The selected strain was cultured in a volume of 5000 mL in MB medium under the above-mentioned conditions. After the incubation period, centrifugation was performed, and then the extract was extracted with ethyl acetate. The extracted metabolite was dissolved in a ratio of 160 mg. mL^{-1} and used for subsequent experiments.

3.7. Determination of MIC and MBC for Selected Strain

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracted metabolite with an antimicrobial effect were determined against some pathogenic microbes. The tubes containing the Müeller-Hinton broth medium, standard suspension of pathogenic microbes, and different concentrations of antimicrobial extract (5, 10, 20, 40, 80, and 160 mg. mL^{-1}) were incubated at 37 °C for 24 hours. The first tube without turbidity was considered MIC. For the determination of MBC, a loop of tubes without turbidity was cultured on the Mueller-Hinton agar and then incubated at 37 °C for 24 hours. The lowest concentration of antimicrobial metabolite that prevented the growth of pathogenic microbes in the environment was considered MBC (31). This test was repeated three times, and its mean was reported.

3.8. Optimization of Antimicrobial Metabolite Production

The optimization of the production medium conditions was performed to produce bioactive antimicrobial compounds for the selected strains (32). The bacterium was grown under different conditions, such as different temperatures (4, 20, 25, 30, 35, 37, 40, and 45 ˚C), pH values (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9) and NaCl concentration (1%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 20%, and 25% w/v), and then the efficiency of the optimized parameters for the strain with the highest antimicrobial activity was discovered using the disk diffusion method against MRSA.

4. Results

4.1. Isolation of Bacteria and Screening for Antimicrobial Compound-Producing Bacteria

In the present study, 23 bacterial species were isolated from the marine samples collected from the northern part of the Persian Gulf in Khuzestan province within the port of Chavibdeh in Abadan. The secondary metabolites of all samples were extracted using ethyl acetate, and their antimicrobial activity was investigated. Only four of them showed antimicrobial activity (17.39%). They differed in morphology and pigmentation. Strains no. 1 (yellow), strains no. 2 and 3 (pale orange-yellow), and strain no. 4 (orange) were reported with different pigments.

Table 1 and **Figure 1** show the antibiotic activity that differed from strain to strain. Of these four samples, one sample had broader and more antimicrobial activity (strain no. 2). The ethyl acetate extract of strain no. 2, out of 12 pathogenic microbes, had an antimicrobial effect on 9 cases (75.00%) and no effect only on *Enterococcus faecalis* PTCC29272, *Pseudomonas aeruginosa* ATCC

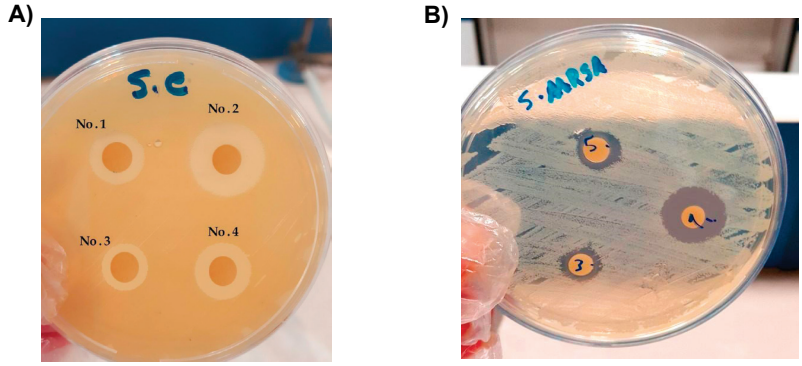

Figure 1. Investigation of antimicrobial activity of microbial extracts by disk diffusion method. A) Effect of ethyl acetate extracts (in 100 mg. mL^{-1}concentration) from four different strain on *Staphylococcus epidermidis* (Clinical). **B)** Effect of different concentrations (30, 50, and 100 mg. mL^{-1}) of ethyl acetate extract (strain no. 2) on methicillin-resistant *Staphylococcus aureus*.

Table 2. Morphological and enzymatic characterization of antibiotic-producing bacterium

Characteristic	No.1	No. 2	No. 3	No. 4
Gram staining	Negative	positive or variable	positive	Positive
Cell Shape	Rod or pleomorphic	Rod	cocci	Rod
Colony color	Yellow	pale orange	orange	Yellow-orange
Colony shape	Circular	Circular	flat	Circular
Motility	+	+	-	+
Specialized structures	Nonsporing	central – sporing	Nonsporing	Nonsporing
Growth on MacConky plates	Weak	-	-	-
Growth on Blood plates	+	+	+	+
Oxidase	+	-	+	-
Catalase	+	+	+	+
α-amylase	-	+	+	+
Protease	-	-	-	-
Lipase	-	-	-	-
Nitrate reduction	+	-	-	+

27853, and *Candida albicans* (25.00%). The only pathogenic bacterium affected by ethyl acetate extract in all four strains was *Staphylococcus epidermidis* (clinical) (**Fig. 1A**). Out of four strains, only strains no. 1, 2, and 3 had antimicrobial effects on *Staphylococcus aureus* PTCC1112; strains no. 2 and 3 had antimicrobial effects on *Bacillus cereus* (clinical); strains no. 1 and 2 had antimicrobial effects on *Klebsiella pneumoniae* (clinical); only strain no. 2 had an antimicrobial effect on MRSA, *Shigella* sp. (clinical), *E. coli* (clinical), *Acinetobacter baumannii* 12156, and *Enterococcus faecalis* (clinical) bacteria and neither had any effect on *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* PTCC 1167, and *Enterococcus faecalis* PTCC 29272. The extract of strain no. 2 showed significant activity against MRSA. So that with increasing the amount of the extract, the inhibition zone around the disc containing extract increased against the MRSA pathogen (**Fig. 1B**).

4.2. Identification of Antibiotic-Producing Bacteria

Table 2 and **Figure 2** show some morphological and enzymatic properties of the strains. For temperature optimization, four bacterial strains were cultured at different temperatures (4, 20, 25, 30, 35, 37, 40, and 45 °C). After incubation, the turbidity of the culture medium was investigated at 600 nm. All the strains were able to grow within the temperature range of 20-40 °C. None were able to grow at 4 °C, and only strains 2 and 4 grew at 45 °C (**Fig. 2A**). For pH optimization, the bacteria were cultured in medium with different pH values (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9). The optimum pH values for strains no. 1, 2, 3, and 4 were 7, 6-7, 6.5, and 6.5-7.5, respectively (**Fig. 2B**). This study investigated the growth rate of the bacteria in culture media with different salt concentrations (1%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 20%, and 25%). The optimum percentages of NaCl for strains no. 1, 2, 3, and 4 were 15%, 2.5-5%, 7.5%, and 5%, respectively (**Fig. 2C**). The bacteria were also tested for three types of alpha-amylase, lipase, and protease, and only strains no. 2 and 4 were able to produce amylase.

The bacterial strains whose metabolites had a stronger inhibitory effect against pathogenic microbes were identified by 16S rRNA sequencing. Strain no. 2 was identified as a novel *Bacillus* (*Rossellomorea) aquimaris* strain Persiangulf TA2 (accession no.: OK235636) and separated from the sediments (**Fig. 3**). Strains no. 1, 3, and 4 were identified as three novel strain*s Halomonas* sp. strain Persiangulf TA1 (OK275643), *Salinicoccus roseus* strain Persiangulf TA4 (OK287340), and *Exiguobacterium profundum* strain Persiangulf TA9 (OK287399), respectively. All three samples were isolated from water. The maximum-likelihood tree

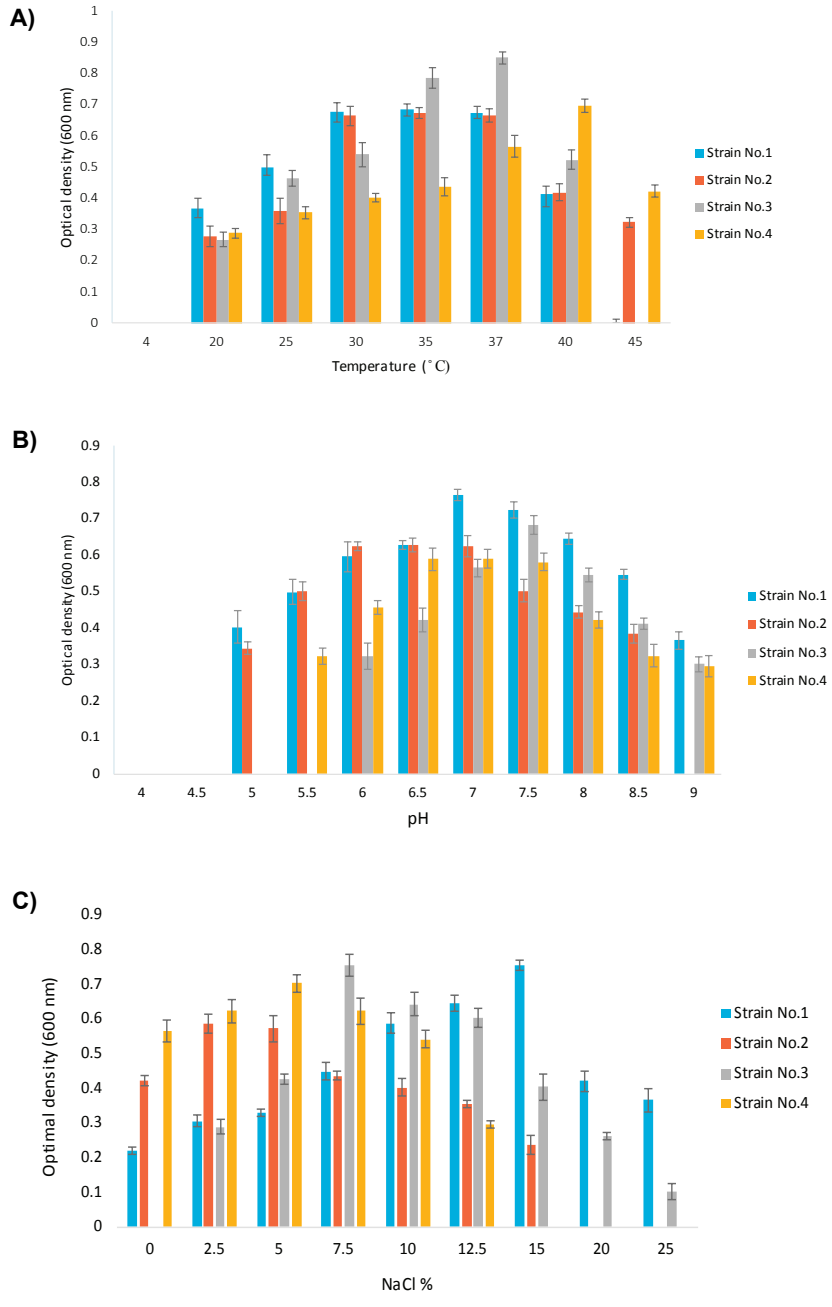

Figure 2. Optimization of growth conditions for antibiotic-producing strains. (A, B, C) Optimal growth conditions in terms of temperature, pH, and salt percentage after 48 hours of incubation by measuring turbidity at 600 nm.

showed the relationship of the TA2, TA1, TA4, and TA9 strains with the related strains of *Bacillus aquimaris* TF-12^T (98.60%), *Halomonas* sp. BH047 (99.92%), *Salinicoccus roseus* strain DSM 5351^T (99.02%), and *Exiguobacterium profundum* strain APBSWPTB105 (97.77%), respectively (**Table 3**).

4.3. Detection and Identification of Bioactive Antimicrobial Metabolites

The ethyl acetate extract of *Bacillus aquimaris* strain Persiangulf TA2 was subjected to GC-MS analysis. Seven peaks were observed in the graph obtained from the GC-MS of the desired extract, the sharpest peaks

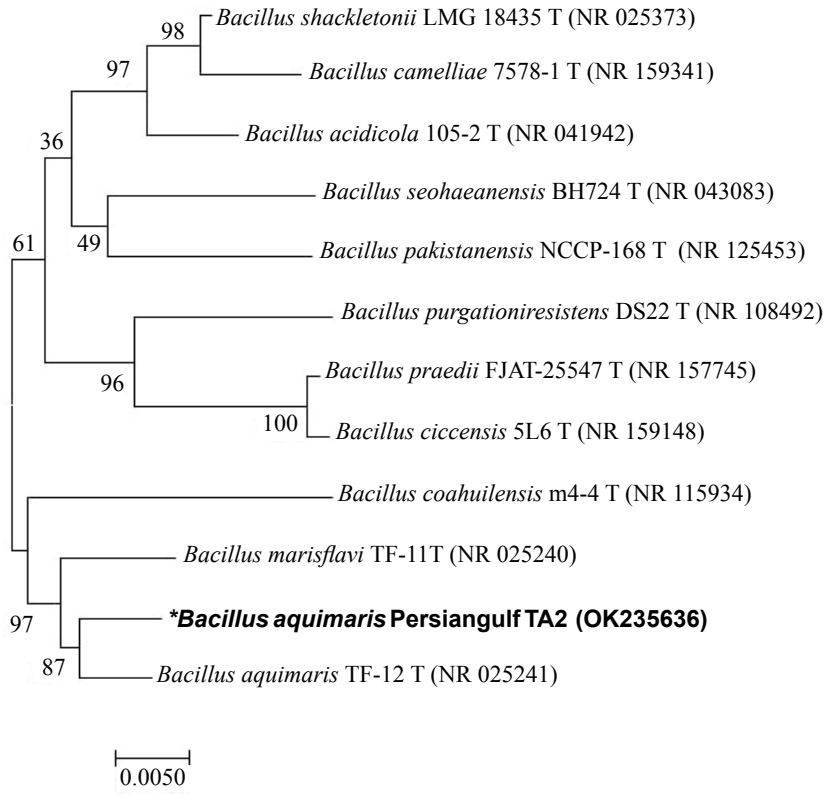

Figure 3. Phylogenetic tree Maximum- Likelihood (of *Bacillus* (*Rossellomorea*) *aquimaris* strain Persiangulf TA2; This tree illustrative of the relationship of strain, *Bacillus aquimaris* sp. starin Persiangulf TA2, with related type strain of *Bacillus aquimaris* TF-12^{T}.

Table 3. Molecular identification of isolates with antimicrobial effect on the closest strain in NCBI

Isolates	Closest strains	Identity (%)	New strain	Accession number*
No.1	*Halomonas* sp. strain BH047 (KM017558)	99.92 %	Persiangulf TA1	OK275643
No.2	*Bacillus aquimaris* TF-12^{T} (NR025241)	98.60 %	Persiangulf TA2	OK235636
No.3	*Salinicoccus roseus* strain DSM 5351^{T} (NR_026311)	99.02 %	Persiangulf TA4	OK287340
No.4	*Exiguobacterium profundum* strain APBSWPTB105 (MG733578)	97.77 %	Persiangulf TA9	OK287399

being observed at 13.322, 21.652, and 19.260 minutes, respectively (**Table 4**). The highest amount of compound was related to a new compound (imidazole,4-fluoro-2-trifluoromethyl). There were also no reports of the identification of compounds 2-hydrazino-4-methyl-6 methylthio-pyrimidine and 3,5-dihydroxybenzoic acid in prokaryotes.

4.4. Determination of MIC and MBC

A fermentation medium in a volume of 5000 mL was prepared for strain Persiangulf TA2, and the antimicrobial compound produced by this strain was extracted with ethyl acetate and dissolved in methanol at a ratio of 160 mg. mL^{-1} to determine the MIC and MBC of used sensitive pathogens. The MBC and MIC for the

Table 4. Compounds detected in antimicrobial extracts of *Bacillus aquimaris* strain Persiangulf TA2 by Gas Chromatography-Mass Spectrometry

Number	Time (min)	Area%	Composition name	Molecular formula	Molecular weight (g/mol)
1	11.149	6.23	Pyrrolo [1,2-a] pyrazine-1,4-dione hexahydro	$C_7H_{10}N_2O_2$	154.1665
2	11.876	7.08	Hexahydro-2H-pyrido(1,2-a)pyrazin- 3(4H)-one	$C_8H_{14}N_2O$	154.209
3	13.015	9.21	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210.2728
4	13.320	29.01	Imidazole 4-fluoro-2-trifluoromethyl 3,5-Dihydroxybenzoic acid	$C_4H_2F_4N_2$ $C_7H_6O_4$	154.066 154.12
5	16.219	4.36	2-Hydrazino-4-methyl-6-methylthiopyrimidine	$C_6H_{10}N_4S$	170.24
6	19.263	15.98	L-Prolyl-L-phenylalanine diketopiperazine (Maculosine 2)	$C_{14}H_{16}N_2O_2$	244.294
7	21.654	21.53	Bis (2-ethylhexyl) phthalate (DEHP)	$C_{24}H_{38}O_4$	390.564

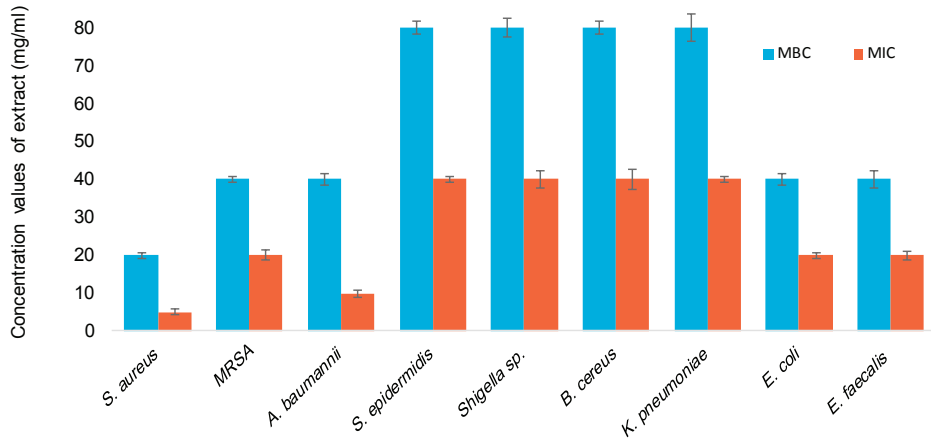

Figure 4. Determination of minimum bactericidal concentration (MIC) and minimum inhibitory concentration (MBC). MIC and MBC values (mg/ml) of extracts of *Bacillus aquimaris* strain Persiangulf TA2 against pathogenic microbes were determined.

ethyl acetate extract of strain Persian Gulf TA2 were 20 and 5 mg. mL^{-1} (*Staphylococcus aureus* PTCC1112), 40 and 20 mg. mL^{-1} (MRSA, *E. coli* [clinical], and *Enterococcus faecalis* [clinical]), 40 and 10 mg. mL^{-1} (*Acinetobacter baumannii* 1256), and 80 and 40 mg. mL^{-1} (*Staphylococcus epidermidis* [clinical], *Shigella* sp. [clinical], *Bacillus cereus* [clinical], and *Klebsiella pneumoniae* [clinical]), respectively (**Fig. 4**).

4.5. Optimization of Antimicrobial Metabolite Production

The strain Persiangulf TA2 was grown at different conditions, such as different temperatures, pH values, and NaCl concentrations. Then, the effect of secondary metabolites produced by strain TA2 in different conditions was investigated against MRSA by the disk diffusion method (**Fig. 5**). The maximum antibiotic

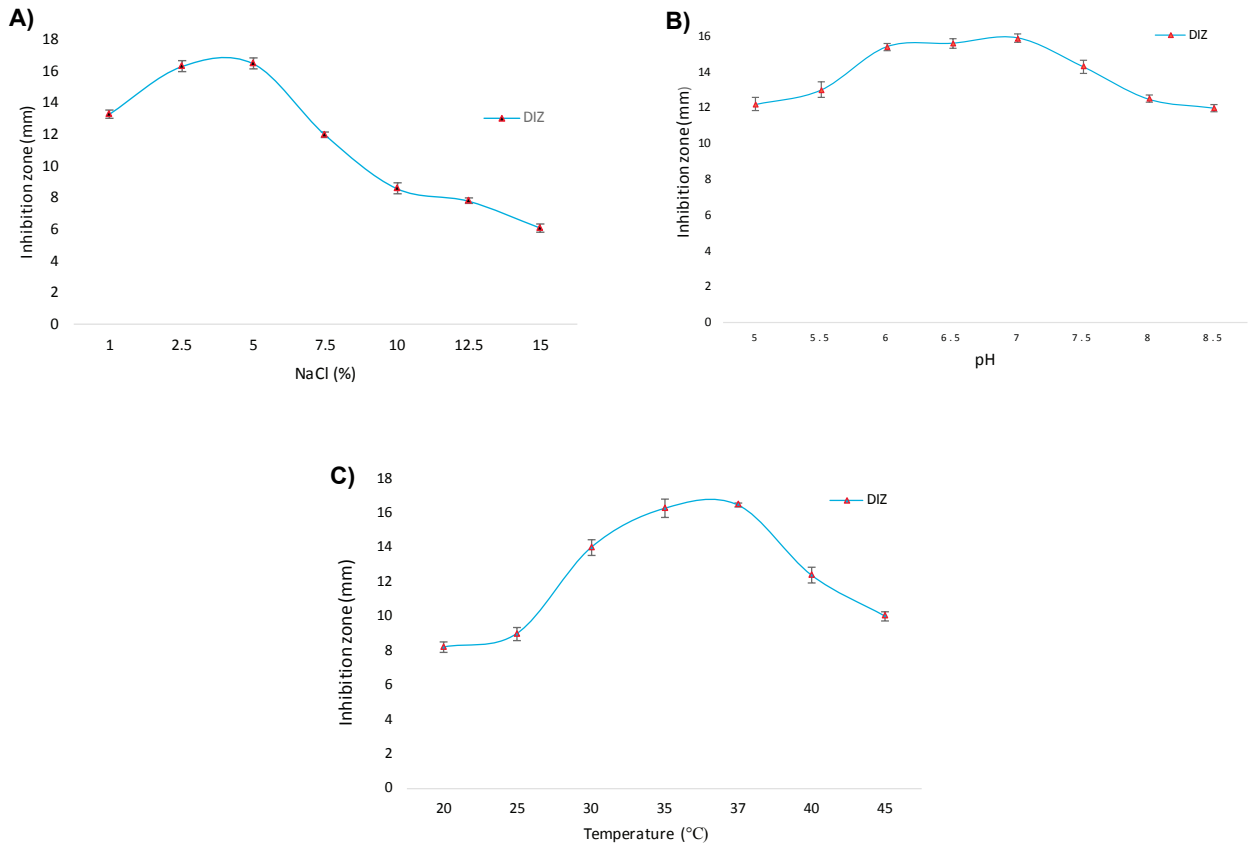

Figure 5. Investigating the effect of different conditions on the production of antimicrobial compounds. (A, B, C) The Effect of salinity, pH, and temperature on the production of antimicrobial compounds by *Bacillus aquimaris* strain Persiangulf TA2 were determined by measuring inhibition zone (mm) of Methicillin-resistant *Staphylococcus aureus* bacteria. DIZ: Diameter of Inhibition Zon.

activity was achieved at NaCl 2.5-5% (w/v) (**Fig. 5A**), initial pH of 6-7 (**Fig. 5B**), and temperature of 35-37 °C (**Fig. 5C**).

5. Discussion

Due to the fact that pathogenic microbes have used new strategies to neutralize the effects of old chemical antibiotics, different antibiotic-resistant strains have emerged that can increase the severity and duration of the disease and even increase human mortality in some cases. Therefore, the emergence of the aforementioned strains is a serious threat. The identification of new bioactive compounds, especially marine microorganisms, can be a good option to deal with this problem (33, 34).

In this study, different bacteria isolated from the water and sediments in the north of the Persian Gulf were screened to identify antimicrobial compounds. Four new marine species with significant antimicrobial effects included *Rossellomorea aquimaris* strain Persiangulf TA2, *Halomonas sp.* Strain Persiangulf TA1, *Salinicoccus roseus* stain Persiangulf TA4, and *Exiguobacterium profundum* strain Persiangulf TA9, respectively; however, among the aforementioned strains, only the Persiangulf TA2 strain had a significant inhibitory effect against both Gram-positive and Gram-negative pathogenic microbes. In most studies, newly identified antimicrobial compounds had minor inhibitory effects on Gram-negatives and often affected Gram-positives (35). Therefore, in the present study, the introduction of TA2 strain with an inhibitory effect on Gram-negative bacteria can be interesting and important.

To date, there have been no reports of the isolation

of *Bacillus aquimaris* with antimicrobial properties in Iran. There are reports of other species of *Bacillus* with antimicrobial properties. This inhibitory effect is related to the newly identified compound in the bacterial antimicrobial extract. The newly identified compound has not yet been reported as a natural antimicrobial metabolite, and only derivatives of this compound (artificially) have been used to treat diseases.

Various studies have shown that members of the genus *Bacillus*, such as *Bacillus aquimaris*, have the ability to produce different types of bioactive compounds with the property of inhibiting pathogenic pathogens. This inhibitory compound can act on different pathogens, or one genus of *Bacillus* can produce different compounds to inhibit various microbes (34). In research to identify *Bacillus* species with antimicrobial activity, *Bacillus equimaris* has been reported less frequently than other *Bacillus* species. It might be due to the needs and growth conditions of this bacterium and might have shown much less antimicrobial properties than other species and, as a result, has not been reported (35). Reported marine *Bacillus* species with the ability to inhibit pathogenic microbes usually include *Bacillus licheniformis*, *Bacillus cereus*, *B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, *B. firmus*, *B. Megaterium*, *Bacillus velezensis*, and *P. polymyxa* (36-39). The methanolic extract of marine *Bacillus aquimaris* has shown significant inhibitory activity against *E. coli*, *Vibrio cholerae*, and *Aeromonas hydrophila* (40). *Bacillus pumilus* extract has shown strong inhibitory effects against pathogenic bacteria, including *Staphylococcus aureus*, *Micrococcus luteus*, *Variant Salmonella gallinarum*, *Pasteurella multocida*, *Swine Salmonella*, *Salmonella enterica*, *Swine E. coli*, and *Chicken E. coli* (41). Marine *Bacillus cereus* SN7 has been shown to have a significant inhibitory effect on aquatic pathogens, such as *Aeromonas*, *Pseudomonas*, and *Vibrio* (37). *Bacillus velezensis* HC6 and a new *Bacillus* strain 2011SOCCUF3 have shown good antimicrobial activity against pathogenic microbes. Additionally, various strains of marine *Bacillus* have shown good inhibitory activity against numerous clinical pathogens and even those that are resistant to several antibiotics (35, 36, 42). The extracts of *Bacillus* strains isolated from the Caspian Sea had a minor inhibitory effect on Gram-negatives but have shown significant inhibitory properties on Gram-positive pathogens (43).

Drug-resistant microorganisms, including methicillin-resistant *Staphylococcus aureus*, have caused serious crises in hospitals and health centers around the world. Few chemical drugs are available to control drug-resistant pathogens, including MRSA. So that most of these drugs can have serious side effects for a person in addition to high costs. For this reason, natural secondary metabolites can be a good alternative for controlling these pathogens (44). In this study, the extract of *Bacillus aquimaris* showed significant activity against MRSA. So that with increasing the amount of the extract, the inhibition zone around the disc containing extract increased against the MRSA pathogen. Therefore, pathogens can be more likely to be inhibited by increasing the amount of antimicrobial compounds until they do not become resistant. Extracts of marine *Streptomyces* (44), *Pseudoalteromonas* (45) and *Bacillus velezensis* (46) strains have also shown significant antimicrobial activity against MRSA. Compounds extracted from a collection of aquatic bacteria associated with marine sponges isolated in Portugal showed weak antimicrobial activity against MRSA strains and their main activity was against *Bacillus subtilis* (49).

In the present study, the growth conditions of the strains and the optimal production of antimicrobial compounds were in almost moderate conditions in terms of temperature, pH, and salinity. The pigments extracted from *Salinicoccus sesuvii* MB597 and *Halomonas aquamarina* MB598 have shown a good inhibitory effect on a large number of pathogenic microbes. Furthermore, the optimal conditions for these two strains in normal and intermediate ranges have been reported (48). The inhibitory activity of marine *Salinicoccus* sp. extract on Gram-positive bacteria, including *Staphylococcus aureus*, has been interesting and has had a minor inhibitory effect on Gram-negative bacteria, such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (49). In the current study, *Salinicoccus roseus* strain Persiangulf TA4 extract had an effect only on *Staphylococcus epidermidis* and *Bacillus cereus*.

Halomonas are salt-loving microorganisms that are abundant as some microorganism rich in bioactive compounds in saline environments, such as seawater; however, there are few studies in this field. The extracts obtained from *Halomonas* sp. EA423 have shown a significant inhibitory effect on plant pathogenic fungi and human *E. coli* (50). The extract of *Halomonas*

salifodinae MPM-TC has shown a significant inhibitory effect on Gram-negative bacteria, such as *Aeromonas hydrophila*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Pseudomonas aeruginosa* (51). In the current study, *Halomonas* sp. Strain Persiangulf TA1 extract had an effect on *Staphylococcus aureus* PTCC1112, *Staphylococcus epidermidis* (clinical), *Shigella* sp. (clinical), and *Klebsiella pneumoniae* (clinical). Moreover, *Exiguobacterium profundum* strain Persiangulf TA9 extract had an effect only on *Staphylococcus epidermidis* (clinical).

Different studies have been performed in different geographical regions, and the ecosystem conditions of each region are different from each other; even the studies are very much affected by the experimental conditions in the laboratory. With regard to all the aforementioned issues, the difference in the results can be explained (35). The *Bacillus aquimaris* strain Persiangulf TA2, *Salinicoccus roseus* strain Persiangulf TA4 and *Exiguobacterium profundum* strain Persiangulf TA9 also had amylolytic activity. Given the importance of microbial amylase in industry, these strains could be candidates for further research in this field. Since marine ecosystems do not have uniform and stable conditions, the enzymes isolated from marine microorganisms should be highly adaptable to unbalanced conditions and can be a good option in different industries (52).

Marine *Bacillus* species can produce a variety of secondary metabolites, such as antimicrobial, antifungal, and anticancer compounds (5). The results of the present GC-MS analysis showed that Persiangulf TA2 strain ethyl acetate extract contains several important chemical compounds. Among the aforementioned compounds, the highest amount was related to imidazole,4-fluoro-2-(trifluoromethyl) composition, which has not been reported to date from the separation of this compound from prokaryotes. Furthermore, the derivatives related to the aforementioned compound have been made artificially, and its antimicrobial effect has been investigated. There are also no reports of the identification of compounds 2-hydrazino-4-methyl-6-methylthio-pyrimidine and 3,5-dihydroxybenzoic acid in prokaryotes. Imidazole is a heterocyclic compound with synthetic derivatives that have various therapeutic applications biologically and are used against bacteria, fungi, viruses, and tumor cells (53).

Some compounds have been previously reported in microbial extracts, including $C_{14}H_{16}N_2O_2$ with anti-microbial and antioxidant properties (54-56) and compounds of $C_{11}H_{18}N_2O_2$ and $C_7H_{10}N_2O_2$ with antioxidant properties (56-58). In addition, $C_{11}H_{18}N_2O_2$ composition from marine *Nocardiopsis* sp. DMS 2 has shown inhibitory activity against biofilm-forming *K. pneumoniae* (59). The secondary metabolites extracted from *Vagococcus fluvialis* and *Bacillus cereus* included compounds of alkaloids, flavonoids, and saponins (37). The identification of new compounds in this strain (Persiangulf TA2) can be a good candidate for further research in the field of pharmacy and treatment.

6. Conclusion

The large parts of the Persian Gulf have a pristine and intact ecosystem; therefore, it is likely to have a very wide diversity of organisms. *Bacillus aquimaris* strain Persiangulf TA2 showed an interesting inhibitory effect against Gram-negative and Gram-positive pathogens. The new compound identified in this bacterium could have an important application in the inhibition of pathogenic bacteria, including important antibiotic-resistant pathogens. Furthermore, the mass production of antibiotics derived from aquatic microorganisms can be important due to their high adaptability to unbalanced sea conditions.

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Not applicable

Data availability

Sequence files for all samples in this study have been deposited at NCBI with accession numbers: OK275643, OK235636, OK287340 and OK287399.

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Conflict of interest

Sara Taghavi, Effat Abbasi Montazeri, Roya Zekavati, laleh Roomiani and Parvaneh Saffarian declare that they have no conflict of interest.

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