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Research Article

Isolation and Antimicrobial Activity of Halophilic Bacteria Isolated from Saline Soil of Shushtar City, Iran

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Abstract

Background: The rise of multi-drug-resistant bacteria seriously threatens human health. Some microorganisms can produce new antimicrobials that have effects on multidrug-resistant bacteria. On the other hand, halophilic bacteria show promise in producing novel bioactive antimicrobial compounds that could benefit drug development. This study aims to investigate the antimicrobial properties of halophilic bacteria recently isolated in soil samples from Shushtar City, Khuzestan Province, Iran.

Methods: In this research, saline soil samples were collected from the salty areas around Shushtar City. The soil sample was then cultured in an enriched culture medium, and in order to isolate the halophilic bacteria, they were cultured in a solid medium. The microorganisms were examined for the production of antimicrobial agents using the agar well diffusion method. Subsequently, the halophilic bacteria were identified through molecular analysis of the 16S rRNA method. The phylogenetic tree was constructed using Mega software through the neighbor-joining method.

Results: Twenty-two strains were isolated in this study. Strain E1, identified as *Alkalihalobacillus* sp, displayed antimicrobial activity against *Enterococcus faecalis*. The MIC and MBC of the *Alkalihalobacillus* extracts against *Enterococcus faecalis* were determined to be 25 µg/mL.

Conclusion: This research highlights the potential therapeutic and preventive advantages of *Alkalihalobacillus* sp. extracts as antibacterial agents. This research report, for the first time, reveals that isolated *Alkalihalobacillus* in Iran has the ability to produce antimicrobial agents. The discovery and isolation of beneficial bacteria from natural sources could have significant implications for future pharmaceutical and industrial applications.

Introduction

Microorganisms can survive in extreme conditions such as salinity, pH, temperature, pressure, light intensity, oxygen levels, and nutrient availability. Hypersaline environments pose significant challenges due to high salt concentrations, fluctuating temperatures, low oxygen levels, and occasionally high pH values. Bacteria and Archaea are the primary organisms that thrive in these harsh environments.[1] Halophilic microorganisms are classified into three groups based on their salt tolerance levels. Slight halophiles, or marine bacteria, are capable of surviving in environments with 1% to 3% NaCl, while moderate halophiles thrive in conditions with 3% to 15% NaCl. On the other hand, extreme halophiles exhibit optimal growth in environments containing 15% to 30% NaCl.[2] Recent research has uncovered the significant capabilities of halophilic microorganisms in various important applications, such as antimicrobials, enzymes, and sources of polymers.[3,4] In the modern era, the rise of multi-antibiotic-resistant pathogens poses a significant health threat within the medical community. Therefore, it is crucial to investigate new sources of antimicrobial compounds to develop effective treatments against these resistant pathogens.[5,6] Halophilic bacteria have been recently identified as a unique source of antimicrobial substances. A wide range of antimicrobial and antitumoral

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agents has been found in moderate and highly halophilic Prokaryotes, including Halocins, a protein-derived antimicrobial substance.[7] The discovery of new antibiotics often originates from the natural products of bacteria.[8] However, there is a shortage of new antibiotic discoveries at the industrial level, which highlights the need to explore new methods for their discovery.[9] Many different microorganisms, including archaea, bacteria, and fungi, have been found to produce a wide variety of antimicrobial substances.[10] This means that discovering and studying new bacteria could be a promising way to find previously unknown sources of natural antibiotics, which could help support our pharmaceutical industry by providing new treatments.[11]

The antimicrobial properties of halophilic microbes from various saline environments, as opposed to numerous pathogens, have increased interest in the pharmaceutical industry for biomolecule applications. Furthermore, many potential bioactivities of halobacteria, halofungi, haloarchaea, and halo-diatoms remain unexplored. More attention should be given to halo-microbial communities as a reliable source of novel drugs against drug-resistant bacteria.[12] This study focuses on the antimicrobial capabilities of halophilic bacteria isolated and identified for the first time from soil samples collected in Shushtar City, Khuzestan Province, Iran.

Methods

Sample collection and growth conditions

All soil samples were collected from Shushtar District, Khuzestan Province, Iran. Shoushtar is located at latitude 32°02–42–48°51–34–E. A shovel was used to collect random soil samples from the undisturbed soil surface (soil height 10 cm) to preserve the soil structure. Samples from eight different locations, each weighing 100 g, were transferred to the laboratory in sterile containers. The soil samples were then incubated with saline nutrient broth containing the following concentrations (g/L): NaCl 81, $MgSO_4.7H_2O$ 9.7, $MgCl_2$.H2O 7.0, $CaCl_2$ 3.6, KCl 2.0, $NaHCO_3$ 0.06, and NaBr 0.026. The pH was adjusted to 7.3 before autoclaving. The cultures were grown in shaker incubator at 25°C, 150 rpm for 72 h. To prepare solid media, 12-15 gL^{-1} agar was added to the new saline nutrient broth, then it was incubated at 25°C for 48 hours. The streak plate method was used to purify single colonies of halophilic bacteria. Enrichment culture was cultured several times under the same conditions with varying NaCl concentrations (0%, 5%, 10%, 15%, 20%, 25%).[13]

Antimicrobial assay

The agar well diffusion method was employed to assess the antimicrobial properties of the isolates. Humans' pathogens were selected for this study. The human pathogenic organisms studied were Bacillus cereus (ATCC 11778), *Escherichia coli* O157 (PTCC 1276), *Klebsiella pneumoniae* (PTCC 10031), *Shigella flexneri* (PTCC 1234), *Pseudomonas aeroginosa* (ATCC 10231), *Streptococcus mutans* (ATCC 35668), and *Candida albicans* (ATCC 10231). Following the method described by Ennahar *et al*.,[14] the bacterial culture supernatant was filtered through a 0.22 µm membrane filter after centrifugation at 5000 rpm for 10 min. The pathogenic microorganisms (10^7 CFU/mL) were then inoculated into a sterile plate containing 20 mL of their selective media. The plate was gently shaken to evenly spread and mixed the microorganisms and media. After solidification, five wells with a diameter of approximately 6 mm were prepared on the agar surface using a sterile cylinder. The plates were then inverted and the wells were labeled. Each well was filled with 0.1 mL of the bacterial extracts. The plates were incubated at 37°C for 24 hours and the inhibition zone was measured. Finally, the results were measured.

Molecular Identification and characterization of isolated species

The genomic DNA extraction was performed using the Cinagene DNA Plus extraction kit from South Korea, following the instructions provided by the manufacturer. For the amplification of the 16S rRNA gene in Gram-negative bacteria, the forward primer 16F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 16R (5'-ACGGCTACCTTGTTACGACTT-3') were used. The amplification reaction mixture consisted of 1µL of each primer, 0.5 µM of dNTPs, 2.5 µL of PCR buffer, 0.75 µL of $MgCl_2$, 1 µL of template DNA, 0.2µL of smartaq DNA polymerase 0.2 µL, and 19.05µL of dH_2O, resulting in a final volume of 25 µL. The amplification protocol for the 16S rRNA gene involved an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes. A final extension step was performed at 72°C for 10 minutes. The PCR product was purified using the Vivantis gel PCR purification kit from Malaysia. The purified product was then sent to Macrogen in South Korea for sequencing.

The obtained sequencing data was compared to the 16S rRNA gene sequences in the GenBank database of the National Center for Biotechnology Information (NCBI) using a BLAST search to determine the isolated bacterial species. The retrieved sequences from NCBI blast search were utilized for drawing Phylogenetic tree. The phylogenetic tree was performed by Mega 11 software with Neighbor-joining method. To validate phylogenetic tree, 100 bootstarp was applied.[15]

Antimicrobial production

To begin, bacterial colonies were cultured in 50 mL of the liquid saline nutrient medium and incubated at a temperature of 30°C while being shaken for 72 hours. Next, the culture was centrifuged at a speed of 4000 rpm for 20 minutes to separate the extract from the medium. Following this, the supernatant was gathered and passed through a 0.22 µm membrane filter.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC of the extract against bacteria was determined using the micro broth dilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS). The MIC value represents the lowest concentration of the extract that completely prevented bacterial growth after 48 hours of incubation at 30°C. To determine the MIC value against the tested pathogens, various concentrations of the extract (ranging from 10^{-1} g/mL to 10^{-8} g/mL) were used. Each well was filled with 16 µl of suitable medium, followed by the addition of 20 µl of a 0.5 McFarland suspension of pathogenic bacteria (diluted 1:10). Subsequently, different concentrations of the extract (20 µl) were added to the same wells, and sterile broth was added to reach a total volume of 300 µl. The positive control wells contained a 0.5 McFarland suspension (OD: 600) of the pathogen and culture medium, while the negative control wells contained an antimicrobial extract and culture media. After cultivation, the microplates were thoroughly mixed and incubated for 24 hours at 30°C. The MIC value was determined by identifying the first well in which the visible growth of microorganisms was inhibited. To determine the minimum bactericidal concentration (MBC), 5 µl of liquid from each well that showed no growth was taken and incubated at 30°C for 24 hours. The lowest concentration that exhibited no visible bacterial growth after sub-culturing was considered the MBC.[13]

Statistical analysis

The statistical analysis involved using MEGA 11 software for designing of phylogenic tree.

Results

Biochemical properties of halophilic bacteria

In this study, 22 halophilic bacteria were isolated from the saline soil of Shushtar City which 10 strains could grow on medium containing NaCl 0-15% and others grow on medium containing NaCl 1-25%. Halophilic bacteria were identified by colony, pigment, and microscopic morphology (Figure 1). The strains were analyzed and their biochemical properties are summarized in Table 1. All the isolated bacteria were Gram-positive rod.

Antibacterial activity of halophilic bacteria

The agar well diffusion technique was performed as previously described to investigate the possible antibacterial activity of halophilic bacteria. *Bacillus cereus* (ATCC 11778), *Escherichia coli* O157 (PTCC 1276), *Klebsiella pneumoniae* (PTCC 10031), *Klebsiella flexneri* (PTCC 1234), *Pseudomonas aeruginosa* (ATCC 10231), *Streptococcus mutans* (ATCC 35668), *Candida albicans* (ATCC 10231) and *Enterococcus faecalis* (ATCC29212) were designated as human pathogens.

In this study, out of 30 isolated halophilic bacteria, only one isolates (E1) were able to form an inhibition zone against pathogenic microorganisms. Bacillus E1 strain showed antibacterial activity against the pathogenic *Enterococcus faecalis* (10 ± 0.1 mm) (Figure 2).

MIC and MBC values show that E1 have antibacterial activity against *Enterococcus faecalis* (ATCC29212). The MIC activity of E1 strain against *Enterococcus faecalis* was 25 µg/ml (Table 2).

Identification of halophilic bacteria

The PCR product bound for E1 strain on gel electrophoresis was showed in Figure 3. To identify the most similar microorganism to E1, a Blast search was conducted against the 16S rRNA database on the NCBI website. The Phylogenetic tree was drowned with Mega software by Neighbor-joining method with 100 bootstarp. The bootstarp more than 60 percent means high quality and validate of that branch in phylogenetic tree. Data showed that E1 was located closely to *Alkalihalobacillus hemicentroti* brach with 81 percent bootstrap (Figure 4). This means that E1 was more likely similar with *Alkalihalobacillus hemicentroti* which characterized as moderate halophile bacteria.

Discussion

In recent years, there has been a significant increase in resistance to antimicrobial agents due to the excessive use of antibiotics.[16] This resistance is now recognized as a major global health issue, threatening human health. Therefore, the importance of discovering new antibiotics cannot be overstated when encountering these challenges.[8] Recent research have revealed that a variety of natural sources,

Figure 1. Colony morphology of E1 strain.

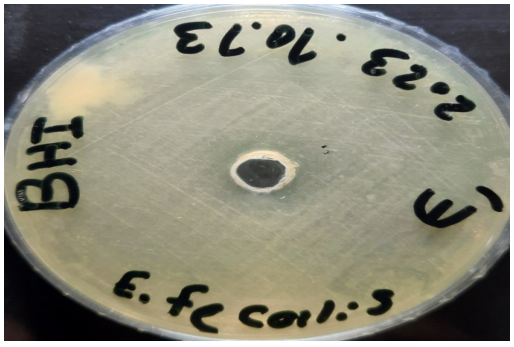

Figure 2. Antibacterial activity of bacterial strain E1 against *Enterococcus faecalis*.

Table 1. Morphology characteristic of isolated halophilic bacteria.

Strains	Cell morphology	Gram reaction	Colony		
			Texture	Size	Colour
A1	Rod	Positive	Smooth	Small	Yellow
A2	Rod	Positive	Smooth	Small	White
A3	Rod	Positive	Smooth	Moderate	Cream
A4	Rod	Positive	Smooth	Small	Milky
B1	Rod	Positive	Smooth	Small	Yellow
B2	Rod	Positive	Smooth	Small	Yellow
C1	Rod	Positive	Smooth	Punctiform	Cream
C2	Rod	Positive	Smooth	Small	White
C3	Rod	Positive	Smooth	Small	White
D1	Rod	Positive	Smooth	Small	Yellow
D2	Rod	Positive	Smooth	Punctiform	White
D3	Rod	Positive	Smooth	Punctiform	White
E1	Rod	Positive	Smooth	Small	Yellow
E2	Rod	Positive	Smooth	Small	Cream
E3	Rod	Positive	Smooth	Small	White
E4	Rod	Positive	Smooth	Small	White
F1	Rod	Positive	Smooth	Small	Gray
F2	Rod	Positive	Smooth	Small	Gray
F3	Rod	Positive	Smooth	Small	Gray
G1	Rod	Positive	Smooth	Small	Yellow- Cream
G2	Rod	Positive	Smooth	Small	Gray
G3	Rod	Positive	Smooth	Small	White

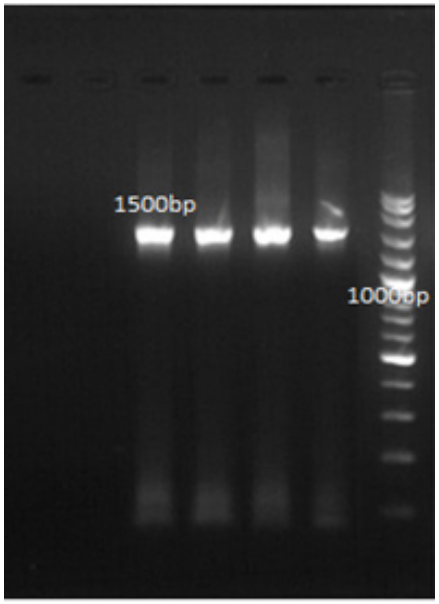

Figure 3. PCR product bound for E1 strain on gel electrophoresis.

such as essential oils, herbal extracts,[17-19] environmental bacteria[20] and non-toxic fungi[21] can be used as innovative antibiotic treatments. Studies indicate that these natural antibiotic therapies are not only safe, but also effective in combating pathogens while causing minimal side effects.[22] Environmental bacteria, as a type of microorganism, display various advantageous characteristics within the medical and pharmaceutical fields. These advantages include the ability to absorb heavy metals,[23] detoxify harmful substances,[24] and produce antimicrobial peptides and antibiotics.[25-27] Halophilic bacteria have great potential as a new source of bioactive substances, including antimicrobial and antitumoral agents.[28] These bacteria encompass a variety of organisms, including moderately halophilic bacteria.[29] The 16S rRNA sequencing technique is crucial for identifying and classifying strains.[30] In the present study, the results of 16S rRNA sequencing determined that the isolates belonged to *Alkalihalobacillus* sp. and *Bacillus* sp. The genus *Alkalihalobacillus* consists of rod-shaped, endospore-forming, and Gram-stain variable bacteria included in the family Bacillaceae. The

Table 2. Inhibition zone, MIC and MBC bacterial extract against Enterococcus faecalis.

Strain	Inhibition zone	MIC	MBC
E1	**10 ± 0.1 mm**	**25 µg/ml**	**25 µg/ml**

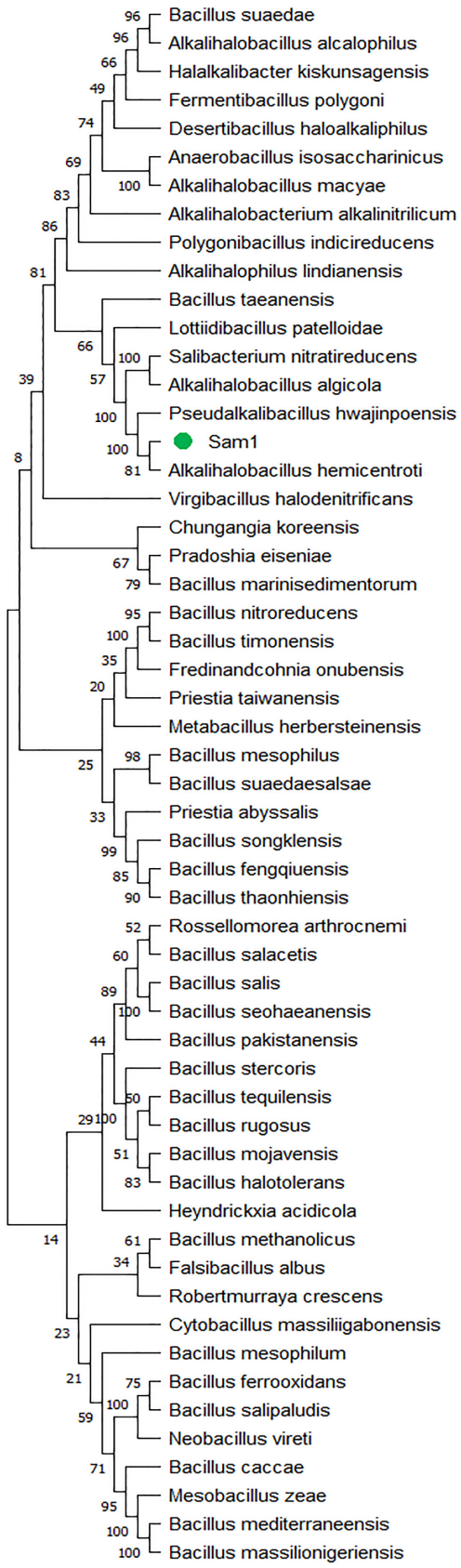

Figure 4. Neighbor-joining phylogenetic tree derived from 16S rRNA gene of E1 strain. The position of E1 strain in the tree showed with green circle.

members are halotolerant or halophilic in nature as they grow in the presence of 1–5% w/v NaCl concentration. According to one research, *Alkalihalobacterium elongatum* isolated from Lonar Lake was able to produce antibiotic.[31] Additionally, *Alkalihalobacillus* was isolated from other saline place in Iran, but there are no reports indicating its ability to produce antimicrobials[32,33] In a previous study, Elyasi Far and colleagues successfully identified *Bacillus*, *Virgibacillus*, and *Halobacillus* from the Haj Aligholi salt desert and Dagh Biarjmand of Shahrood in Iran.[13] In a separate research conducted by Hashemi and colleagues[34] the identification of *Bacillus* from Maharlu Salt Lake in Iran was documented. Furthermore, moderately halophilic bacteria such as *Halomonas*, *Salinicoccus*, *Planococcus*, *Bacillus*, and *Halobacillus* were detected in the Weihai Solar Saltern in China.[28]

In a separate study, Irshad and colleagues[35] successfully isolated various bacteria, such as *Bacillus*, *Streptomyces*, *Microbacterium*, *Micrococcus*, *Planococcus*, and Marinobacter, from foreshore soils in Korea. *Bacillus* was found to be the dominant bacteria in all the experiments.[28,34-36]

Additionally, only the E1 strains showed an inhibition zone against *Enterococcus faecalis*, with exhibiting a maximum inhibitory zone of 10 mm. *Enterococcus faecalis* is a type of Gram-positive, facultative anaerobic coccus that is commonly found in the intestines of healthy individuals. This bacterium can also be found in places like soil, water, and food products. *Enterococcus faecalis* is considered an opportunistic pathogen that can potentially cause serious and even fatal illnesses by taking advantage of weaknesses in the host's immune system.[37,38] It is crucial to prioritize the exploration of various environments to identify new and powerful antimicrobial treatments against these harmful bacteria.

The similar research from the Haj Aligholi salt desert and Dagh Biarjmand of Shahrood in Iran indicated that the D6A, Dar, and D8B strains have antimicrobial properties against various pathogens such as *Candida albicans*, *Fusarium oxysporum*, *Aspergillus flavus*, *Neurospora crassa*, *Botrytis cinerea*, and *Pseudomonas syringae* pv. Syringae. Additionally, a phylogenetic analysis revealed that the D6A and Dar strains belong to *Bacillus subtilis* species, while the D8B strain is classified under *Virgibacillus olivae*.[13]

These findings suggest that exploring new environments could lead to the discovery of novel antimicrobial substances that target specific pathogens.

Conclusion

According to recent studies, further investigation is necessary to understand the composition of antimicrobial agents using spectroscopic methods to determine their mode of action. The main objective of this study was to isolate potent antibiotic-producing halophiles from the Shushtar District in Khuzestan Province, Iran. For the first time, the research reveals that isolated *Alkalihalobacillus* in Iran has the ability to produce antimicrobial agents.

The results from the antibacterial assessments on these halophiles suggest that the saline soil in this region may serve as a valuable source of new antimicrobial substances.

Ethical Approval

The Ethics Committee of Dezful University of Medical Science sapproved this study (ethical code: IR.DUMS.REC.1401.058).

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Author Contributions

Abbas Moridnia: Conceptualization, Formal Analysis, Project Administration, Writing - Original Draft Leyla Hoseinitabar: Investigation, Alireza Khosropanah: Formal Analysis, Ladan Mafakher: Investigation, Marzieh Anaam: Investigation. Babak Elyasi Far: Conceptualization, Investigation, Formal Analysis, Project Administration, Supervision, Writing - Original Draft.

Conflict of Interest

The authors claim that there is no conflict of interest.

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