

ORIGINAL ARTICLE

Isolation and identification of halophilic and halotolerant bacteria from the Red Sea coast at Yanbu Region

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ABSTRACT

Background: Halophilic and halotolerant bacteria are potential sources of industrially important enzymes because of their ability to survive under hypersaline environmental conditions. Interest in extremophilic microorganisms for biotechnology has increased in recent years; however, limited information is available regarding the diversity and extracellular enzyme activity of halophilic bacteria along the Red Sea coastline, particularly in the Yanbu region of Saudi Arabia.

Methods: Thirty-five bacterial isolates were recovered from coastal seawater samples collected from the Red Sea coast of Yanbu, Saudi Arabia. The isolates were screened for extracellular enzyme activity, including amylase, cellulase, gelatinase, and protease production, using agar plate assays at 22°C and 37°C. Enzyme activity was evaluated using a semi-quantitative scoring system based on hydrolysis zone formation. Ten selected enzyme-producing isolates were subjected to partial 16S rRNA gene sequencing for molecular identification and phylogenetic analysis.

Results: Most isolates demonstrated at least one detectable enzyme activity and several exhibited multiple enzyme activities. Gelatinase activity was the most frequently observed enzyme activity, followed by protease activity. Molecular identification revealed that most selected isolates belonged to marine-associated bacterial genera within the class Gammaproteobacteria, including *Alteromonas*, *Pseudoalteromonas*, and *Vibrio*, with sequence similarities ranging from 97% to 100%.

Conclusion: This study demonstrates the diversity of halophilic and halotolerant bacteria along the Yanbu coastline and highlights their extracellular enzymatic potential. The findings support further molecular and biotechnological investigations of marine halophilic bacteria from the Red Sea region. The observed extracellular enzyme activities may also have biomedical relevance in future enzymatic and biofilm-related applications.

Keywords: Halophilic bacteria, Red Sea microbiome, enzyme activity, 16S rRNA sequencing, Yanbu.

Introduction

The Red Sea is characterized by elevated sea surface temperatures, high salinity, intense solar radiation, and limited freshwater input, creating a distinctive marine environment that supports specialized microbial communities [1]. These extreme environmental conditions favor the growth and persistence of halophilic and halotolerant microorganisms that are adapted to survive under osmotic stress and saline conditions through unique physiological and genetic mechanisms [2-4]. The ability of these microorganisms to produce enzymes that remain active under high salinity and fluctuating environmental conditions has attracted increasing scientific and biotechnological interest [4,5].

The central coastal region of the Red Sea, including the Yanbu coastline of western Saudi Arabia, is influenced

by both natural environmental processes and human activities such as urbanization, industrialization, and recreational development [6,7]. These environmental influences may affect the composition and functional diversity of microbial communities inhabiting coastal

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marine ecosystems [8,9]. Although molecular and metagenomic studies have considerably expanded current knowledge regarding microbial diversity in the Red Sea, relatively few studies have focused on the culture-based isolation and characterization of halophilic and halotolerant bacteria from coastal marine environments [9-11]. In particular, baseline information regarding extracellular enzyme-producing halophilic bacteria from the Yanbu coastal region remains limited.

Halophilic and halotolerant bacteria are increasingly recognized as valuable sources of extremozymes that remain functional under harsh environmental conditions, including elevated salinity and temperature [4,12,13]. These enzymes have attracted attention because of their potential applications in biotechnology, environmental processes, food production, and industrial systems [4,5,14,15]. In addition, extracellular enzymes such as proteases and gelatinases may have biomedical and pharmaceutical relevance because of their possible applications in enzymatic processing systems, biofilm-related studies, and biotechnological product development. Despite the rapid advancement of culture-independent molecular techniques for studying marine microbial diversity, culture-dependent approaches remain important for the recovery of viable bacterial strains and for the investigation of their functional and enzymatic properties [15-17].

The Yanbu coastal region remains relatively underexplored as a source of cultivable halophilic and halotolerant bacteria. Improved characterization of these microorganisms may contribute to understanding the microbial diversity of saline coastal ecosystems and support future ecological and applied microbiological investigations [12,14]. In this context, the present study provides a culture-based characterization and extracellular enzyme profiling of halophilic and halotolerant bacteria recovered from the Yanbu coastline of the Red Sea. Furthermore, with the rapid development of the NEOM region and other coastal projects in Saudi Arabia, future studies integrating cultivation-based approaches with advanced molecular methods such as metagenomics may provide deeper insights into the diversity and functional potential of marine microbial communities [16,17]. These efforts are also aligned with Saudi Arabia's Vision 2030 objectives related to sustainable biotechnology and environmental research [18].

Main objective

To isolate and characterize cultivable halophilic and halotolerant bacteria from the Red Sea coast of Yanbu, Saudi Arabia, with emphasis on their extracellular enzyme-producing potential.

Specific objectives

1. To collect and analyse coastal seawater samples from selected sites along the Yanbu coastline and determine their physicochemical characteristics.
2. To isolate halophilic and halotolerant bacterial strains using selective culture techniques under varying salt concentrations.

3. To screen the isolated bacteria for extracellular enzyme activities, including protease, amylase, cellulase, and gelatinase, under controlled temperature conditions.
4. To identify selected enzyme-producing isolates using 16S rRNA gene sequencing.

Materials and Methods

Research design

This study utilized a quantitative, culture-dependent experimental design to isolate and characterize halophilic and halotolerant bacteria from seawater samples collected from the coastal area of Yanbu City, Saudi Arabia. The main focus of the study was to analyze the extracellular enzyme activity of the isolated culturable bacteria as well as identify them through molecular methods.

Study area and sample collection

To obtain baseline microbial data, seawater samples were taken from four different coastal locations along the Yanbu coastline, which have differences in terms of human and environmental impact. Sampling was performed during a single field collection period in May 2025. One independent surface seawater sample was collected from each sampling site. At each of the four sampled locations, surface seawater (0-30 cm deep) was taken in sterile 1-l polycarbonate bottles and was delivered in ice to be processed in the lab within 6 hours of collection.

Physicochemical analysis of seawater

The use of a calibrated portable multiparameter meter facilitated the collection of in situ temperature, Potential of Hydrogen (pH), and salinity data. The total dissolved solids (TDS), electrical conductivity, turbidity, and dissolved oxygen were then analyzed using American Public Health Association standard testing procedures [15]. These measurements provided information about the environment in which the samples were collected to assist in understanding the microbial growth trends.

Isolation of halophilic and halotolerant bacteria

Seawater samples were serially diluted (10⁻¹ to 10⁻⁶) using sterile saline solution. Aliquots (100 µl) were spread-plated onto nutrient agar supplemented with NaCl at concentrations of 5%, 10%, and 20% (w/v). These salt concentrations were selected to recover a broad range of halotolerant and moderately to highly halophilic bacteria, as previously described in marine halophile isolation studies [12]. Plates were incubated at 37°C for 48-72 hours. Distinct colonies were sub-cultured repeatedly to obtain pure isolates, which were stored in glycerol stocks (20%, v/v) at -80°C.

Screening for extracellular enzyme activity

Using culture media that contained 5% sodium chloride and additional substrates: 1% starch (for amylase), 1% carboxymethyl cellulose (for cellulase activity), 3% gelatin (for gelatine), and 1% skimmed milk (for proteases), all isolates were screened for extracellular

enzyme activity via traditional agar plate methods that had been previously established with marine halotolerant bacteria [10]. After inoculating with 150 µl of bacterial cultures, the plates were incubated at 22°C and 37°C for 48-72 hours, respectively.

Extracellular enzyme activity was determined by observing the presence of clear hydrolysis zones surrounding bacterial colonies on substrate-specific media. Enzyme activity was evaluated using a semi-quantitative scoring system based on hydrolysis zone formation around each colony, as previously described for preliminary screening of halophilic bacteria [12]. Activity scores were categorized as follows: 0 = no activity, 1 = weak activity, 2 = moderate activity, and 3 = strong activity. For each plate tested, an equivalent negative control (uninoculated media) was included. Because the enzyme assays were exploratory and semi-quantitative in nature, the observed hydrolysis zone activities should be considered preliminary indicators of extracellular enzymatic potential rather than definitive quantitative measurements. The enzyme activity assays were intended as a preliminary screening approach to identify enzyme-producing isolates rather than to provide detailed kinetic or quantitative characterization.

Selection of isolates for molecular identification

Based on the results of the identification of enzymes, we selected ten isolates that showed stronger extracellular enzyme activity or multiple enzyme activities for molecular identification. The isolates we selected were intended to be from different environmental sampling sites and from environments that were sampled at two different temperature regimes.

Rationale for dual-temperature enzyme assays

Enzyme assays have been performed using two different temperatures, 22°C, in order to simulate natural coastal water conditions, and 37°C, to assess optimal temperature requirements for enzyme expression during laboratory growth of marine microorganisms. This method of using two different temperatures to assess enzyme activity compares the effect of the environment with that of the laboratory on the enzymatic activity of the marine bacterium [16].

Deoxyribonucleic Acid (DNA) extraction, polymerase chain reaction (PCR) amplification, and sequencing

Genomic DNA was extracted from bacterial cultures using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA) according

to the manufacturer's instructions. The quality and concentration of the extracted genomic DNA were assessed using a NanoDrop™ spectrophotometer and confirmed by agarose gel electrophoresis. The 16S rRNA gene was amplified using universal primers 27F and 1492R under standard PCR conditions previously described for bacterial molecular taxonomy [17]. PCR amplification was performed under the following conditions: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. The PCR products were verified by agarose gel electrophoresis, purified, and sequenced using traditional Sanger sequencing methodology. Comparative analysis of the obtained 16S rRNA gene sequences was performed using the Bioinformatics Search Tool (BLAST) tool in the National Center for Biotechnology Information (NCBI) GenBank database.

Phylogenetic analysis

Multiple sequence alignments were made with the Molecular Evolutionary Genetics Analysis (MEGA) program. Phylogenetic trees were made using the neighbor-joining method with 1,000 bootstrap resampling to evaluate the support of the branches. This methodology is commonly adopted to support the evolution of organisms [18]. In this analysis, only the best quality sequences were analyzed.

Statistical analysis

Descriptive statistics were used to summarize physicochemical parameters and semi-quantitative enzyme activity scores. Statistical analyses were exploratory in nature and intended to support descriptive comparisons rather than formal hypothesis testing. All analyses were performed using IBM SPSS Statistics (version 25).

Results

Physicochemical characteristics of seawater samples

The physicochemical properties of seawater samples collected from the four coastal sites along the Yanbu coastline are summarized in Table 1. Surface water temperature across the sampling sites ranged from 24.1°C to 24.5°C, showing minimal variation among locations. The pH values were slightly alkaline, ranging from 8.1 to 8.3. Electrical conductivity values ranged from 58,100 to 58,550 µS/cm, while TDS varied between 67,590

Table 1. Physicochemical parameters of water samples from four coastal sites of Yanbu.

Site	Temperature (°C)	pH	Turbidity (%)	TDS (ppm)	Conductivity (µS/cm.)
Site A	24.2	8.2	1.4	67,710	58,170
Site B	24.1	8.1	3.2	67,590	58,100
Site C	24.3	8.3	1.1	68,350	58,320
Site D	24.5	8.3	1.5	67,630	58,550

Note: Values represent single environmental measurements obtained during sample collection.

and 68,350 ppm. Turbidity levels were low at all sites, ranging from 1.1% to 3.2%.

Overall, the measured parameters indicated relatively consistent physicochemical conditions across the four coastal sampling locations during the study period.

Isolation and morphological characteristics of bacterial isolates

A total of 35 distinct bacterial isolates were recovered from the four coastal sampling sites. The distribution of isolates by sampling site is summarized in Table 2. Site D yielded the highest number of isolates ($n = 10$), followed by Sites B and C ($n = 9$ each), while Site A yielded the lowest number ($n = 7$).

The isolates displayed diverse colony characteristics, including variations in color (cream, white, yellow, orange, and beige) and morphology, such as round, circular, irregular, raised, and flat forms, as summarized in Table 2. Growth of the isolates was observed across a range of NaCl concentrations (5%-20%), indicating that the recovered bacteria exhibited halophilic or halotolerant growth characteristics.

Extracellular enzyme activity screening

All 35 bacterial isolates were screened for extracellular enzyme activity, including amylase, cellulase, gelatinase, and protease production, using agar plate assays. Enzyme activity was evaluated using a semi-quantitative scoring system based on hydrolysis zone formation around bacterial colonies.

At 37°C, the extracellular enzyme activity profiles of the isolates are summarized in Table 3. Most isolates exhibited at least one detectable enzyme activity. Gelatinase activity was the most frequently observed enzyme activity, followed by protease activity, whereas amylase and cellulase activities showed greater variability among isolates.

At 22°C, the extracellular enzyme activity profiles are summarized in Table 4. Similar trends were observed, with gelatinase activity remaining the predominant enzymatic activity detected among the isolates. Several isolates demonstrated activity for more than one enzyme under both incubation temperatures.

No hydrolysis zones were observed in uninoculated control plates, confirming that the observed extracellular enzyme activities were associated with viable bacterial growth.

Representative agar plate images illustrating hydrolysis zones for the enzyme assays are presented in Figure 1.

Molecular identification and phylogenetic analysis

Phylogenetic analysis based on partial 16S rRNA gene sequences was performed for ten selected bacterial isolates together with closely related reference sequences retrieved from the NCBI GenBank database. BLAST sequence similarity analysis demonstrated high sequence identity ($\geq 97\%$) with previously reported marine bacterial taxa, and the corresponding molecular identifications are summarized in Table 5.

Table 2. Summary of halophilic and halotolerant bacterial isolates by sampling site and colony characteristics.

Sampling site	No. of isolates	Colony colors observed	Colony morphologies
Site A	7	Cream, yellow	Round, circular
Site B	9	Yellow, orange	Circular, irregular
Site C	9	Cream, white	Round, raised
Site D	10	Orange, beige	Irregular, flat

Table 3. Semi-quantitative extracellular enzyme activity scores of bacterial isolates at 37°C.

Isolate code	Amylase	Cellulase	Gelatinase	Protease
S1	1	1	3	1
S2	0	1	1	1
S3	0	1	1	0
S4	2	2	3	2
S5	0	1	2	0
S6	0	2	2	0
S7	1	1	2	1
S8	1	1	2	1
S9	0	1	1	1
S10	1	1	3	1
S11	0	0	1	1
S12	1	1	3	1

Activity scores were categorized as follows: 0 = no activity; 1 = weak activity; 2 = moderate activity; 3 = strong activity based on hydrolysis zone formation.

Table 4. Semi-quantitative extracellular enzyme activity scores of bacterial isolates at 22°C.

Isolate code	Amylase	Cellulase	Gelatinase	Protease
S13	1	1	3	1
S14	0	1	1	1
S15	0	1	2	0
S16	1	1	2	0
S17	0	1	2	1
S18	1	1	2	1
S19	1	1	3	1
S20	0	1	1	1
S21	0	1	1	1
S22	0	1	1	0
S23	0	1	2	1
S24	1	1	1	1
S25	0	1	1	0
S26	1	1	1	0
S27	0	1	1	1
S28	0	1	1	1
S29	1	2	2	2
S30	0	1	2	1
S31	1	1	1	1
S32	1	0	1	1
S33	1	1	1	1
S34	1	1	1	0
S35	1	1	3	1

Activity scores were categorized as follows: 0 = no activity; 1 = weak activity; 2 = moderate activity; 3 = strong activity based on hydrolysis zone formation.

The Neighbor-Joining phylogenetic tree (Figure 2) demonstrated clustering patterns consistent with the BLAST-based taxonomic assignments. Bootstrap support values generated from 1,000 replicates are presented at the branch nodes to indicate the reliability of the inferred phylogenetic relationships.

Phylogenetic analysis was performed using the Neighbor-Joining method implemented in MEGA X following Multiple Sequence Comparison by Log-Expectation alignment of partial 16S rRNA gene sequences. Evolutionary distances were calculated using the Kimura two-parameter model with pairwise deletion of gaps. Node support was assessed using 1,000 bootstrap replicates, and the resulting phylogenetic tree is presented in Figure 2.

Molecular identification and phylogenetic consistency

Partial 16S rRNA gene sequencing enabled molecular identification of the selected bacterial isolates at the genus level. BLAST sequence analysis demonstrated high sequence similarity ($\geq 97\%$) with previously reported marine bacterial taxa, with most isolates belonging to the class Gammaproteobacteria. Phylogenetic analysis using the Neighbor-Joining method demonstrated clustering patterns consistent with the BLAST-based taxonomic identifications presented in Figure 2 and Table 5. Overall, these findings support the usefulness of molecular

approaches for the identification and classification of halophilic and halotolerant bacteria from marine environments.

Discussion

This study examined the isolation and characterization of halophilic and halotolerant bacteria recovered from coastal seawater along the Yanbu coastline in the Red Sea. The physicochemical analyses indicated fairly stable environmental conditions with sampling sites featuring slightly alkaline pH, high salinity, and elevated conductivity values; all characteristic of marine environments of the Red Sea, known to support halophilic and halotolerant bacteria [3].

Thirty-five distinct bacterial isolates were obtained from sampled coastal sites that showed variation in colony pigmentation and morphology. Variations in colony color and morphology among marine bacterial isolates are known to suggest differences in physiological adaptation, pigment production, and metabolic activity under saline environmental conditions [19,20]. The ability of the isolates to grow on media containing 5% -20% NaCl indicates that they may be halophilic or halotolerant, reflecting adaptations to hypersaline marine environments [2,3]. Screening for extracellular enzymes revealed that many of the isolates exhibited enzymatic activity-particularly gelatinase and protease activity, gelatinase being the most frequently detected enzymatic activity

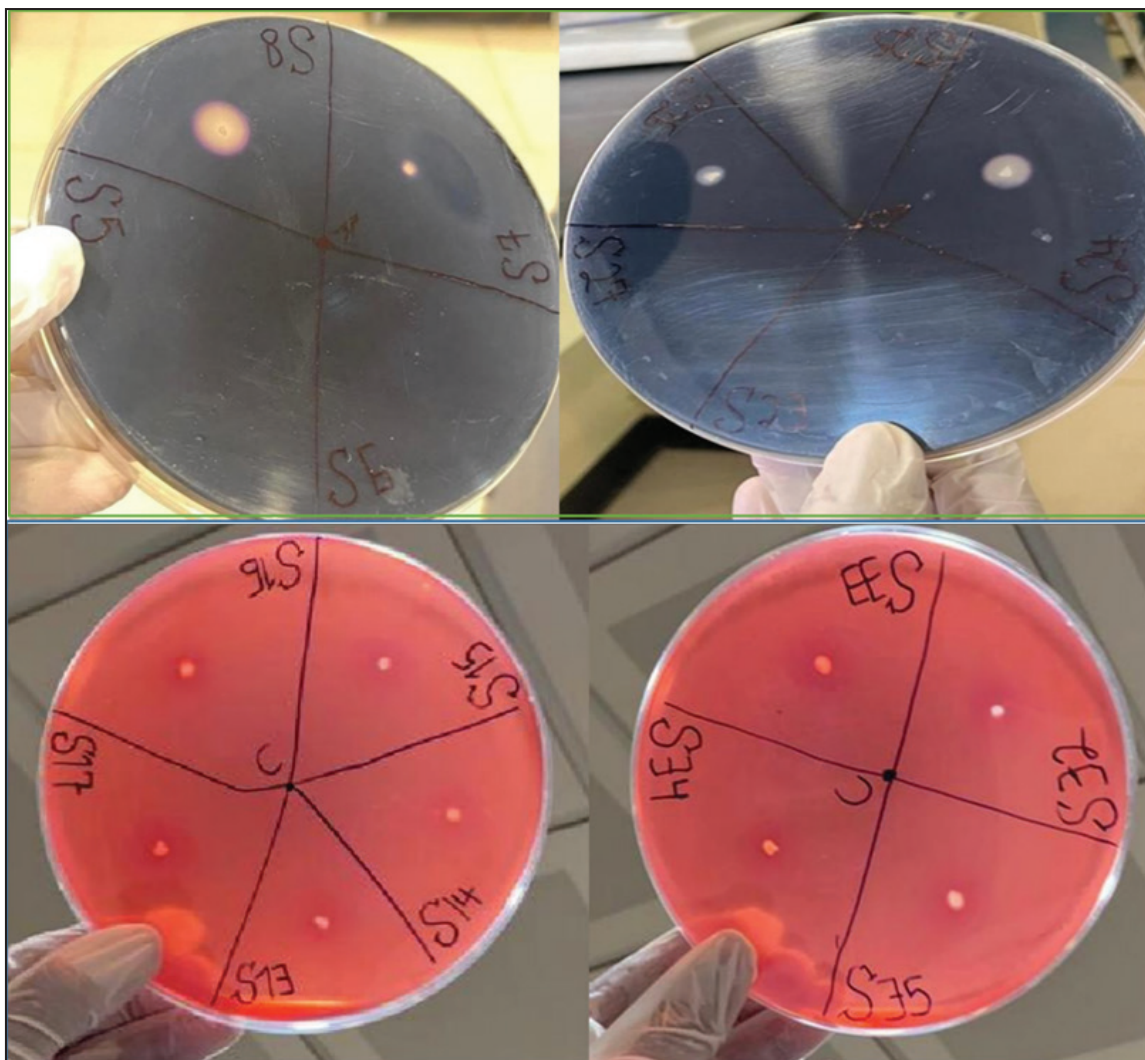


Figure 1. Representative agar plate images showing extracellular enzyme activity produced by halophilic bacterial isolates. (A) Gelatinase activity, (B) protease activity, (C) amylase activity, and (D) cellulase activity, demonstrated by hydrolysis zone formation surrounding selected bacterial colonies grown on substrate-specific media.

Table 5. Molecular identification of selected halophilic bacterial isolates based on partial 16S rRNA gene sequencing and BLAST analysis.

Isolate code	Closest identified taxon (16S rRNA)	GenBank accession no.	Query coverage (%)	E-value	Max identity (%)
S1	<i>Alteromonas mediterranea</i>	OK175572	100	0.0	99.28
S4	<i>Pseudoalteromonas ruthenica</i>	OK175573	100	0.0	100.00
S6	<i>Oceanicaulis alexandrii</i>	OK175574	100	0.0	99.85
S10	<i>Alteromonas mediterranea</i>	OK175575	100	0.0	98.67
S12	<i>Pseudoalteromonas profundus</i>	OK175576	100	0.0	99.26
S13	<i>Alteromonas mediterranea</i>	OK175577	100	0.0	99.56
S19	<i>Alteromonas mediterranea</i>	OK175578	100	0.0	99.56
S23	<i>Alteromonas australica</i>	OK175579	100	0.0	99.27
S29	<i>Vibrio rumoiensis</i>	OK175580	100	0.0	99.85
S35	<i>Alteromonas mediterranea</i>	OK175581	100	0.0	99.12

at both 22°C and 37°C; amylase and cellulase activities were less commonly observed and showed variability among the isolates. These results suggest that marine halophilic and halotolerant bacteria may contribute to the breakdown of macromolecular organic material

within marine ecosystems, consistent with findings reported in previous studies [12,22].

The semi-quantitative screening method for determining enzyme activity indicated the presence of extracellular enzymatic potential among the isolates, with a

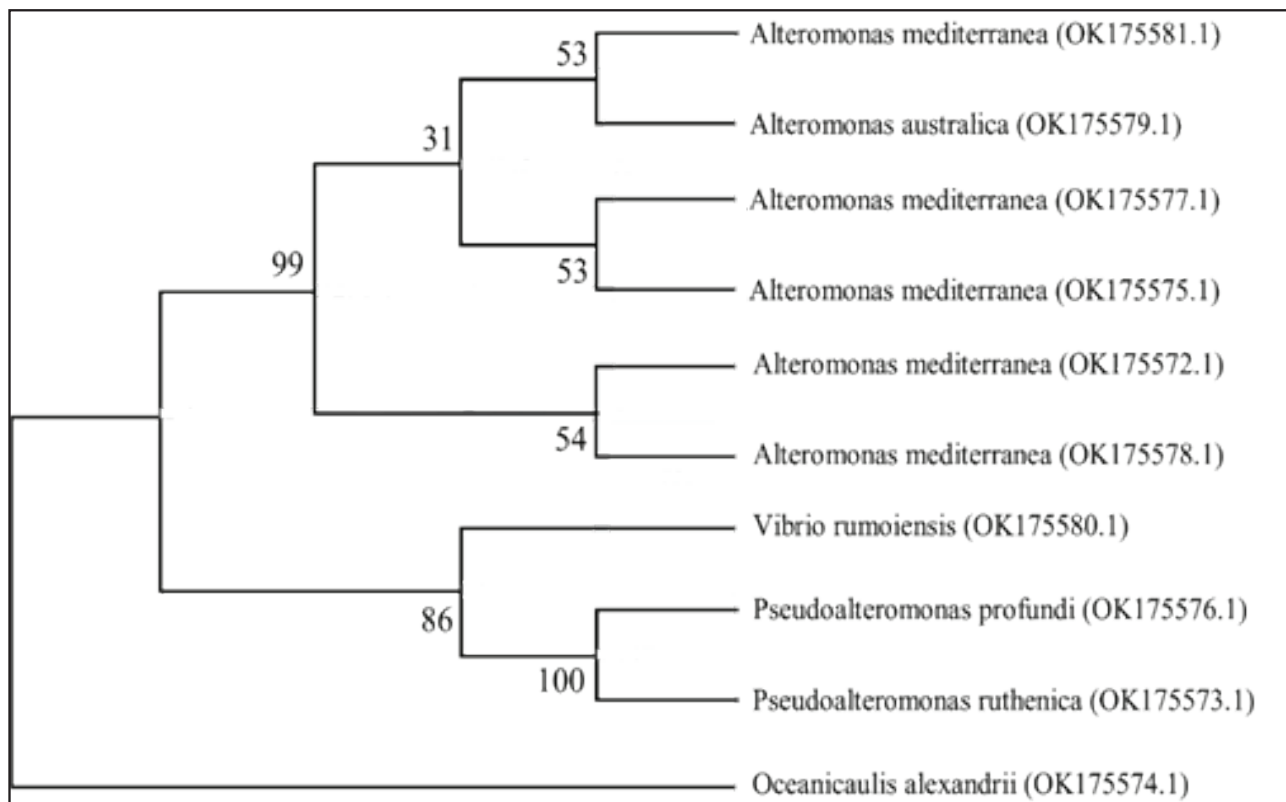


Figure 2. Neighbor-Joining phylogenetic tree based on partial 16S rRNA gene sequences of selected halophilic bacterial isolates and closely related reference sequences retrieved from GenBank. The phylogenetic tree was constructed using the Kimura two-parameter model with pairwise deletion of gaps in MEGA X software. Bootstrap support values generated from 1,000 replicates are shown at the branch nodes. Lower bootstrap values observed in some branches may reflect the close phylogenetic relatedness among the analyzed marine bacterial isolates.

number showing activity for multiple enzymes at both temperatures, suggesting metabolic versatility and/or adaptation to changing environmental conditions. Marine-derived enzymes from halophilic bacteria are considered of increasing interest in biotechnology as they are likely to retain functional stability under conditions of high salinity and variable physicochemical environments, with potential applications in biotechnology, environmental processes, and marine bioproducts [4,12-15,21].

In addition to their ecological and industrial significance, extracellular proteases and gelatinases produced by halophilic bacteria may also have potential biomedical relevance. Proteolytic enzymes derived from extremophilic microorganisms have attracted attention because of their possible applications in wound debridement, biofilm disruption, and enzymatic processing systems under harsh environmental conditions. The predominance of gelatinase and protease activity observed among the Yanbu isolates may therefore support future investigations into the medical and pharmaceutical potential of marine-derived extremozymes.

Molecular identification, based on partial sequences for the 16S rRNA gene, showed that most of the selected isolates belonged to marine-associated genera of the class Gammaproteobacteria. e.g., *Alteromonas*, *Pseudoalteromonas*, and *Vibrio*. These genera are commonly found in marine environments and have previously been associated with enzyme production and/

or adaptations to a saline environment [22,23]. The high sequence similarity observed in the BLAST analysis further supports the molecular identification results [24,25].

Phylogenetic analysis using the Neighbor-Joining method revealed clustering patterns generally in line with the BLAST-based taxonomic assignments. The lower bootstrap support values evident on a few branches may reflect the close phylogenetic relatedness of the analyzed isolates, and the conserved nature of the partial 16S rRNA gene sequences, although similar observations have been made for closely related marine taxa [24,25].

In summary, the results of this study point to the diversity of halophilic and halotolerant bacteria present along the Yanbu coastline, as well as their extracellular enzymatic capacity. A combination of culture-based characterization, semi-quantitative screening for important hydrolytic enzymes, and molecular identification yielded useful preliminary information on the diversity and functional characteristics of isolates along the Red Sea coast.

Limitation

This study has several limitations. First, the investigation relied on culture-dependent methods, which may underestimate the total microbial diversity present in the sampled marine environment. Second, extracellular enzyme activity was evaluated using semi-quantitative

agar plate assays rather than detailed quantitative enzymatic analyses. Third, molecular identification was based on partial 16S rRNA gene sequencing, limiting taxonomic resolution at the species level. Finally, the study focused on preliminary environmental and biotechnological characterization, and no direct clinical or biomedical applications were experimentally investigated.

Conclusion

Several diverse halophilic and halotolerant bacteria were isolated from the Yanbu coastline of the Red Sea, Saudi Arabia, in relatively stable physicochemical conditions. Several isolates exhibited extracellular enzyme activity, particularly gelatinase and protease activity. These findings support further studies on the ecological and biotechnological significance of these bacterial genera. Molecular identification based on 16S rRNA gene sequencing indicated that most selected isolates belonged to marine-associated bacterial genera within the class Gammaproteobacteria. Overall, the findings provide a good insight into some of the diversity and functional potential of marine halophilic bacteria from the Red Sea and indicate the need for further studies using molecular, enzymatic, and other biotechnological approaches. The extracellular enzyme activities observed in several isolates may also provide a basis for future biomedical and pharmaceutical investigations involving marine-derived extremozymes.

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List of Abbreviations

BLAST	Bioinformatics Search Tool
DNA	Deoxyribonucleic Acid
MEGA	Molecular Evolutionary Genetics Analysis
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
ph	Potential of Hydrogen
RNA	Ribonucleic Acid

Conflict of interests

The author declares that there are no competing interests, financial or otherwise, that could have influenced the conduct or outcomes of this study.

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Consent to participate

Written informed consent was obtained from all participants before participation in the study.

Ethical approval

This study did not involve human participants or animal subjects. Ethical approval and informed consent were therefore not required.

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Supplementary content (If any) is available online.

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