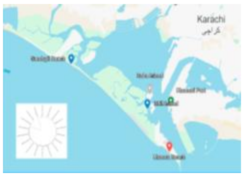

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Culturable Diversity of Halophilic Bacteria from Soil and Saline Water Samples of Arabian Sea, Karachi Port, Pakistan

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ABSTRACT: *Microorganisms capable of survival in extremely high salt concentration are termed as halophiles and a significant source of various enzymes such as hydrolases. Many hydrolases e.g., proteases, amylases and lipases are widely employed in industrial applications. In the current study, we isolated, screened and characterized halophilic bacteria from the shoreline of Arabian Sea, Karachi Port, Pakistan. The water and soil samples collected from Karachi Port; Pakistan were tested for the presence of halophilic bacteria using increasing salt concentrations. Isolated strains were described using various biochemical assays i.e., KOH production, ammonia production, catalase activity, and oxidase activity. For amylase and protease activity, formation of clearance zones – around bacterial growth – was observed, on starch and gelatin agar plates, respectively. Two bacterial strains, N20 (PQ460959) and M3 (PQ460960), with notable amylase and protease activities, were identified via 16S rRNA sequencing as Halobacillus salinus and Halobacillus fulvus, respectively. Further exploration of saline environments is crucial for discovering microbiota with potential applications in biotechnology and environmental remediation*

Keywords: Halophile, Saline water, Protease, Amylase, Hydrolase

INTRODUCTION

The Arabian Sea, with its unique saline properties and proximity to Karachi Port, offers a distinct habitat that may harbour diverse halophilic bacteria with unique adaptations. The bacterial species in such habitats that thrive in the presence of salt are referred to as halophiles (Kushner, 2020). Halophilic bacteria grow optimally in high salt concentrations and are catalytically efficient in surviving other extreme conditions i.e. high temperature, pH, etc. (Ahmed et al., 2020). According to the growth promotion showed by these strains on salt consumption, they are classified as moderate and extreme halophiles (Oren, 2024). Archaea capable of growth in up to 30% w/v salt concentration are termed as extreme halophiles (Kasirajan et al., 2021). While most bacterial domain members capable of growth in up to 15% w/v salts are termed as moderate halophiles.

For survival in such extreme habitats, halophiles produce various unique enzymes that are necessary for survival in such extremes (Wang et al., 2023). Halophiles are significant sources of amylase, protease and are being utilized for various biotechnological applications (Ahmed et al., 2020). Since, the production of hydrolases is an adaptation mechanism, these enzymes help in breakdown of complex macromolecules. Under extreme osmotic pressure, hydrolases also aid in nutrient acquisition (Cui et al., 2017). Various hydrolases have been found to confer cellular stability and flexibility, allowing halophiles to function in their salt-rich niches (Kushner, 2020). Exploration of other saline settings may lead to identification of novel halophiles with promising biotechnological applications (Oren, 2020).

This study was designed to isolate indigenous microbiota from the water samples of Arabian Sea and coastal soils of Karachi Port. Isolated bacterial strains were screened for presence of halophiles via KOH hydrolysis, ammonia production and amylolytic, proteolytic activity. Promising strains were identified via 16S rRNA sequencing.

MATERIALS AND METHODS

Sample Collection and Characterization

Halophilic water and sand samples were collected from Arabian Sea, Port of Karachi, Sindh, Pakistan. Samples were collected from eleven different sites at the Port of Karachi, i.e., Sandspit Beach, Manora Beach, Keamari Port, Baba Bit Island and labelled from 1-11 (Fig. 1).

Fig. 1. A map showing the location of the sampling sites used in the diversification study. Location tags mark the sites; Sandspit Beach, Baba Island, Bhitt Island, Keamari Port, and Manora Beach

Samples were collected aseptically, labelled and stored at 4^{o}C. Sample pH, temperature, colour, texture, odour, turbidity, appearance, etc. were observed and tabulated.

Isolation of halophilic bacteria

Dilutions in series, up to 10^{-5} were used to prepare samples. Pure water and 100 µl portions from also applied to nutritional agar (NA) plates in dilution. Then incubated for 72 hours at 30 °C. In terms of morphology, different bacterial colonies were chosen for additional research. Specific bacterial isolates were stored at -20 °C in a 20% glycerol solution.

Screening for Halophilic Behavior

Halophilic media was prepared using salt concentration of 1M NaCl and 2M NaCl. Isolated strains were streaked on plates and incubated at 37 °C. After 24 h incubation, plates were checked for absence and presence of culture growth.

KOH hydrolysis test

Two drops of a 3% KOH solution were dispensed on a glass slide. One loopful of bacterial culture was added to the KOH solution using a sterile stick. The suspension thickened in 10-20 s. Formation of thin slime thread on pulling the suspension with sterile toothpick indicated a positive result (MMM Ahmed et al., 2020).

Ammonia production

Isolated bacterial strains were cultured in 5 mL peptone broth. Media was incubated at 30 °C for 72 h. After incubation, 0.5 mL Nessler's reagent was added to the media. The change in colour from brown to yellow signified the formation of ammonia (James et al., 2014).

Screening for hydrolase producing bacteria

For amylase production activity, bacterial strains were streaked on N. Agar plates containing 20 g/L starch. After incubation, plates were flooded for 10-15 min with iodine solution. The zone of clearance was observed. Screening for protease production activity was observed by streaking bacterial strains on N. Agar plates containing 10 g/L gelatin. After incubation, plates were treated with mercuric chloride solution and zone of clearance was observed (Ahmed et al., 2020).

Characterization of bacterial isolates

Bacterial isolates were characterized via gram staining, catalase and oxidase activity (James et al., 2014).

1.1.1 16S rRNA Sequencing of Promising Strains

Strains were grown on Nutrient Agar medium. The 16S rRNA sequencing of samples was done commercially at Macrogen Inc., Seoul, Korea (https://dna.macrogen.com/eng/support/ces/guide/universal_primer.jsp). Sequences were attained from chromatograms using Chromas 2.6.6. software. Maximum homology was inspected against GenBank using BlastN. Sequences were submitted to NCBI GenBank. Evolutionary relationship of selected strains with related taxa were mapped out using MEGA 10.1.7 for dendrogram construction (umar et al., 2018). Neighbour-joining method was used for computing evolutionary distances, based on the Maximum Likelihood Composite (Saitou et al., 1987).

RESULTS

1.1.2 Isolation and Screening of Halophiles

Samples were characterized based on pH, temperature, colour, odour, texture. Microbial flora of samples collected from Arabian Sea; Karachi Port was enumerated by spread plate method. Calculations of log CFU/mL showed that highest diversity was obtained in samples 8 and 10 taken from Baba-Bhitt Island. The comparative enumeration on media N Agar and Halophilic media showed that almost all isolates were halophiles except for samples from Keamari Port 6, and 7 that showed no halophilic growth. While one sample from Manora Beach (5), showed no growth on either media (Fig. 2). A total of 107 strains were isolated form all samples and named as N1-40, M1-M67.

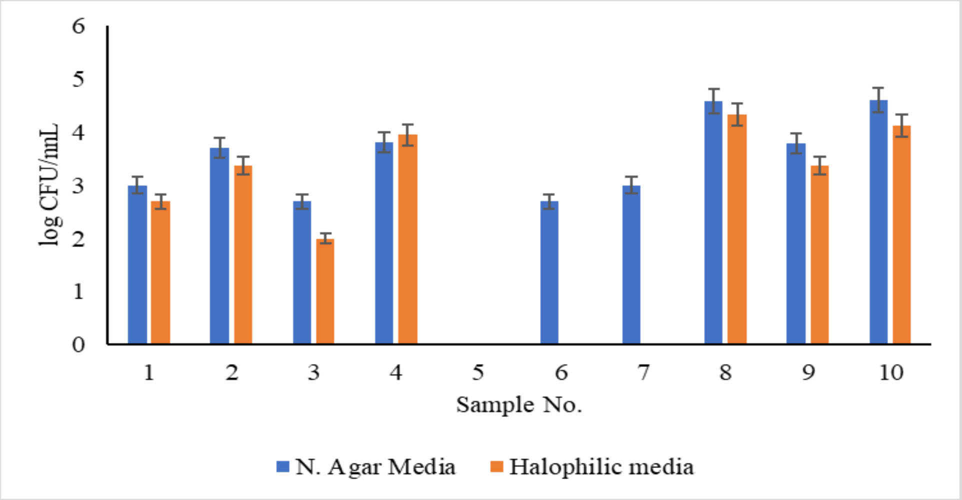

Fig. 2. Comparative Sample Enumeration Karachi Port samples on N. Agar and Halophilic Media

1.1.3 Screening of Halophiles

Screening for halophilic behaviour was done by observation of various parameters i.e., growth on 1M and 2M NaCl to screen moderate and extreme halophiles showed that out of isolates only 14 were moderately halophilic. Further screening tests showed only six among these strains, were capable of gelatin and starch hydrolysis (Fig. 3).

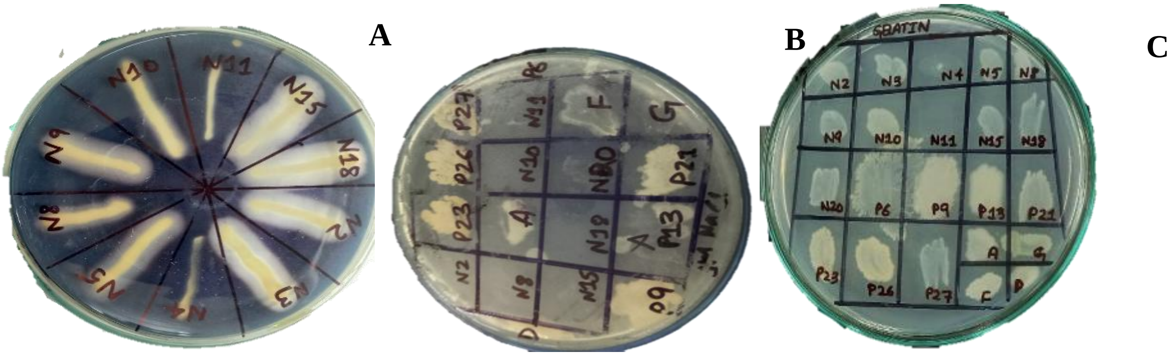

Fig. 3. Screening for Halophiles; A) Starch Hydrolysis, B) Growth on 1M and 2M NaCl, C) Growth on Gelatin

While only strains N20 and M3 were capable of KOH hydrolysis and ammonia production (Table 1).

Table 1: Screening of Pure Cultures

Strain name	Growth on 1M NaCl	Growth on 2M NaCl	Gelatin Hydrolysis	Starch Hydrolysis	KOH Hydrolysis	Ammonia Production
N2	+	+	+	-	+	-
N3	-	-	+	+	+	-
N4	-	-	-	-	-	-
N5	-	-	+	-	+	-
N8	+	+	+	+	+	+
N9	-	-	-	-	+	+
N10	-	-	-	-	+	-
N11	+	+	-	-	-	-
N15	+	+	+	-	+	+
N20	+	+	+	+	+	+
M2	+	+	+	-	-	-
M3	+	+	+	+	+	+
M4	+	+	+	-	-	-
M5	+	+	-	-	-	-
M10	+	+	-	-	-	-
M11	+	-	-	-	-	-
M12	+	+	+	+	-	-
M16	+	+		-	-	-
M18	+	+	+	+	-	-
M21	+	+	+	-	-	-

1.1.4 16S rRNA Sequencing of Promising Strains

Strains N20 and M3 were identified via 16S rRNA sequencing as *Halobacillus salinus* (PQ460959) and *Halobacillus fulvus* (PQ460960), respectively. Neighbor-Joining method was used for dendrogram mapping. The optimum tree is shown to scale, and Maximum Composite Likelihood was used to calculate the branch lengths corresponding to evolutionary distances.

The evolutionary distance for computation of phylogenetic tree is expressed in terms of the base substitutions per site. This test included 18 nucleotide sequences while there was total 1599 sites in the complete dataset. Ambiguous sequences were eliminated using pairwise deletion (Fig. 4).

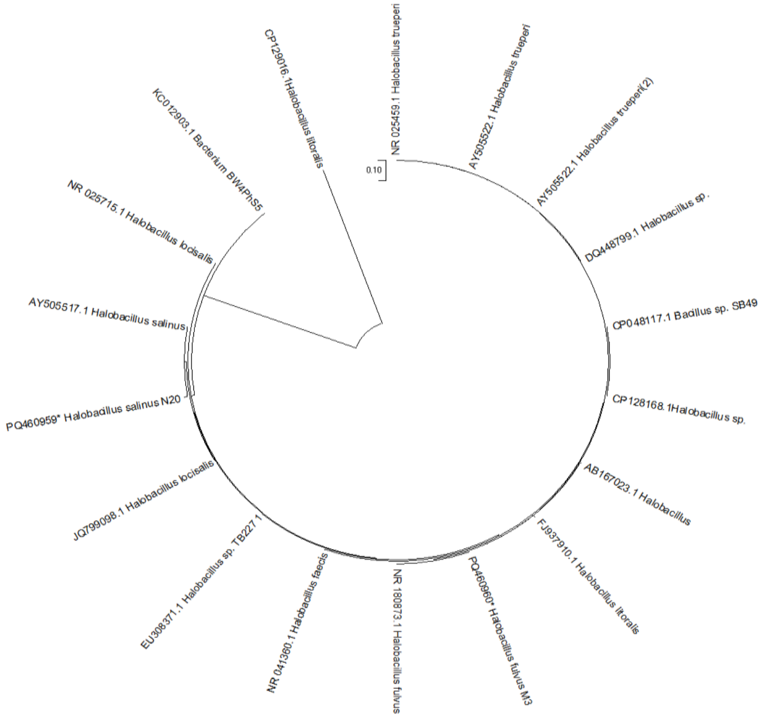

Fig. 4. Evolutionary relationship of closely related taxa with Strain N20 and M3. Neighbor-Joining approach was used to infer the evolutionary history in terms of the number of nucleotide substitutions per site. The optimal tree is presented to scale, and the branch lengths correspond to evolutionary distances determined with the Maximum Composite Likelihood approach. The analysis contained 18 nucleotide sequences while the codon sites given were first, second, third, and noncoding. All ambiguous sites in each sequence pair were removed (using the pairwise deletion option). The entire dataset had a total of 1599 sites.

DISCUSSION

The surface of Earth is covered with 70% water, most of which is comprised of extremely saline environments. Microbiota of various kinds thrive in such extreme environments of temperature, pH, and salinity, by adapting different survival techniques (Dutta et al., 2022). Halophiles thriving in high salt concentrations store potassium ions in their cytoplasm and counteract the osmotic pressure of their environment (Qiu et al., 2021). These halophile have also developed many osmo-protectants for maintenance of osmotic pressure (Oren, 2024).

Among the notable saline waterbodies on Earth surface, Arabian Sea has been the focus of many explorative studies for discovery of novel microbiota (Khan et al., 2021). Like many other saline waterbodies i.e., the Dead Sea, saltern crystallized ponds and hypersaline sun lakes, the Arabian Sea has been crucial in isolation of many industrially important halophiles (Didari et al., 2020; Dutta et al., 2022). In this study, the saline water samples collected from Arabian Sea and coastal soil samples from Karachi Port, were found to be an excellent source of halophiles. Approximately 107 different bacterial strains were isolated form these samples, ranging from moderate to extreme halophiles. These strains were culturally characterized and a variety of gram-positive, gram-negative, spore-formers etc. were observed. Similar results were observed in explorative studies conducted on Oman Sea (Hashemzahi et al., 2020), South China Sea (H Zhang et al., 2022), Southwest Indian Sea (Qiu et al., 2021) and many others.

Studies have also shown that halophile employ different adaptation mechanisms to overcome the harsh growth conditions in their extreme habitats (Deosthali et al., 2022). These include production of enzymes capable of bioremediation in oil contaminated waters (Ullah et al., 2021), plant growth promotion (F Orhan et al., 2020), potential in bio saline agriculture (Ghafar et al., 2022), etc. Strains isolated from various location of Karachi Port underwent further screening for production of hydrolases. Among 107 isolates, many strains showed strong amylolytic, and proteolytic activities. Studies have shown that hydrolases exhibit significant catalytic activity (Drissi Kaitouni et al., 2020). Recent studies have shown the impact of such hydrolases on the biogeochemical cycle via aid in nitrogen recovery

(Zhang et al., 2021), removal of nitrogen from saline waste water via various nitrification processes (Cui et al., 2021), and potential in bio-cleaning (Ruginescu et al., 2022).

Two strains N20 and M3 with promising results were sequenced via 16S rRNA sequencing. These strains belonging to *Halobacillus* genus have been known to survive in highly saline environments (Kong et al., 2024; Wang et al., 2019). During this study, strains M3 and N20 were grown on high concentration of NaCl up to 2M. Similar studies have shown that *Halobacillus* is capable of growth on 5 to 10% NaCl (Y Kim et al., 2023). Isolation of *Halobacillus* from saline samples of Arabian Sea water and coastal soils, is corroborated by similar studies conducted in such environments. *Halobacillus* species have been found in saline and hyper saline lakes (Didari et al., 2020; Ruginescu et al., 2020), solar salterns (Ali et al., 2024; Ding et al., 2020), salt lakes, etc. In their niche ecosystems, these *Halobacillus* species have been reported to contribute in organic matter decomposition (Ibrahim et al., 2020), nitrogen cycling (Yaradoddi Jayachandra et al., 2020), etc. Studies have also shown the importance of Halobacillus in various biotechnological industries i.e., detergent and food processing, (Xu et al., 2023) and bioremediation (Huang et al., 2006), and therapeutics (Sadaf Wajahat, 2024). The strains N20 and M3, therefore, have high potential for utilization in various biotechnological processes. This study broadens our understanding of microbial diversity in saline environments and highlights potential applications in industry, particularly for enzymes requiring high salt tolerance.

CONCLUSION

In this study, the Arabian Sea – an extremely hyper-saline environment – was explored for isolation of halophilic bacteria. Isolation of two halophilic strains N20 (*Halobacillus salinus*) and M3 (*Halobacillus fulvus*) with hydrolase activity, showed the importance of screening such extreme environments. Future studies should focus on optimizing enzyme production and exploring additional biotechnological applications of these strains, such as in saline wastewater treatment.

ACKNOWLEDGMENT

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ETHICAL STATEMENT

This study ????

CONFLICT OF INTEREST

Authors declare no conflict of interests.

DATA AVAILABILITY

The data that supports the findings of this study are available on request by the corresponding author.

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