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Research Article

MOLECULAR AUTHENTICATION AND GENETIC DIVERSITY ANALYSIS OF HALOFEROX VOLCANII AND HALOBACTERIUM SALINARIUM FROM SALT BRINES

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ABSTRACT

Objective - Halophilic bacteria present in various high salt locations have shown to possess various commercially viable properties. An understanding of the microbial flora present in salt brines will help in the proper exploitation of this resource.**Methods -** In the current study, bacteria were isolated from salt brines and characterized. Followed by the morphological and biochemical characterization, the strains were subjected to molecular characterization. Genomic DNA was isolated and the 16s rRNA gene was amplified and sequenced. The sequence so obtained was subjected to sequence alignment analysis. The genetic diversity of the isolated strains were also analysed by RAPD PCR and phylogenetic relationship was established by dendrogram construction.**Results –** The isolated strains were found to be *Haloferax volcanii* and *Halobacterium salinarium.* The genetic diversity analyses of *H. salinarium* and *H. volcanii* reveal the resistance of the former to genetic variations.**Conclusion -** This study analysed the impact of environmental stress on the genotypes of *H. salinarium* and *H. volcanii.*

Keywords: *Halobacterium salinarium, Haloferax volcanii*, Halophilic Archaea, RAPD.

INTRODUCTION

Over the past decade, marine microorganisms have become recognized as an important and untapped resource for novel bioactive compounds. One among them is the Archaea bacteria. At the end of 20th century, microbiologists realized that the Archaea are a large and diverse group of organisms, widely distributed in nature and are also common in less extreme habitats, such as soils and oceans [1]. Most of the Archaea are highly adapted to cope with extreme chemical and/or physical environments (temperature, pH, salinity, etc.).

The halophilic Archaea are characterized as organisms capable of growing from around 8% (1.5 M) sodium chloride (NaCl) to approximately 36% (5 M) NaCl, which is at saturation for NaCl. They are chemoorganotrophic, utilizing amino acids or carbohydrates as carbon source and occur ubiquitously in nature where the salt concentration is high [2, 3]. These halophilic microorganisms leading life in saline environments offer a multitude of potential applications in various fields. Their current commercial uses include fermentation of soy and fish sauce and β-carotene production. They are likely to be used in bioremediation of contaminated hypersaline brine. These organisms are known to produce stable hydrolytic enzymes such as DNAases, lipases, amylases, gelatinases and proteases capable of functioning under high salt conditions at which other proteins usually precipitate or denature. Also, they are the source of novel halophilic biomolecules having specialized applications e.g. bacteriorhodopsin for biocomputing, pigments for food colouring and compatible solutes as stress protectants [4]. Though they are well explored for their possible use in diverse areas like wastewater treatment, agriculture, bioplastics, and in medical field, their use in human nutrition is less explored.

In the current study, an attempt was made to isolate halophiles from salterns, characterize them and understand the genetic variations within them.

MATERIALS AND METHODS

Sampling sites and sampling

Brine samples were collected from different ponds in the solar salterns at Chennai and Tuticorin salt works, Tamil Nadu, India. The samples were collected in sterile poly ethylene sample bottles and labeled. The brine samples were filtered through 0.45µ membrane filters [5]. The samples were packed in ice packages and transported to the laboratory for further analysis.

Enumeration of halophilic bacteria

The collected samples were inoculated in the halophilic broth and the serial dilution was carried out using same broth. Further the plating was performed using the same media composition which included 25% w/v and 12% w/v salt concentration. The inoculated plates were incubated at 37°C for 7-10 days [6].

Morphological and Biochemical Characterization of isolates

The colonies showing specific morphology on the primary isolation plates were selected and further analyzed. From the screened isolates 8 bacterial strains were selected for the characterization based on the difference in the morphological appearance. The isolates were tested for characterization including morphological and biochemical features [7]. All tests were carried out in the Halophile medium [8]. From the identified isolates two bacterial strains, one from each concentration of salt, were selected for molecular characterization, on random basis. Molecular characterization was performed for the screened isolates by amplifying the Archaea specific 16s rRNA region.

Genomic DNA isolation

The genomic DNA was extracted from the isolated strains using standard phenol: chloroform method [9]. 16S rRNA sequence was amplified using Archaea specific primers by PCR. Amplification was carried out in a 20µl reaction set up containing 0.3µM of each primer, 0.2mM deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 100ng of template DNA sample and 1 U of Prime TaqDNA polymerase (Genetbio, Korea). The reaction tubes were subjected for Thermal cycling reactions consisted of an initial denaturation (5 min at 94°C) followed by 32 cycles of denaturation (1 min at 94°C), annealing (45 s at 49°C),and extension (1 min at 72°C), with a final extension(10 min at 72°C). The PCR product was purified (QIAquick PCR purification kit, Qiagen, Madrid, Spain) and analyzed by Sanger's DNA sequencing method. The nucleotide sequences obtained were

compared to the 16S rRNA sequences from the GenBank database using BLAST tool.

Random Amplification of Polymorphic DNA

The primers screened for RAPD included OPN6, OPN8, OPN10, OPN11, OPN12, OPN13, OPN15, of which 2 (OPN11 and OPN15) were selected for genetic diversity studies. Amplification was carried out in a 25µl reaction set up containing 0.3mM of each primer, 0.2mM deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 100ng of template DNA sample and 1 U of Prime TaqDNA polymerase (Genetbio, Korea). The reaction tubes were subjected for Thermal cycling reactions consisted of an initial denaturation (3 min at 94°C) followed by 40 cycles of denaturation (45 sec at 94°C), annealing (1 min at 36°C),and extension (1 min at 72°C), with a final extension(7 min at 72°C). The results were analyzed using 1.5% agarose gel stained with ethidium bromide. DNA bands were scored as 1 for the presence and 0 for its absence. Scoring table was generated for the obtained scoring data. Based on the scoring data Dendrogram was constructed for the different halophilic isolates obtained from salt brines using SPSS software [10].

RESULTS

Sampling and Screening of Halophilic bacteria

Collected samples were subjected for screening and isolation of Halobacteriaceae. Screening and isolation of bacteria was performed using serial dilution and plating techniques in halophilic medium. Samples subjected to serial dilution were incubated for the observation of red/pink colour colonies in Halophilic medium. Bacterial colonies with specific morphology were isolated and confirmed for pure colony and pigment production.

Microbial and Biochemical characterization

All the 8 pure colony isolates were subjected to biochemical and microbial identification. From the biochemical and microbial results, the isolated cultures A, B, C, D and E were identified as *Haloferax sp.*, isolated culture F, G and H were identified as *Halobacterium sp*.

All the isolates exhibited similar morphology being rod shaped, motile and negative for gram stain. They were positive for catalase and oxidase. Gelatin and casein were hydrolysed by cultures F, G and H which means that the isolates could be good sources of proteases. Among the 8 isolates, 5 isolates (culture A, B, C, D, E) hydrolysed tween 80 which revealed the presence of esterase activity. Utilization of carbohydrates was found to be variable among the isolates. Strains A, B, C, D, E showed identical pattern of different sugars, whereas the isolate H did not utilize any of the sugars. Rest of the isolates showed a variable pattern of acid production in presence of carbohydrates.

Molecular Characterization

Cultures A and F were selected for Molecular characterization. Whole genomic DNA was extracted from the selected strains using Phenol: Chloroform method. The isolated nucleic acids were subjected to qualitative and quantitative analysis using Agarose gel electrophoresis and UV Spectrophotometry. Amplification of the 16s rRNA region of the DNA was carried out in a thermocycler. The amplicons were visualized at 900bp (Fig 1) and subjected to DNA sequencing. The sequences so obtained were used to identify the organisms at species level, and similarity was analyzed using the BLAST tool. The sequences were submitted to the GenBank database with the accession number HM804587.1 and HM80458.1. Isolates subjected for molecular characterization were found to be *Haloferax volcanii* and *Halobacterium salinarium.*

Genetic diversity analysis

Primer screening for RAPD PCR was done with 6 Random primers using *Haloferax volcanii* and *Halobacterium salinarium* DNA samples. From these 6 primers, 2 primers (OPN11 and OPN15) gave maximum amplicons and thus were selected to develop a fingerprint. 18 amplicons were observed for 5 strains of Haloferax volcanii using OPN11 and OPN15 primers, while 18 amplicons were also observed for 3 strains of *Halobacterium salinarium* using OPN11 and OPN15 primers. Individual bands was converted into binary data matrices where 0 represent the absence of a fragment and 1 its presence of the fragment for the strains of *Haloferax volcanii*. The procedure is repeated for the strains of *Halobacterium salinarium* and the scoring table is generated. The genetic relationships among the isolates of *H. salinarium* and *H. volcanii* individually were analyzed and the phylogenetic tree (Fig 2 and 3) was developed by using SPSS software.

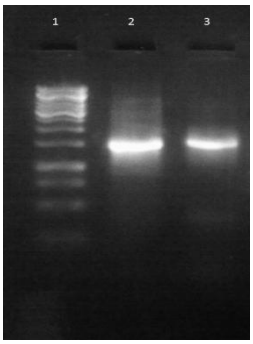

Figure 1: Amplification of 16S rRNA Gene for molecular characterization – Isolate A and F. Lane 1: amplified product of 16s rRNA gene – Strain A, Lane 2: amplified product of 16s rRNA gene – Strain F, Lane 3: 1kb DNA ladder

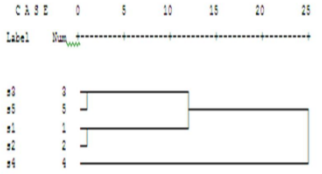

Figure 2: Hierarchical cluster analysis for closely related samples Dendrogram using Average Linkage (Between Groups). S1: culture A, S2: culture B, S3: culture C, S4: culture D, S5:culture E

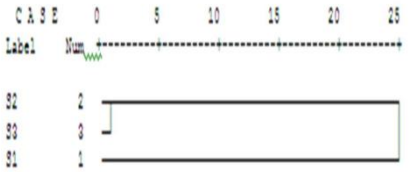

Figure 3: Hierarchical cluster analysis for closely related samples Dendrogram using Average Linkage (Between Groups). S1: culture F, S2:culture G, S3: culture H

DISCUSSION

The extremely halophilic archaea are a diverse group of euryarchaeota that inhabit hyper saline environments such as salt lakes, salt ponds and marine salterns [11].

In the current study, 8 Archaeal strains were isolated from four distinct parts of Tamil Nadu, India. All isolates examined belong to the Archaeal domain, family Halobacteriaceae. The genus *Halobacterium* is usually isolated from different ecosystems such as saline lakes [12], Dead Sea [13], halite brine inclusion and Saltern Ponds [14]. Microbial, biochemical and molecular characterization of the isolated strains in the current study revealed the presence of

Haloferax volcanii and *Halobacterium salinarium* strains in the salt brines.

RAPD technique was used to document the genetic variation among *Halobacterium salinarum* and *Haloferax volcanii* from different parts of Chennai and Tuticorine, India. RAPD amplified fragments are unique because the procedure does not amplify two distinct fragments that co-migrate on gels owing to a similar size. RAPD markers are dominant, the presence or absence of bands produced with a single primer is often assumed to be independent. The bands are identified on the basis of their intensity and separation from other products [15]. In the current study, three strains of *Halobacterium salinarium* showed relatively low genetic variation among themselves. 12.2% variation or dissimilarity was observed between Chennai and Tuticorin samples. The genetic similarity index value – F value for the two strains were 0.615.This proves to be significant value as suggested by Nei's algorithm. The estimated genetic similarity based upon the F-value was 77.8% in *Halobacterium salinarum*. This RAPD data indicates that the genome of *Halobacterium salinarium* remained conserved and didn't show much intraspecific genetic diversity among the strains.

Haloferax volcanii showed high genetic variation amongst them. 61.1% of polymorphism was noted between them. Thus the intraspecific variation was high in comparison with *Halobacterium salinarium.* The genetic similarity index value – F value for the two individuals were 0.76. The estimated genetic similarity was 39%. This proves to be significant value as suggested by Nei's algorithm. This clearly throws light on the genetic changes that has taken place amongst the strains of *Haloferax volcanii.*

Dendrogram was constructed from Similarity matrix values using UPGMA algorithm. A Statistical software package SPSS v 16 was used to develop dendrogram. The dendrogram divides into two major clusters containing *Halobacterium salinarium* and *Haloferax volcanii* together. *Halobacterium salinarium* strains were closely related to each other. *Haloferax volcanii* strains shows 23% of variation from dendrogram scale. This again validates the RAPD results.

CONCLUSION

The current study was an attempt to understand the prevalence of halophilic bacteria in salt brines and also understand their genetic diversity. This study comes in the light of the current commercial exploitation of these halophilic strains [4]. The isolated strains were subjected to molecular characterization by amplifying the 16srRNA region of the bacteria [16,17]. The genetic diversity analyses of *H. salinarium* and *H. volcanii* reveal the resistance of the former to genetic variations, probably hinting at the high tolerance level of these microorganisms, while *H.salinarium* genome was found to be highly conserved. This study analysed the impact of environmental stress on the genotypes of *H. salinarium* and *H. volcanii.*

Researches like the current one will aid in better understanding and conservation of the commercially useful strains of microorganisms.

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