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Compatible solutes from halophilicbacteria from saltpans of Goa, India

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ABSTRACT
Hypersaline environments in Goa harbour diverse microorganisms capable of thriving in a salinity range of 0-300 psu (0-30%) and elevated temperatures (20 – 50°C). Samples of solar salt collected from the salt pans ofCurca, Ribandar, and Agarwado, Goa, India. were used for isolating extremely halophilic bacteria with modified Zobell Marine Agar (ZMA) with 20% crude salt from the respective salt pans. Among the 22 isolated strains, six isolatesو could proliferate on ZMA with 25% salinity. The main aim of the present study was to explore the presence and the type of compatible solutes in hypersaline bacteria. Different methods were used to lyse the cells to extract the compatible solutes which were intracellular metabolites. Morphological and biochemical analysis, and 16S rRNA sequencing used for identification, revealed a similarity to Chromohalobactersalexigens. The isolated strain was further assessed for the presence of different compatible solutes using chemical methods and High-Performance Liquid Chromatography (HPLC). Chromohalobactersalexigens was found to have multiple compatible solutes like glutamic acid, glutamine and betaine hydrochloride which enabled this organism to survive in high salt concentrations irrespective of the location of the salt pan and the salinity.

Keywords: Solar salt, halophilic bacteria, compatible solutes.

INTRODUCTION

Salt pans, exist as both artificial man-made salt ponds and naturally occurring desert landscapes and contain various salts and minerals depending on whether they originate from sea water or other sources. In Goa, salt primarily originates from seawater (thalassohaline) through a process of evaporation, result in the production of crude solar salt. (1). Hypersaline environments are extreme habitats characterized by high salinity, elevated temperatures, low oxygen and nutrient levels, and alkaline pH values (2). Halophilic and halotolerant organisms have adapted to these extremophilic conditions. Over the years, adaptation of halophilic microorganisms to their environment has been the subject of increasing interest, with methodology for culturing, manipulation, and genetic engineering steadily advancing. (3). Understanding the adaptation of halophiles to high salinity includes several mechanisms for balancing the osmotic stress of the external medium. HalophilicArchaea (Haloarchaea) primarily use a 'salt-in' strategy, accumulating concentrations of KCl equal to NaCl in their environment, and where examined, their enzymes tolerate or require 4–5 M salt. In contrast, most halophilic Bacteria and Eukarya, largely use a 'salt-out' strategy, excluding salts and accumulating or synthesizing de novo compatible solutes (e.g. glycine betaine and other zwitterionic compounds for Bacteria, and glycerol and other polyols for Eukarya). However, a combination of adaptive mechanisms may operate.

The extreme conditions in salt pans, foster unique microbial communities capable of adapting to these extreme habitats, by mechanisms such as the production or accumulation of compatible solutes inside their cells, also known as osmoprotectants. Compatible solutes, help the microorganisms maintain their cell integrity and function under high salinity stress (4). These solutes counteract the harmful effects of osmotic stress caused by elevated salt concentrations by balancing osmotic pressure within their cells.

Compatible solutes are low molecular weight compounds reported as biomolecules such as polyols, sugar, amino acid, cyclic derivative of amino acids like mannitol, trehalose, glutamine and ectoine. These compatible solutes have many applications: ectoine [(4S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] was discovered first in the extremely halophilic phototrophic purple sulfur bacterium (5), followed by the detection of a hydroxylated derivative of ectoine, 5-hydroxyectoine in a Gram-positive soil bacterium, Streptomyces parvulus(6), ectoine and hydroxyectoine are low molecular weight solutes produced by microbes under stress conditions of salinity and temperature. These compatible solutes have a variant application including protection against heating, freezing, and drying that help in storage of enzymes and protein (7). use in skin care products and denaturing solutes, have protein-stabilizing properties, therapeutic potentials, PCR amplification and many more (8,9), Glutamic acid is used in many biochemical processes, including the synthesis of proteins, the metabolism of carbohydrates, and the regulation of pH. It is also used as a flavor enhancer, Betaine hydrochloride has been used as a dietary supplement to help with digestion and to reduce the risk of heart disease. It is a zwitterionic compound, with both a positive and negative charge, which makes betaine compatible with a variety of biological systems, and varied applications.

Hypersaline prokaryotes which produce compatible solutes, are generally either Bacteria, Archaea or Cyanobacteria and have a peculiar cell wall which sometimes cannot be lysed with the conventional methods available, or sometimes easy to lyse by suspending them in media devoid of salt. In the present study, we have isolated bacteria from 3 salt pans which were stubborn to lyse using physical methods.Therefore, the option of lyses was by chemical methods. We have further found similar compatible solutes in the same identified isolate from three different salt pans, of Goa depending on salinity from 10 to 25 %.

2. MATERIALS AND METHODS

Sampling

Samples of salt crystals were collected from salt pans of Curca, Ribandar and Agarwado in North Goa, India.during the peak of salt manufacturing season. Samples of salt crystal from each salt pan were collected in sterile bottles and transported to the laboratory for processing within a 24h period. The physio-chemical parameters,pH, temperature and salinity were measured during sample collection.

Isolation of Hypersaline Bacteria

Serial dilutions were performed using sterile 200psu saline water prepared with the natural salt from the respective salt pans, to isolate hypersaline bacteria. Subsequently, 0.2 ml of the sample from dilutions of 10^{-1}, 10^{-2} and 10^{-3}were plated (100 µl each) on Zobell Marine Agar (ZMA) supplemented with crude salt to achieve a salinity of 200 psu. Plates were then incubated at 34 ± 2°C for two weeks. Morphologically distinct bacterial colonies were selected and subcultured onto respective isolation media at 200 psu. Isolates were preserved on ZMA by storing them at 4°C on slants and as glycerol stocks at -20°C.

Media and Chemicals

The isolation media used in this study was supplemented with solar sea salt sourced from salterns of Goa to simulate the ecobiome conditions. All media and chemicals utilized in the experiments were procured from Hi_Media, Mumbai, India (10,11).

Identification: Identification was carried out through morphological characterization, microscopic observation, biochemical tests, and 16S rRNA gene sequencing. Genomic DNA was extracted using a DNA isolation kit (Hi Media).

Salinity Test

Bacterial isolates were spot-tested by streaking them on MD medium (Soya Peptone 5g/L, Tryptone 15g/L, NaCl 5g/L, distilled water 1000 ml, final pH (at 25°C) 7.2+ 0.2) with salinities ranging from 5 psu to 300 psu (50, 100, 150, 200, 250, 300psu_crude salt) and on MD agar prepared with varying salinities (0 to 300psu using crude salt). The isolates were streaked on the respective agar plates and incubated at 36 ± 2 °Cfor 48 hours.

Extraction of Compatible Solutes

Bacterial isolates (a loopful) were inoculated into Zobell Marine broth (ZMB) and incubated at 36 ± 2°C for 72 hours. Subsequently pellets were collected by centrifugation, and extraction was performed using two-phase extraction methods:

1-Ethanol extraction:

Bacterial cells were inoculated in Zobell Marine Broth (ZMB) adjusted to 200psu with crude salt and incubated for 24hr at 37°C on a temperature controlled shaker incubator at 150 RPM (Rivotek, India.). The pellet was collected after centrifugation (20 min at 10000 RPM at 4°C, and washed with saline water of the same salinity. The pellet was suspended in 80% of ethanol and mixed well and boiled till dry. The dry pellet was mixed with (water: chloroform) (2:1 v/v) shaking it for 2 minutes and kept in a separating funnel to separate the aqueous part which was used for further tests. (12)

2-Methanol, chloroform and water (lipid extractions)
Bacterial cells were inoculated in 100ml of ZMB at 200psu of salinity and incubated for 24hr at 37°C at 10000 rpm. The pellet was collected after centrifugation at 10000 rpm for 20 min at 4°C, and washed at same salinity .4 ml of methanol - chloroform (2:1 v/v) was added to the cell pellet with 1 ml of distilled water, incubated at room temperature (30+ °C) for 4 hrs and centrifuged to remove the cell debris. 2 ml of chloroform and 2 ml water were added to the supernatant, and vortexed, which was used for further tests. The upper (aqueous) layer was collected for further test (13).

Characterisation of Compatible Solutes
Qualitative tests were carried outto characterise the compatible solutes. The presence of **Proline** was checked by adding 2-3 drops of ninhydrin solution to 1mlof the aqueous sample, resulting in a yellow coloration (14). **Glycine** was identified by adding 5 drops of dilute hydrochloric acid and 1 ml of sodium nitrite solution to the1 ml of sample. The generation of a colourless gas indicated its presence. Glutamic acid was detected by mixing the 1ml of sample with 1 drop of ninhydrin, sodium acetate, and NaOH, resulting in a dark bluish-violet colour after heating for 5 mints. **Betaine hydrochloride** (BHCL) was identified by mixing the sample with bromocresol green solution, producing a yellow colour. **Choline chloride** was determined by the formation of a red precipitate upon adding 0.5 ml iodine solution to 0.5ml of sample and followed by 0.5 ml of NaOH.
High Performance Liquid Chromatography (HPLC):
The aqueous extract (20) from the ethanol extraction was further used for the Identification and quantification of compatible solutes on an Agilent 1260 Infinity HPLC system with a reverse-phase column (Agilent Advance Bio AAA, USA.).(15, 16, 17)

Morphological Studies and Molecular Identification
Pure bacterial colonies were observed and examined for pigment production, morphology: form, elevation, margin, etc. Gram character, and motility was assessed using hanging drop method(REF). For molecular identification, the genomic DNA from six bacterial strains was extracted using the HiPur ATM Bacterial Genomic DNA purification kit (Hi-Media, India) as per the manufacturer's instructions. This purified DNA served as a template for amplifying the 16S rRNA gene via Polymerase Chain Reaction (PCR). The PCR employed Universal Bacterial primers: forward primer 27F (AGAGTTTGATCCTGGCTCCAG) and reverse primer 1492R (TACGGTTACCTTGTTACGACTT). The reaction mixture comprised 20 µLMilliQ water, 3 µL template, 1 µL each of forward and reverse primers, and 25 µL 2X Taq polymerase mix. Cycling conditions included an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. The resulting PCR products were run on a 0.8% low melting agarose gel alongside a Step Up™ 500 bp DNA ladder (Genei, India). The anticipated 1.5 kb band was excised, purified using the AxyPrepTM DNA Gel Extraction Kit (Axygen Biosciences, USA), and sequenced by AgriGenome Labs, Pvt. Ltd, India. Raw sequences were assembled into contigs using BioEdit software (version 7.2.5) and aligned. The contigs were compared to the GenBank database using NCBI-BLAST, and a phylogenetic tree of the 16S rRNA gene sequences was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software (version 11) via the Neighbour-joining method(18,19,20). Evolutionary distances were computed using the Tamura-Nei method with 1000 bootstrap replicates, involving 21 nucleotide sequences. Positions with less than 95% site coverage were excluded, allowing for fewer than 5% alignment gaps, ambiguous bases, or missing data at any position. The bacterial isolate sequence has been deposited in GenBank.

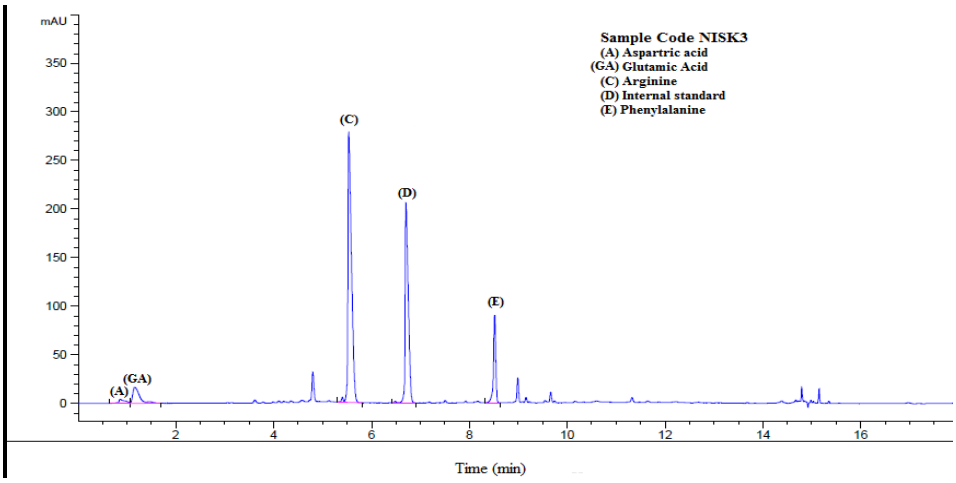
RESULTS
Isolation of Hypersaline Bacteria
A total of 24 morphologically different hypersaline bacterial colonies were isolated at 200 psu, from the salt crystals collected from the salt pans. These 24 strains also grew on ZMA at salinities ranging from 25 to 300 psu. Six strains exhibited growth at 250 psu salinity. viz. NSK3, NSK4, NSK6, NSK51 and CH.H did not growth in the absence of salt with a minimal requirement of at least 50psu ofsalt.

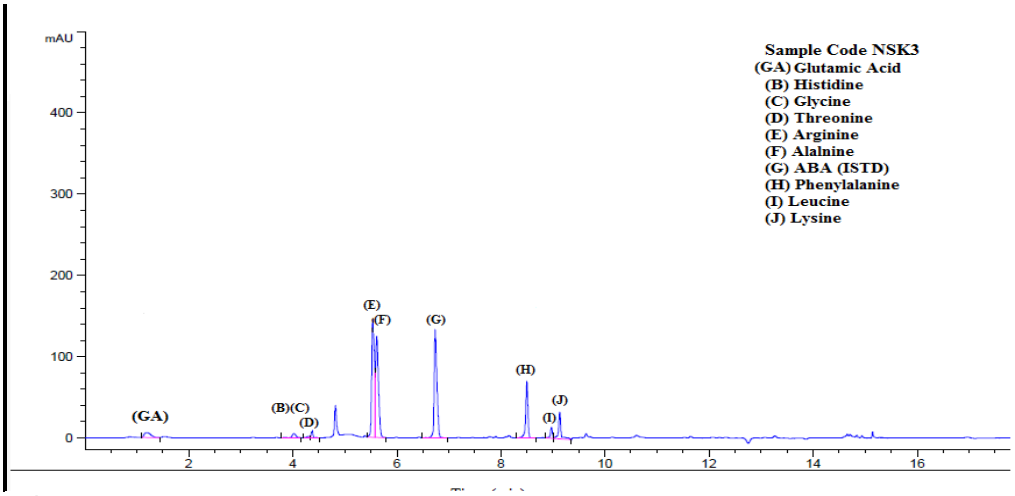
Production of Compatible Solutes
Qualitative analysis of the 24 isolates showed the production of Glutamic Acid by isolates NISK3, NSK3, NSK4, NSK6, NSK51 and CH.H. CU tested positive for choline chloride, and Betaine Hydrochloride tested positive in NISK3.
High-Performance Liquid Chromatography (HPLC) analysis indicated the production of these compatible solutes in different concentrations. (Table 1 for HPLC analysis results.).
Out of 24 isolates,6 of them showed best production and high concentrations of glutamic acid, Arginine and Alanine.Fig (1).

Table 1: High Performance Liquid Chromatography(HPLC) analysis of intracellular amino acids.

Amino acid ↓	Sample code →concentration in µg /mg					
	NSK3	NSK4	NSK6	NSK51	NISK3	CH.H
Aspartic acid	0.000	0.456	0.467	0.549	0.143	0.877
Glutamic acid	1.120	2.283	7.780	0.887	0.638	6.787
Asparagine	0.000	0.000	0.097	0.028	0.000	0.046
Serine	0.000	0.072	0.016	0.043	0.000	0.033
Glutamine	0.000	0.246	9.248	1.323	0.000	0.660
Histidine	0.798	0.354	0.269	10.819	0.000	0.111
Glycine	0.080	0.155	0.112	0.191	0.000	0.255
Threonine	0.489	0.069	0.019	0.503	0.000	0.056
Arginine	10.982	3.015	1.628	9.368	5.624	4.679
Alanine	5.245	22.420	9.140	1.836	0.000	17.177
Tyrosine	0.000	0.000	0.021	0.087	0.000	0.000
Cysteine	0.000	0.000	0.105	0.033	0.000	0.134
Valine	0.000	0.000	0.007	0.320	0.000	0.032
Methionine	0.000	0.000	0.007	0.263	0.000	0.162
Tryptophan	0.000	0.000	0.031	1.368	0.000	0.000
Phenylalanine	3.508	0.000	0.009	0.806	1.039	0.082
Isoleucine	0.000	0.077	0.156	0.258	0.000	0.069
Leucine	0.503	0.492	0.006	0.755	0.000	0.948
Lysine	1.088	1.477	0.075	0.294	0.000	0.027
TOTAL	23.815	31.115	29.192	29.728	7.444	32.135

From the selected sixisolates, which were grown at a salinity of 200psu to screen for the presence of compatible solutes, we found the presence of glutamic acid in concentrations ranging from 0.6 to 7.78 □ g/ml in all the 6 isolates, since this was also previously confirmed in the qualitative test carried out Fig(1).Further, along with glutamic acid NSK3 showed 10.98 □ g/ml concentration of arginine and 5.24□ g/ml alanine. Similarly, NSK4 showed 10% higher concentration of alanine 22.42□ g/mlas compare to glutamic acidconcentration. NSK6 also showed a high concentration of alanine 9.140□ g/ml as compare to glutamic acid which was 7.78□ g/ml similarly NSK51 had a very low concentration of glutamic acid 0.887 □ g/ml and very high concentration of histidine and arginine being 10.8□ g/mland 9.36□ g/mlrespectively.
Similarly,NISK3 contained a fivefold increase in arginine concentration that is 5.62□ g/mland CH.H had 17.17 □ g/ml of alanine, 4.67□ g/mlof arginine and 6.78□ g/mlof glutamic acid.




mAU
Sample Code NSK3
(GA) Glutamic Acid
(B) Histidine
(C) Glycine
(D) Threonine
(E) Arginine
(F) Alalnine
(G) ABA (ISTD)
(H) Phenylalanine
(I) Leucine
(J) Lysine
(GA)
(B)(C)
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(F)
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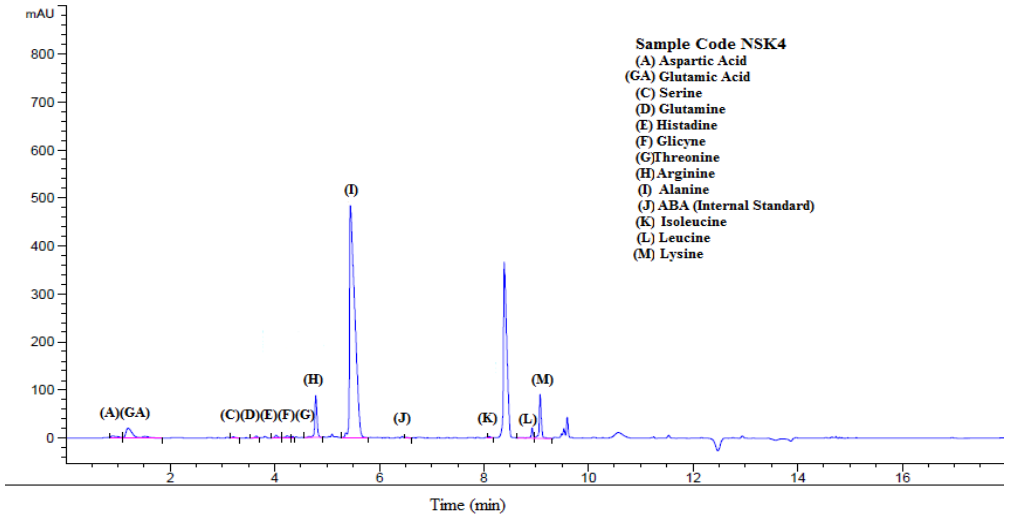
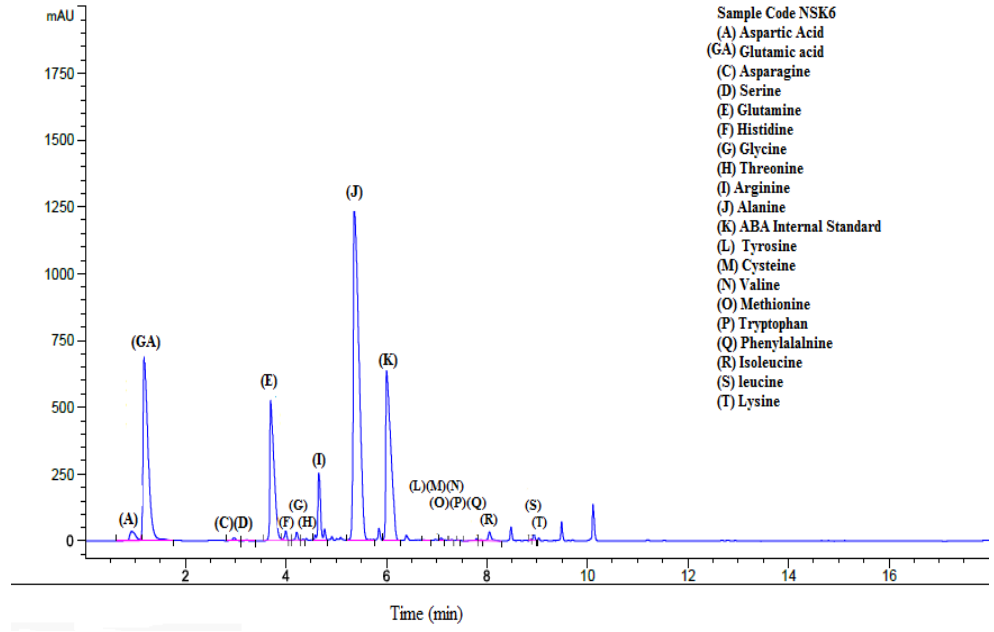
(H)
(I)
(J)


mAU
Sample Code NSK4
(A) Aspartic Acid
(GA) Glutamic Acid
(C) Serine
(D) Glutamine
(E) Histadine
(F) Glicyne
(G)Threonine
(H)Arginine
(I) Alanine
(J) ABA (Internal Standard)
(K) Isoleucine
(L) Leucine
(M) Lysine
(A)(GA)
(C)(D)(E)(F)(G)
(H)
(I)
(J)
(K)
(L)
(M)
Time (min)


mAU
Sample Code NSK6
(A) Aspartic Acid
(GA) Glutamic acid
(C) Asparagine
(D) Serine
(E) Glutamine
(F) Histidine
(G) Glycine
(H) Threonine
(I) Arginine
(J) Alanine
(K) ABA Internal Standard
(L) Tyrosine
(M) Cysteine
(N) Valine
(O) Methionine
(P) Tryptophan
(Q) Phenylalalnine
(R) Isoleucine
(S) leucine
(T) Lysine
(A)
(GA)
(C)(D)
(E)
(F) (H)
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(T)
Time (min)

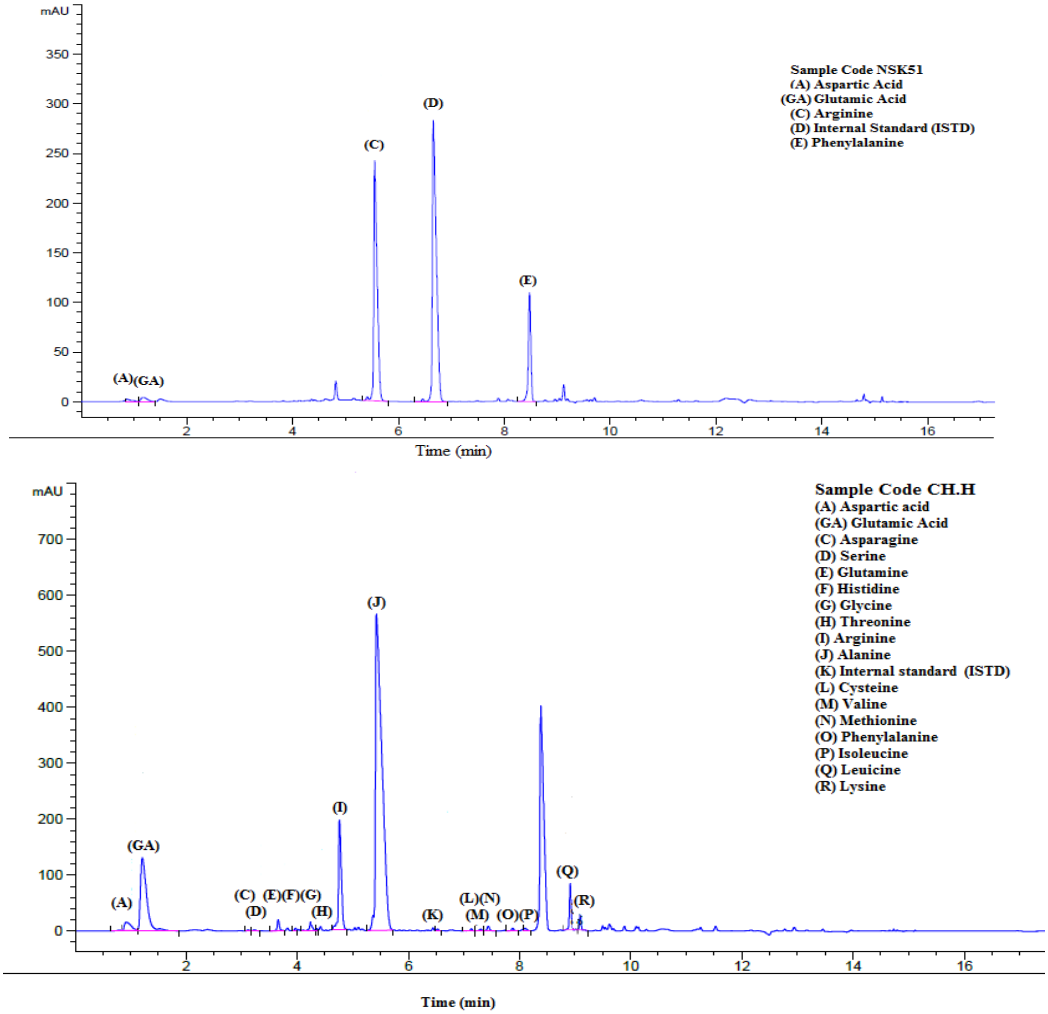

Fig 1. Chromatogram of NISK3, NSK3, NSK4, NSK6, NSK51 and CH.H by using High PerformanceLiquid Chromatography HPLC.

Characterization of Isolated Strains and Phylogenetic Analysis

The six isolates which grew on 250 psu, showed minor variations in the biochemical characteristics and one isolate (NSK6) was distinct in its morphology. The 5 similar isolates which were isolated from 3 different salt pans, revealed creamy white to light yellow colour, were short rods and were found to be Gram negative and NSK6 was orange in colour and exhibited elongated rod-shaped forms under oil immersion 100X microscope and was Gram positive. Five isolates that grew at 250 psu were similar toChromohalobacter sp., and one isolate was similar to Halobacillus sp. after 16S rRNA sequencing.

Table 2. Halophilic bacterial Compatible solute from different salt pans of Goa produced from 100 psu and 240 psu.

Bacterial sp. And Code	Location	100 psu	250psu
Bacillus thuringiensis NSK3	Curca	Glutamic acid	Glutamic acid
ChromohalobacterspNSK10	Curca	Glutamic acid	Glutamic acid
ChromohalobacterspNSK4	Curca	Glutamic acid	Glutamic acid
ChromohalobacterspSH1	Ribandar	Glutamic acid	Glutamic acid
ChromohalobacterspNSK51	Agarwado	Glutamic acid	Glutamic acid
ChromohalobacterspNISK3	Ribandar	Betaine hydrochloride, Glutamic acid	Betaine hydrochloride, Glutamic acid, Glutamine
Halobacillus sp. NSK6	Curca	Glutamic acid	Glutamic acid + Glutamine

Table 3. Characteristics of isolated hypersaline bacteria based on 16S rRNA gene sequencing.

Culture code	GenBank accession number	Length of the sequence	Percentage similarity	Culture identification (Sequence ID)
NISK3	PQ282527	1400	96%	Chromohalobactersalexigens
NSK3	PQ282514	1316	99.39%	Bacillus thuringiensis
NSK4	PQ270472	1403	96%	Chromohalobactersalexigens
NSK6	PQ282505	1383	99%	Halobacillusalkaliphillus
NSK10	PP854578	1359	96.40%	Chromohalobactersalexigens
NSK51	PQ282526	1400	96.10%	Chromohalobactersalexigens
Sh1	PQ282515	1402	96.00%	Chromohalobactersalexigens

Phylogenetic Analysis

Six strains which were identified by 16S rRNA gene sequencing the sequences have been submitted to the Gen Bank database under accession numbers as shown in (Table3). A phylogenetic tree based on the 16S rRNA gene sequences was constructed for NSK3 using MEGA software by the Neighbour-joining method, with Paucibactertoxinivorans used as an outgroup.The five isolates with similar morphology were identified as Chromohalobactersalexigens, NSK6 was similar to Halobacillusalkaliphilusand NSK3 was similar to Bacillus thuringiensis.

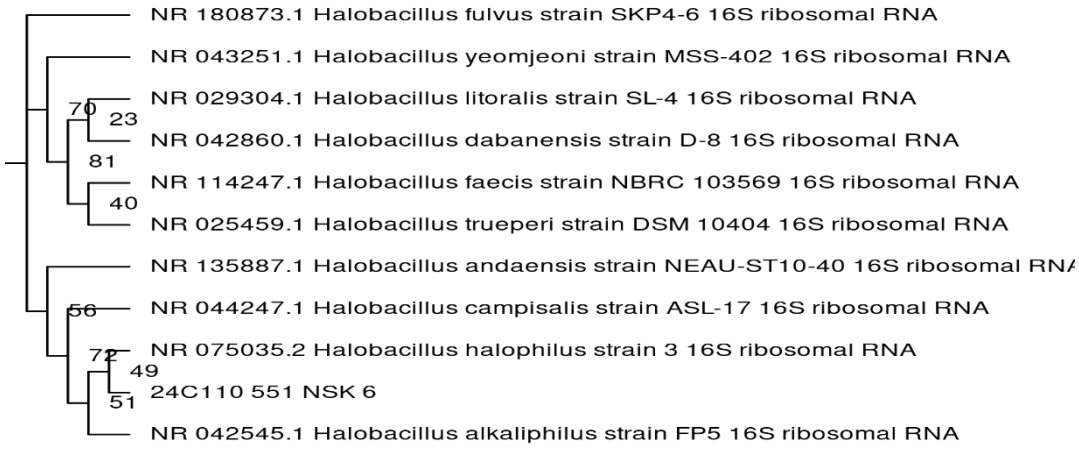

Fig. 2: Phylogenetic tree describe the relationship of the Halobacillusbacteria NSK6 with other isolates based on similarity 16S rRNAgene sequence.

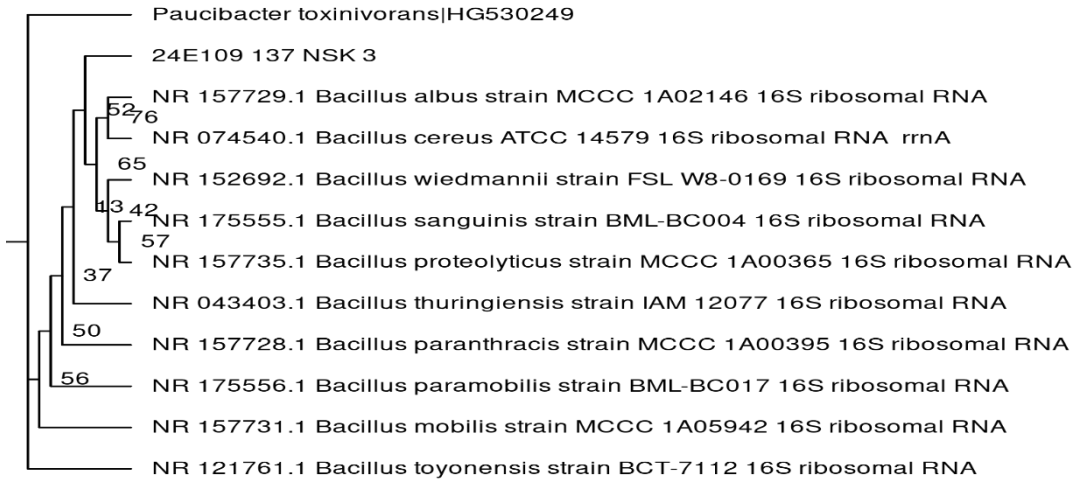

Fig. 3: Phylogenetic tree describe the relationship of the Halobacillus bacteria NSK3 with other isolates based on similarity 16S rRNAgene sequence.

DISCUSSION

Compatible solutes are generally produce by hypersaline bacteria in osmotically stress conditions. Compatible solutes are osmolytes which are sensitive to salinity of growth media, in the present study we have checked up the production of different compatible solutes in the same chromohalobactersp from different salterns of Goa, India. The same compatible solute were produce at 100 psu and 250 psuthe presents of glutamic acid, betaine hydrochloride, choline chloride, alanine, glutamine and histidine.

The isolation of bacterial strains from salt crystals collected at a salinity of 200 psu highlights the adaptation of these microorganisms to the challenging conditions prevailing in the salt pans. This finding is consistent with previous studies of Oren indicating the presence of specialized microbial communities in hypersaline environments (21).

The present investigation contributes valuable insights into the microbial ecology and the innate behaviour of adaptation to the hypersaline conditions in salt pans ecosystems. The study demonstrates the production of similar intracellular compatible solutes by similar strains located in different salt pans irrespective of the location of the salt pan in Goa and irrespective of the high salinity, enabling these bacteria of thriving in these extreme environmental conditions which is attributed to the synthesis of osmolytes such as betaine hydrochloride, glutamic acid andcholine chloride.

In the present study similar bacteria viz.Halobacillussp.Chromohalobactersp. were isolated from three geographically distinctly located salt pans of Goa viz.Curca, Ribandar and Agarwado.Halophilic microorganism in India have been isolated from the sediment and water of salt pans.Gawas and Kerkar,(22) have also reported Chromohalobactersalexigens and Halobacillus sp. from Curca and Agarwado salt pans of Goa.In the present study we isolated the bacteria from crystals of crude salt at 200 psu. We have isolated 24 cultures at 200 psu and 6 of them grew up to 250 psu, producing 3 different typesof compatible solutes. These isolates fall under extreme halotolerant bacteria. Our resultshave also been compared to Fritze (23) recommendation that phenotypic analyses is substantiated with 16SrRNA gene sequence data.

Kushneret al. (24) reportedhalophilic bacteria grouped by using salt tolerance tests from 1**0** to 320 psu. Our result agrees with Rodriguez-Valera, F. (25) who reported bacteria isolated from 15 psu and the interaction of halophilic microorganisms with ecological extremes of salinitywhere the maximum salinity used by him was 180 psu whereas in the present study, we used 250 psuas the maximum salinity for growth.

The salinity tolerance tests conducted in this study revealed varying degrees of tolerance among the isolated bacterial cultures (5% to 30%), with six cultures exhibiting growth even at a salinity of 250 psu. This underscores the remarkable adaptability and resilience of these bacteria to survive and proliferate under extreme salinity conditions, which is crucial for their ecological niche in salt pan ecosystems (26).

From the selected six isolates, which were grown at a salinity of 200psu to screen for the presence of compatible solutes, we found the presence of glutamic acid in concentrations ranging from 0.6 to 7.78 □ g/ml in all thesix isolates, since this was also previously confirmed in the qualitative test carried out. Fig (1). Further, along with glutamic acid, NSK3 showed 10.98□ g/ml, concentration of arginine and 5.24□ g/mlalanine. Similarly, NSK4 showed 10% higher concentration of alanine 22.42□ g/mlas compare to glutamic acidconcentration. NSK6 also showed a high concentration of alanine 9.14□ g/mlas compare to Glutamic acid which was 7.78□ g/mlsimilarly NSK51 had a very low concentration of glutamic acid 0.8□ g/mland very high concentration of histidine and arginine being 10.8 and 9.36□ g/mlrespectively. Similarly,NISK3 contained a fivefold increase in arginine con that is 5.62□ g/ml and CH.H had 17.17 □ g/ml of alanine, 4.67□ g/ml of arginine and 6.78□ g/ml of glutamic acid.

From the above results, it is evident that the same Chromohalobactersp isolated from multiple salt pans of Goa, at the salinity of 200psu, accumulates different types of compatible solutes as well as it is observed that there is also adistinct intracellular amino acid profile which is remarkably high.We have currently no reasonable explanation for this observation. It is possible that accumulation of high concentration of a particular amino acid can act as a distresses in hypersaline conditions?

Follow up studies are required to define the precise reason for the accumulation of these amino acid intracellularly.

Moreover, the identification of compatible solute production by the isolated bacterial strains through biochemical tests and HPLC analysis indicates their potential biotechnological applications. Compatible solutes play a crucial role in osmotic adaptation by maintaining cell integrity and function under high salinity stress (27). The production of compatible solutes by hypersaline bacteria suggests their utility in various biotechnological applications, including stress tolerance enhancement in crops, bioremediation of saline soils, and pharmaceutical production (28).

The morphological and biochemical characterization of the isolated strains, coupled with their identification through 16S rRNA gene sequencing, enhances our understanding of the diversity and taxonomy of hypersaline bacteria. The identification of Chromohalobacter and Halobacillus species among the isolated strains underscores their prevalence and ecological importance in hypersaline ecosystems (29).

Phylogenetic analysis based on 16S rRNA gene sequences further supports the taxonomic classification of the isolated strains and provides insights into their evolutionary relationships. The construction of a phylogenetic tree elucidates the genetic relatedness of the isolated strains to known bacterial species, thereby contributing to our understanding of microbial diversity and evolution in hypersaline environments (30).

In conclusion, this study advances our knowledge of microbial adaptation and diversity in hypersaline environments of Goan natural salt and highlights the biotechnological potential of hypersaline bacteria by the presence of these compatible solutes and elevated concentrations of specific amino acids. Further research on the metabolic pathways involved in compatible solute synthesis and the ecological roles of these bacteria in salt pans ecosystems will deepen our understanding of their biotechnological applications and ecological significance.

Despite the significance of compatible solutes, other studies, report marine bacteria from water and sediments of the salt pans of Goa, capable of producing these compounds. This study focuses on isolating bacteria from the salt crystals of the marine salterns of Curca, Ribandar, and Agarwado, Goa. The present bacterial strains produce betaine hydrochloride, choline chloride and Glutamic acid at both the salinities of 100 psu and 250 psu with crude salt collected from the respective salt pans which was added to the growth media. (ZMB). Understanding the mechanisms underlying compatible solute synthesis and tolerance in these bacteria offers insights into cellular adaptation strategies under extreme environmental conditions.

CONCLUSION

Salt pans of Goa, India contain a diversity of bacteria potentially capable to survive in different ranges of salinity from 3.5psu to 500psu, high temperature upto 45°C, pH range from 7.7-8.25 and produce different kinds of compatible solutes.

This is first study about diversity of compatible solutes from a Chromohalobacter spp. isolated from the salt crystals of geographical distinct salt pans in Goa, India. These hypersaline environments are inhabited with different bacterial species that produce compatible solutes which are potential moisturisers and also have various other applications.

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REFERENCES

1. Kerkar, S. (2004). Ecology of hypersaline microorganisms.
2. Ventosa A.,(2006). Unusual microorganisms from unusual habitats: Hypersaline environments. In: logan N.A., Lppin-scott H.M., Oyston P.C.F., editors. Prokaryotic Diversity-Mechanism and significance. Cambridge University Press; Cambridge, UK.223-253.
3. Martínez-Espinosa, R. M., Kumar, S., Upadhyay, S. K., &Orhan, F. (2023). Adaptation of halophilic/halotolerant microorganisms and their applications. Frontiers in Microbiology, 14, 1252921.
4. DasSarma S., DasSarma P. (2015) Halophiles and their enzymes: negativity put to good use. Current Opinion in Microbiology. 25: 120-126.
5. María de Lourdes Moreno, Dolores Pérez, María Teresa García, EncarnaciónMellado (2013) Halophilic Bacteria as a Source of Novel Hydrolytic Enzymes. Life (Basel). 3(1), 38–51.
6. Galinski, E.A.; Pfeiffer, H.P.; Trüper, H.G. 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid. A novel cyclic amino acid from halophilic phototrophic bacteria of the genus Ectothiorhodospira. Eur. J. Biochem. 1985, 149, 135–139.
7. Inbar, L.; Lapidot, A. The structure and biosynthesis of new tetrahydropyrimidine derivatives in actinomycin D producer Streptomyces parvulus. Use of 13C- and 15N-labeled l-glutamate and 13C and 15N NMR spectroscopy. J. Biol. Chem. 1988, 263, 16014–16022.
8. Lippert, K.; Galinski, E. A. Enzyme stabilisation by ectoinetype compatible solutes: protection against heating, freezing and drying. Appl. Microbiol. Biotechnol. 1994, 37, 61-65.
9. Da Costa, M. S., Santos, H., &Galinski, E. A. (1998). An overview of the role and diversity of compatible solutes in Bacteria and Archaea. Biotechnology of extremophiles, 117-153.
10. Shivanand, P., &Mugeraya, G. (2011). Halophilic bacteria and their compatible solutes–osmoregulation and potential applications. Current science, 1516-1521.
11. Deng, Y., Xu, G., & Sui, L. (2015). Isolation and characterization of halophilic bacteria and archaea from salt ponds in HanguSaltworks, Tianjin, China. Chinese journal of oceanology and limnology, 33(4), 862-868.

12. Sowers, K. R., D. E. Robertson, D. Noll, R. P. Gunsalus, and M. F. Roberts. 1990. N,-acetyl-p-lysine: an osmolyte synthesized by methanogenicarchaebacteria. Proc. Natl. Acad. Sci.USA 87:9083-9087.
13. Purohit, M. S., Tipre, D. R., Shah, M. B., & Dave, S. R. (2016). Isolation of halotolerant and halophilic bacteria and screening of their poly enzyme potential.
14. Smith, A. M., &Agiza, A. H. (1951). The determination of amino-acids colorimetrically by the ninhydrin reaction. Analyst, 76(908), 623-627.
15. Xu, W., Zhong, C., Zou, C., Wang, B., & Zhang, N. (2020). Analytical methods for amino acid determination in organisms. Amino Acids, 52(8), 1071-1088.
16. Lai, M. C., Sowers, K. R., Robertson, D. E., Roberts, M. F., &Gunsalus, R. P. (1991). Distribution of compatible solutes in the halophilicmethanogenicarchaebacteria. Journal of bacteriology, 173(17), 5352-5358.
17. Gauthankar, M., Khandeparker, R., Shivaramu, M. S., Salkar, K., Sreepada, R. A., &Paingankar, M. (2021). Comparative assessment of amino acids composition in two types of marine fish silage. Scientific Reports, 11(1), 15235.
18. Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular biology and evolution, 24(8), 1596-1599.
19. Docking of vaginal lactobacillus isolates as a potential bactericidal agent in Kirkuk 2022.
20. Thaker, H. M., Tawfeeq, A. A., & Ali, A. A. (2023). Docking of vaginal Lactobacillus isolates as a potential bactericidal agent in Kirkuk 2022. Medical Journal of Babylon, 20(Supplement 1), S136-S140.
21. Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology, 37(8), 911-917.
22. Oren A. (2013) Life at high salt concentrations, intracellular KCl concentrations, and acidic proteomes. Front. Microbiol. 4: 315.
23. Gawas, P., &Kerkar, S. (2023). Bacterial diversity and community structure of salt pans from Goa, India. Frontiers in Microbiology, 14, 1230929.
24. Fritze, D. 2002. Bacillus identification- traditional approaches. In: Applications and Systematics of Bacillus and Relatives, (Eds.): R. Berkeley, M. Heyndrickx, N. Logan and P. De Vos. pp. 100-122. Oxford: Blackwell.
25. Kushner, D.J. 1993. Growth and nutrition of halophilic bacteria. In: The Biology of Halophilic Bacteria. (Eds.): R.H. Vreeland and L.I. Hochstein. pp. 87-89. Boca Raton: CRC Press.
26. Rodriguez-Valera, F. (2020). Introduction to saline environments. In The biology of halophilic bacteria (pp. 1-23). CRC Press.
27. Empadinhas N., da Costa M.S. (2011) Osmoadaptation mechanisms in prokaryotes: distribution of compatible solutes. IntMicrobiol. 14(3): 151-161.
28. Cánovas D., Vargas C., Csonka L.N. et al. (2017) Osmoprotectants in Halophilic Bacteria: Vast Potential for Applications in Biotechnology and Biomedicine. Front. Microbiol. 8: 1575.
29. Ventosa A., de la Haba R.R., Sánchez-Porro C. et al. (2014) Halophiles and their biomolecules: recent advances and future applications in biomedicine. Mar Drugs. 12(1): 5168-5183.
30. Ludwig W., Schleifer K.H. (1994) Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. FEMS Microbiol Rev. 15(2-3): 155-173.