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Isolation and identification of Ectoine-producing Halophilic Bacteria from the Deccan Trap Region of India

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Abstract

The aim of this study was to isolate and identify microorganisms that can produce ectoine from the Deccan trap area of the states of Hyderabad and Karnataka.

The Microorganism that produce ectoine were found in the soil samples from the Deccan Trap region. A 16S rDNA sequencing analysis that was done to identify the strain. The biological characteristics of the strain were also investigated. Studies were conducted on the capacity to accumulate ectoine at various concentrations of NaCl. The effect of an osmotic downshock solution affected the release of ectoine and rate of survival.

An isolate (strain DSH-3) that produced ectoine was selected for further study, a total 33 bacterial colonies that were isolated from soil samples. Strain DSH-3 was identified as *Halomonas spps* and it was found to be closely related to *Halomonas daqingensis,* with 99.21% identity based on 16S rDNA sequencing. At a concentration of 15% (w/v) NaCl, the strain DSH-3 showed a total cell dry weight (CDW) of 7.8 wt%. Maximum total ectoine synthesis of 0.5 wt% was attained by strains DSH-3 at 15% NaCl concentration. After incubation the bacterium DSH-3 for 30 min in a 6% of sodium chloride the effect of downshock solution, with a survival rate of 96% for bacteria and a 40% release of ectoine, it showed impressive results.

Some bacteria appear to use ectoine accumulation and secretion as a common adaptation strategy to survive in a saline milieu. The level of our understanding, for the first time the soil sample collected from a Deccan trap area revealed the presence of halophilic bacteria that produce ectoine.

Key words : Deccan trap, Compatible solutes, Halophilic bacteria, Halomonas and Ectoine.

One of the most volcanic landscapes on Earth, the Deccan Traps are a massive igneous province located between 17° and 24°N and 73° and 74°E on the Deccan Plateau of west-central India. Plate tectonics and erosion together decreased the Deccan Trap region to its present extent[28]. Different type of rocks occur naturally in the Deccan Plateau like Cinder, China clay, Coquina, Blue rock, Granite, Grit stone, Gypsum a mineral, Limestone, Marble, Sandstone, and Slate. Limestone is one of the chief available sedimentary rocks of this region and 10% of the total volume of all sedimentary rocks is made up of this rock type. According to Dunham[6] and Folk[8], limestone is a sedimentary rock mostly made up of the aragonite, minerals calcite, and Calcium carbonate ($CaCO_3$) exists in a wide variety of crystal shapes. These are mostly utilised as raw ingredients in the manufacture of cement. Huge quarries with typical habitat are left behind after their excavation for the aforementioned purpose from the soil. Extreme environments stand for distinct ecosystems that support novel biodiversity.

Extremes in temperature, salinity, water scarcity, and pH are only a few of the many different situations where microbial communities can be discovered. These organisms, known as extremophiles, have evolved adaptive characteristics that allow them to thrive at their best in a number of extreme conditions, whereas polyextremophiles thrive ideally under a variety of environments[26]. According to Van den Burg (2003), these extremophiles may survive in some of the least hospitable environments on Earth in terms of salinity (halophiles for 2-5 M NaCl), temperature (2 to 20°C for psychrophiles and 60 to 115°C for thermophiles) and pH (4 for acidophiles and >9 for alkaliphiles). Two separate methods are used by organisms that may flourish in habitats with high concentrations of salt to maintain osmotic equilibrium across the cell membrane. The first approach, commonly observed in archaeal *Halobacteriaceae*, involves using non-organic molecules like K+ and Cl- ions to maintain Osmotic balance. However, this strategy is limited in its adaptability and is mostly suited for specific high-salt environments. On the other hand, the second approach, widely adopted by eubacterial halophiles, involves the accumulation of small, low-molecular-weight organic molecules to balance the concentration of salt in their condition. This second method allows for a more flexible and versatile adaptation to varying salinity levels. These minuscule molecules can be zwitterionic, neutral, or anionic (K+ is usually utilised as a counterion). Compatible solutes include sugars (trehalose), inositols (myo-inositol), amino acids (such as ectoine, proline, amino sulfone acids, and taurine), and betaines (glycine betaine). These substances are all highly polar and have relatively small molecules. Osmolytes like proline can be found in the first metabolic pathways. The use of osmolytes as a mechanism has the benefit of being more adaptable and suited for use over a larger range of salinity. Organic osmolytes serve an important function in preserving and safeguarding intracellular equilibrium.

Osmolytes play a crucial role in responding to changes in extracellular osmolarity. These compounds help cells adjust to increased salinity by accumulating within the cell, causing a decrease in their intracellular concentration. Osmolyte concentrations can vary from

millimolar to 1-2 M, indicating that certain osmolytes, known as compatible solutes, can be tolerated by the cell's macromolecular machinery across a wide concentration range. Galinski *et al.*,[9] Ectoine, initially discovered in Halorhodospira halochloris, a halophilic phototrophic bacterium, has been found in various halophilic and halotolerant microbes. Notably, species like *Escherichia coli,* Bacillus subtilis, Corynebacterium glutamicum, and Sinorhizobium melilotii are unable to synthesize ectoine (Jebbar and et al., 2005). Another derivative of ectoine, called hydroxyectoine, was identified in Streptomyces bacteria along with actinomycin-D. Interestingly, the concentration of hydroxyectoine often increases with higher temperatures in Streptomyces. Overall, these osmolytes, including ectoine and hydroxyectoine, act as compatible solutes, enabling cells to adapt to varying external osmolarity, particularly in high-salinity environments.

Ectoine, a natural compound derived from extremophilic microorganisms, is making significant contributions in diverse industries. In cosmetics and skincare, it's known for hydrating the skin and shielding it from UV radiation and pollution. In pharmaceuticals, ectoine stabilizes proteins and enzymes in formulations. Biotechnology harnesses ectoine for stress protection during fermentation. Ectoine's potential for food preservation is being explored and it's used in dermatology for its anti-inflammatory properties. In medicine, ectoine aids wound healing and manages inflammation. Ectoine-producing microorganisms are studied for environmental remediation. These applications highlight the versatility and growing importance of ectoine in various fields.

The specific objectives of the study:

a. Isolate halophilic bacteria from soil samples collected from the Deccan trap region.
b. Identify the isolated bacteria using 16S rRNA sequencing.
c. Investigate the ability of the isolated bacteria to accumulate ectoine.
d. Assess the potential applications of the isolated bacteria for the production of ectoine.

Selection of the site and collection of samples :

The Lime stone quarries and lime powder dwelling habitats covering the part of north Karnataka and boarder area of Telangana and Andhra Pradesh states (Hyderabad-Karnataka region) surveyed and selected the spot and area for the collection of the soil sample from harsh habitat. The zip lock bags containing the 41 soil samples were collected and taken to the lab for further testing. These samples are segregated based on its texture, color, pH and graded as Normal profile sample (NPS), Moderate profile sample (MPS), High Profile sample (HPS). The soil samples are cleaned and submitted to analysis for pH and *organic matter content in the samples* (O.C %) (Table-1).

Isolation of halophilic bacteria :

Selective isolation method is used for halophilic microorganisms. The medium Salt pretreated starch casein broth culture method (SCB broth). Starch casein broth (SCB) medium containing (g/l) Soil samples - 5gm, CaCo3 - 5gm, NaCl - 15gm, pH – 7. Upon completion of five days of cultivation at 40 °C and 150 rpm in a rotary shaker. In order to disperse 100µl of the diluted culture broth onto a Nutrient

agar plate, It serially diluted 1ml of the culture broth. Five days were spent incubating the plates at 40 °C. The pure cultures are more than 200 isolated colonies were subcultured onto fresh NA plates. (Table-1).

Screening of halophilic bacteria :

A range of different concentrations of salt (with a 3% (w/v) increase from 3 to 30%), were used to grow bacterial isolates on NA plates. Following a 48-hour incubation period, the selected isolates that thrived in a variety of saline concentrations were chosen. On NA medium containing 15% NaCl, the chosen bacterial strains were subsequently cultivated. One culture of all 33 cultures shows 15% NaCl tolerance. (Table-2).

Identification of halophilic bacteria :

Morphological characteristics :

According to colony morphology and cell morphology, microorganisms are categorised. Bacterial colonies are made up of millions of cells and develop from a single cell. Every colony has a unique shape, size, edge, texture, level of opacity, and colour. These characteristics can help with the preliminary identification of a bacterial species as they define the morphology of a single colony. (Table-3).

The environmental scanning electron microscope (ESEM) was used to determine the size and form of the chosen bacterial strain. The bacterial cells were collected by centrifugation after a 24-hour cultivation in a 15% NaCl nutrient broth medium, and then fixed with 2.5% glutaraldehyde and incubated at room temperature for an overnight period before being washed three times with 0.1-M phosphate buffered saline. Centrifugation is used to collect the pellet, and the sample is then dehydrated using increasing concentrations of ethanol for 10 minutes at a time, commencing at 30%, 50%, 70%, 80%, 90%, and 100%. Application of adhesive tape, followed by placement of the bacterial sample on the tape, to prepare the SEM stub. (Fig. 2).

Biochemical test :

The VITEK® 2 Gram-Negative identification card (GN) was used to analyse the biochemical characteristics of the chosen bacterial strain. It is designed to be used with VITEK® 2 Systems for the automated identification of the majority of clinically important fermenting and non-fermenting Gram-negative bacilli. This is a standardised method for identifying non-fastidious Gram-negative rods and Enterobacteriaceae. It is made up of recently created substrates for assessing resistance, enzymatic activity, and carbon source utilisation. Gram-negative rods are used in a list of biochemical tests. The Decarboxylase test wells are compared to the Decarboxylase Negative Control Well (well 52) as a baseline. Miniaturised biochemical tests and a particular database provide final results in 10 hours or less.

Effects of salt on the potential bacterium:

The selected strain was grown in Nutrient broth containing (g/l): peptone, 5.0; beef extract, 3.0; pH- 7.2 and varying Sodium chloride concentrations, 0.1%, 3%, 5%, 10%, 15%, 20%, 25% and 30%. Kept for cultivation for 24hrs, 48hrs and 72hrs at 40 °C with 170 rpm, Centrifugation was used to collect the

bacterial cells for CDW.

Production of Ectoine by two-step fermentation :

To initiate the cultivation process, the selected bacteria were cultivated in Erlenmeyer flasks (250-ml) filled with 100 ml of NB medium containing 6% NaCl. These conditions were maintained at a temperature of 40°C and an agitation speed of 170 rpm for a period of 15 hours. This initial growth phase provided an optimal environment for the bacteria to thrive and establish their metabolic activities. Once the desired cultivation time elapsed, the next step involved separating the bacterial cells from the culture broth. This was achieved through a centrifugation step, where the culture was spun at a force of 5000xg for a duration of 10 minutes. This centrifugation process effectively separated the cells from the liquid medium, allowing for further manipulation and analysis. Following the separation step, the bacterial cells were resuspended in a fresh batch of NB medium. However, this time, the medium was supplemented with varying concentrations of NaCl, specifically 15%. The cells were reintroduced into 250 ml Erlenmeyer flasks, each containing 100 ml of the modified NB medium. These adjusted conditions aimed to induce the production of ectoine, a valuable compound of interest.

The flasks containing the resuspended bacterial cells were then incubated for an extended period of 48 hours. This prolonged incubation allowed for sufficient time for the bacteria to synthesize and accumulate ectoine within their cellular structures. After the incubation period, the bacterial cells were harvested once again. This was accomplished by subjecting the cultures to centrifugation, with the resulting force causing the cells to pellet at the bottom of the flasks. The collected cells were then used to determine the cell dry weight (CDW), which provided valuable information about the biomass yield and ectoine production. Overall, this experimental procedure involved a series of carefully orchestrated steps, including cultivation, separation, resuspension, incubation, and cell harvest. These steps were designed to optimize the production of ectoine from the selected bacteria and enable further analysis and applications of this valuable compound.

Survival efficacy of bacterium during the production of ectoine :

Based on the provided information, the series of experiments conducted evaluated the viability and tolerance of a specific bacterium during ectoine production. Here is a breakdown of the experimental procedure and the results obtained.

Bacterial Culturing: The bacterium was initially cultured in a medium containing 6% NaCl at 40°C and 170 rpm for 15 hours. After the cultivation period, the cells were separated from the culture broth by centrifugation at 5000xg for 10 minutes. The resulting pellet (containing the bacterial cells) was then resuspended in a fresh medium containing 15% NaCl.

Ectoine Release Induction: After 48 hours of cultivation in the new medium with

15% NaCl, the cells were collected. The collected cells were treated with different solutions: distilled water, 2.5% NaCl, and 5% NaCl. These solutions were used to induce ectoine release. The suspensions were incubated for 30 minutes. After the incubation period, the suspensions were centrifuged at 10,000xg for 10 minutes to separate the supernatants, which contained the released ectoine. The ectoine concentration in the supernatants was quantified using high-performance liquid chromatography (HPLC).

Assessment of Cell Survival: The cell pellet obtained from the initial centrifugation step (before ectoine release induction) was suspended in a 6% NaCl solution. The suspension was diluted and spread onto plates containing 6% NaCl. Similarly, cells from the culture broth that did not undergo osmotic downshock were also diluted and spread onto plates. After 48 hours of cultivation at 40°C, the bacterial colonies on the plates were counted. By comparing the colony counts before and after osmotic downshock, the percentage of cell survival was determined, serving as an indicator of the bacterium's tolerance to ectoine production.

Overall, these experiments aimed to assess the ability of the specific bacterium to produce ectoine and determine its tolerance to the osmotic downshock associated with ectoine release. The ectoine concentration in the supernatants provided information about the efficacy of different NaCl solutions in inducing ectoine release. The colony count comparison before and after osmotic downshock helped evaluate the bacterium's survival rate during the process.

Molecular characterization of the potential bacterium :

The methodology described involves analysing bacterial 1200base pairs of 16S rRNA gene sequences using PCR, Sanger sequencing, and BLAST. The 16S rRNA gene is amplified using PCR and then purified. The purified amplicons are sequenced using Sanger sequencing. The resulting sequencing files are edited for quality using CHROMASLITE. A BLASTN search is performed to compare the edited sequences to a database and identify closely related sequences. Pair-wise alignment is conducted to assess sequence similarity. The top hits from the BLASTN search are retrieved for further analysis. Multiple sequence alignment and phylogenetic analysis are recommended to determine evolutionary relationships. This methodology enables the identification of bacterial species and the inference of functional and evolutionary links between sequences. "The DNA sequence was obtained from GenBank (accession number: OR544414)."

Determination of bacterial biomass :

To calculate the Cell Dry Weight (CDW) of a chosen bacterial strain, 15 ml of the culture sample is centrifuged and the pellet is rinsed and dried. The final weight of the centrifuge tube containing the filter and dried residue (W3) is obtained. The weight of the empty tube (W2) is subtracted from W3, and the result is divided by the volume of the culture sample (W1) multiplied by 1000. This calculation gives the CDW, representing the biomass of the bacterial strain per unit volume.

$$CDW = \frac{(W_3)\text{ Weight of filter + dried residue (mg)} - a\ (W_2)\text{ Weight of filter (mg) X1000}}{(W_1)\text{ Volume (ml)}}$$

The given equation represents the calculation of Cell Dry Weight (CDW) for a chosen bacterial strain based on the described methodology. Here's a breakdown of the variables used in the equation :

W3: Final weight of the centrifuge tube containing the filter and dried residue.

W2: Weight of the empty centrifuge tube (prior to adding the culture sample).

W1: Volume of the culture sample (in milliliters).

Note: CDW is a measure of the biomass of the bacterial strain and is typically reported in units such as milligrams per milliliter (mg/ml) or grams per liter (g/l).

Extraction and analysis of Ectoine :

According to the method developed by Kunte *et al.*,[16], 10 mg of cellular biomass was suspended in a 6% NaCl solution for 30 minutes as part of the ectoine extraction procedure. Following centrifugation, the pellet was recovered and shaking vigorously with a 3:1 mixture of chloroform and methanol. Centrifugation was used to separate the phases, allowing the hydrophilic top layer containing ectoine and other suitable solutes to be collected. A rota vapour system was then used to evaporate the solvent from the supernatant. High-Performance Liquid Chromatography (HPLC) was used to further analyse and quantify the concentrated substance that was produced and may have contained ectoine.

TLC analysis :

To do an initial identification of the crude extract of ectoine, thin-layer chromatography was used. With acetonitrile, acetic acid, and water (12:5:5), potpourri is separated. The developer is 0.2% ninhydrin that is dissolved in acetone, while standard item ectoine. After 3 minutes of heating at 130 °C, the sample spot that is separated to takes on a brown colour, but because the collection of illustrative plates sharpness is not high enough that obtains, separating effect is lessened. The computation Rf value (distance at former dot centre to spot center/former dot centre to solvent front) is 0.77 as a result, indicating the necessity for a higher analytical method with more sensitivity. The thin layer chromatography's pretreatment is rather straightforward and should only be used for potentially qualitative analyses.

HPLC anaylsis :

To quantify the concentration of ectoine, High-Performance Liquid Chromatography (HPLC) was employed. The analysis was performed using an UltiMate 3000 Standard Dual System, which consisted of a Bio-Rad Aminex HPX-87C column and a UV detector set at 65°C. To ensure accurate measurements, ectoine and hydroxyectoine (Sigma) were used as standard compounds for calibration. Onraedt *et al.*, (2005). For the HPLC analysis, a mobile phase consisting of a mixture of Acetonitrile and Water in a ratio of 70:30 was utilized, with a flow rate of 1 ml/ min. The concentration of ectoine was

determined by expressing the total ectoine concentration as ectoine per liter of culture broth (g/l), while the intracellular ectoine content was represented as grams of ectoine per gram of biomass (wt%). Van-Thuoc *et al.*, in 2010. This approach allowed for precise determination of ectoine concentration. The mobile phase composition and flow rate provided optimal separation and detection, while the calibration using standard compounds ensured accurate quantification. The expression of ectoine concentration in different units facilitated the assessment of its presence in the bacterial biomass.

HR-LCMS Orbitrap :

In order to verify the synthesis of ectoine, the excreted compound was dissolved in methanol solvent and subjected to OHR-LC-MS and MS/MS studies. Thermo Scientific's Q-Exactive Plus Biopharma equipment with Xcalibur version 4.2.28.14 was used for data acquisition, while Compound Discoverer 3.2 SP1 served as the data processing software. Chromatographic separation was achieved using an SB-C18 RRHD column with a run time of 0 to 35 minutes. The mobile phases consisted of a gradient elution program of 0.1% formic acid in miliq water and acetonitrile. LC-MS analysis covered a mass scan range of m/z 70 to 1000, while OHR-LC-MS/MS analysis involved a product ion scan of m/z 143. The obtained spectra signals, including the pseudo-molecular ion and product ions, matched the data of authentic ectoine. Thus, the compound in the conversion mixture was confirmed to be ectoine. Thermo Scientific's Q Exactive hybrid quadrupole-Orbitrap mass spectrometer played a crucial role in the analysis.

Selection of sites and collection of sample:

Physicochemical characteristics of fourty one samples were collected from Deccan trap area (Table-1). Organic carbon and pH values of these samples are in a range of O.C% 0.1 to 0.6 and 6.6 to 9.4 pH; respectively.

Table-1 Collection of soil samples from harsh habitats

Sl.No	U.NO	P^H	O.C%
1.	NPS1	7.6	1.23
2.	NPS2	7.9	0.1
3.	NPS3	7.8	0.56
4.	NPS4	7.9	0.53
5.	NPS5	8.4	0.44
6.	NPS6	7.7	0.53
7.	MPS2	7.9	0.25
8.	MPS3	8	0.2
9.	MPS4	7.9	0.33
10.	MPS6	7.4	0.27
11.	MPS7	8.5	0.16
12.	MPS8	8.6	0.13
13.	MPS9	7.8	0.18
14.	MPS10	7.9	0.13
15.	MPS11	7.8	0.19
16.	MPS12	7.7	0.33
17.	MPS13	7.6	0.1
18.	MPS15	7.6	0.21
19.	MPS18	7.8	0.42
20.	MPS20	7.7	0.67
21.	MPS21	8.1	0.14
22.	HPS1	7.9	0.16
23.	HPS2	8.8	0.15
24.	HPS3	9.1	0.14
25.	HPS4	7.7	0.11
26.	HPS5	7.9	0.05
27.	HPS6	8	0.17

Table-1 Continued

28.	HPS7	8.2	0.073
29.	HPS8	7.8	0.15
30.	HPS9	7.9	0.1
31.	HPS10	9.4	0.7
32.	HPS11	7.9	0.23
33.	HPS12	8	0.24
34.	HPS13	8.2	0.18
35.	HPS14	8.1	0.53
36.	HPS15	7.9	0.39
37.	HPS18	7.6	0.24
38.	HPS20	8.2	0.15
39.	HPS21	7.4	0.44
40.	HPS23	6.6	0.64
41.	HPS24	8.1	0.22

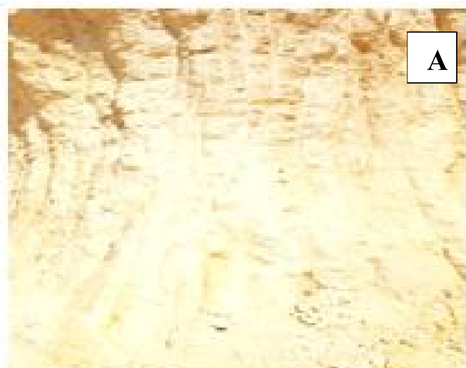


Image:1 Lime powder quarry (A and B) and Limestone quarry (C)

Screening of halophilic bacteria :

Pure cultures obtained from soil samples of the Deccan trap area were cultivated on NA plates supplemented with varying concentrations of NaCl (ranging from 3% to 30%) at a temperature of 40°C. After incubation for 24 hours, a total of 33 bacterial strains were isolated. Among these, eight strains demonstrated robust growth across various salt concentrations. These selected cultures were designated as DSK-1, DSK-25, PS-4, DGR-48, P2-25, HPS-2, HPS-3, and DSH-3. Among these strains, DSH-3 exhibited promising characteristics and was chosen for further experimentation.

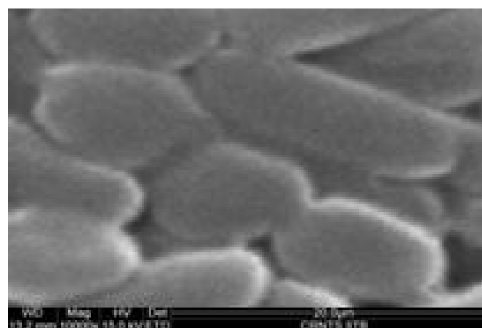


Fig: 2. The strain was determined by Environmental scanning electron microscopy (ESEM)

Table-2. Screening of halophilic bacteria with varying %NaCl concentration

Sl. no	Isolates	%Nacl concentration							
		3%	6%	9%	12%	15%	20%	25%	30%
1.	DMR-17	+	+	-	-	-	-	-	-
2.	DSK-16	+	+	+	-	-	-	-	-
3.	DSK-25	+	+	+	-	-	-	-	-
4.	DSK-28	+	+	-	-	-	-	-	-
5.	DGR-28	+	+	-	-	-	-	-	-
6.	DGR-24	+	+	-	-	-	-	-	-
7.	DGR-26	+	+	-	-	-	-	-	-
8.	DGR-48	+	+	+	-	-	-	-	-
9.	A-DBT(108)	+	+	-	-	-	-	-	-
10.	PSH-5	+	+	-	-	-	-	-	-
11.	PSH-4	+	+	+	+	-	-	-	-
12.	PSH-8	+	+	-	-	-	-	-	-
13.	H-10	+	+	-	-	-	-	-	-
14.	H-3	+	+	-	-	-	-	-	-
15.	FS-7	+	+	-	-	-	-	-	-
16.	DSM-1	+	+	-	-	-	-	-	-
17.	P2-25	+	+	+	-	-	-	-	-
18.	HPS-1	+	+	-	-	-	-	-	-
19.	HPS-2	+	+	+	-	-	-	-	-
20.	HPS-3	+	+	+	-	-	-	-	-
21.	HPS-4	+	+	-	-	-	-	-	-
22.	HPS-5	+	+	-	-	-	-	-	-
23.	HPS-7	+	+	-	-	-	-	-	-
24.	HPS-9	+	+	-	-	-	-	-	-
25.	HPS-24	+	+	-	-	-	-	-	-
26.	DSH-1	+	-	-	-	-	-	-	-
27.	DSH-3	+	+	+	+	+	-	-	-
28.	DSH-5	+	-	-	-	-	-	-	-
29.	DSH-6	+	-	-	-	-	-	-	-
30.	DSH-7	+	-	-	-	-	-	-	-
31.	DSH-8	+	-	-	-	-	-	-	-
32.	DSH-9	+	-	-	-	-	-	-	-
33.	DSH-10	+	-	-	-	-	-	-	-

Identification of halophilic bacteria :

Tables 3 and 4 give a summary of the structural and biochemical features of the selected microorganisms.

Table-3. Morphological characteristic of a bacterial strains

Sl.no	Isolates	Colour	Shape	Margin	Elevation	Size	Surface of colony
1	DSK-1	Whitish brown	Irregular	Undulated	Umbonated	0.9mm	Rough
2	DSK-25	Light pink	Round	Circular	Raised	0.1mm	Smooth
3	PS-4	Light orange	Round	Entire	Raised	0.1mm	Smooth
4	DGR-48	White	Curled	Entire	Raised	0.5mm	
5	P2-25	Greyish green	Round	Entire	Flat	0.6mm	Rough
6	HPS-2	White	Round	Entire	Raised	0.2mm	Rough
7	HPS-3	White	Round	Entire	Flat	0.3mm	Smooth
8	**DSH-3**	**Yellowish creamy**	**Round**	**Entire**	**Raised**	**0.3mm**	**Smooth**

Table-4. Biochemical test

Sl. no	Isolates	Indole test	MR test	VP test	Citrate test	Catalase test	Oxidase test	Gram staining
1	DSK-1	+	_	_	_	_	_	Gram +ve
2	DSK-25	+	+	+	+	_	_	Gram +ve
3	PSH-4	_	_	+	_	_	_	Gram +ve
4	DGR-48	+	_	_	+	_	_	Gram +ve
5	P2-25	_	+	+	+	_	_	Gram –ve
6	HPS-2	_	+	_	_	_	_	Gram-ve
7	HPS-3	+	_	_	_	_	_	Gram –ve
8	DSH-3	+/_	_	_	_	_	_	Gram –Ve

The selected potential bacterium, DSH-3, exhibits several characteristic features. It is a Gram-negative bacterium that belongs to the group of strictly aerobic microorganisms. Morphologically, it appears as short rods measuring approximately 0.7-0.8 mm to 1.0-1.2 mm in length. Optimal growth conditions for DSH-3 include temperatures ranging from 37ºC to 50ºC, with the ideal temperature being 40ºC. The bacterium thrives in a pH range of 6.0 to 8.0, with the optimum pH being 7.0. It can tolerate NaCl concentrations between 1% to 15% (w/v), with the most favorable growth occurring at 15% NaCl.

DSH-3 exhibits various metabolic capabilities. It produces indoles and has the ability to oxidize and ferment D-glucose, casein, and DNA. The strain is capable of hydrolyzing D-glucose and D-galactose.

Table-5. The selected strain's and the reference strain's characteristic features *H. daqingensis* (Gang Wu et al., 2008)

Characteristics	***Halomonas daqingensis***	**DSH-3**
Cell size (µm)	0.7-0.8 x 1.0 – 1.2	0.5-0.8 x 1.0-1.2
Flagellation	Peritrichous	-
Optimum temperature (ºC)	30ºC	40ºC
Temperature range	10ºC – 50ºC	37ºC -50ºC
Optimum pH	9.0	7.0
pH range	8.0 – 10.0	6.0-8.0
Optimum NaCl (%)	5.0 – 10.0	15.0
NaCl range (%)	1 – 15.0	1 – 15.0
Cellulobiose-D	-	-
Ala-Phe-Pro-Arylamidase	-	-
L-Ayrrolydonyl-Arylamidase	-	-
L-Arabitol	-	-
β-n-acetyl-Glucosaminidase	-	-
β-Galactosidase	-	-
H_2S production	-	-
Gamma-Glutamyl-Transferase	-	-
Fermentation/ glucose	-	-
Glutamyl arylamidase pNA	-	-
β-Alanine Arylamidase pNA	-	-
L-Proline Arylamidase	-	-
β-xylosidase	-	-
Lipase	-	-
β-glucosidase	-	-
Tyrosine Arylamidase	+	+
Palatinose	-	-
Urease	+	+
Saccharose/Sucrose	-	-
D-sorbitol	-	-
D-Tagatose	-	-
5-keto-D-Gluconate	-	-
Alpha-Glucosidase	+	+
Malonate	-	-
Citrate (Sodium)	-	-
L-Lactate Alkalinization	-	-
Glycine Arylamidase	+	+
Succinate alkalinization	-	-
Phosphatase	-	-

α-Galactosidase	-	-
Lysine Decarboxylase	-	-
β-n-Acetyl-Galactosaminidase	-	-
L-Histidine Assimilation	-	-
Ornithine Decarboxylase	-	-
Decarboxylase base	-	-
β-glucuronidase	-	-
coumarate	-	-
GLU-GLY-ARG-ARYLAMIDASE	-	-
O/129RESISTANCE (comp.vibrio.)	-	-
Ellman	+	+
D-Malate Assimilation	-	-
Oxidation/fermentation of D-Glucose	Oxidation	Oxidation
L-Lactate Assimilation	-	-
Indole Production	+	+
Hydrolysis Of Casein & DNA	+	+
Malonate	+	+
D-Glucose	+	+
myo-Inositol	-	-
D-Galactose	+/-	+
D-mannose	+	+
D-mannitol	+	+
Maltose	+	+
Dextrin	+	+
Tween 80	+	+
SALICYLIC Acid	-	-
L-Cysteine	+	+
Aerobic NO^-_3 Reduction	+	+
L-Isoleucine	+	+
Aerobic NO^-_2 Reduction	+	+
DL-methionine	+	+
L-Histidine	+/-	+
L-Serine	+	+
L-lysine	+	+
D-Galatose	+	+
L-valine	+	+
Trehalose	+	+
Lactose	-	+
Sucrose	+	+
Maltose	+	+

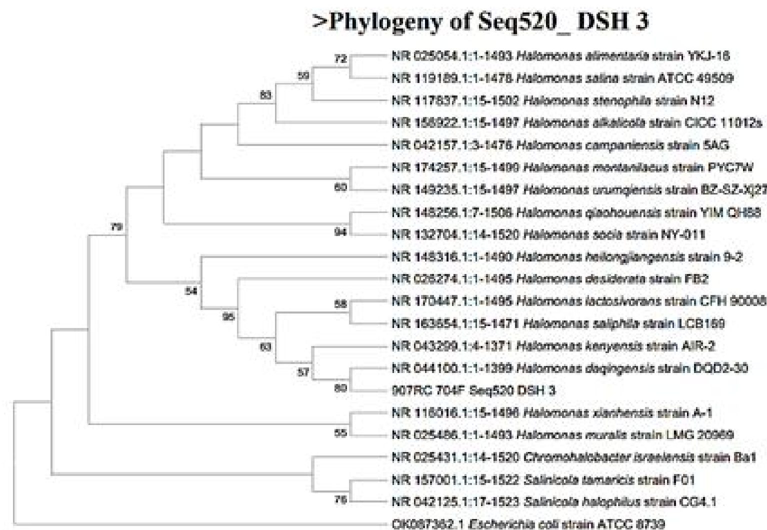
However, it does not produce certain enzymes, including adonitol, beta-galactosidase, l-pyrrolydonyl-arylamidase, ala-phe-pro-arylamidase, d-cellulobiose, H_2S production, l-arabitol or glutamyl arylamidase pNA. It has the ability to utilize a wide range of substances such as Dextrin, D-Mannitol, Maltose, D-Mannose, L-Isoleucine, L-Histidine, L-Cysteine, DL-Methionine, L-Lysine, L-Valine, L-serine, Sucrose, D-Galactose, Trehalose Maltose and Lactose. It is worth noting that the characteristics of DSH-3 often resemble those of *H. daqingensis DQD2-30*.

Effects of salt on the potential bacterium :

The impact of various salt concentrations on the development and accumulation of ectoine in the chosen bacteria that cannot grow in the absence of NaCl. The growth of the bacteria was most ideal at salt concentrations of 3 to 6%, and at NaCl concentrations greater than 15%, the growth of the bacteria rapidly declined. Additionally, the maximum CDW for strain DSH-3 at 6% NaCl concentration was 7.85 (Fig. 3). However, in 48 hours, 12%–15% of NaCl was the ideal salt concentration for the growth of cells.

Molecular characterization of the potential bacterium :

Phylogenetic analysis of the selected strain was conducted using the 16S rDNA sequencing method to elucidate its taxonomic characteristics. Comparison of the strain's 16S rDNA sequences with those available in public databases revealed significant similarities to various Halomonas species. Notably, the highest similarities were observed with *Halomonas daqingensis* (99.21%), *Halomonas lactosivorans* (99.13%), and *Halomonas saliphila* (98.63%). These findings suggest a close phylogenetic relationship between the selected strain and these Halomonas species. The utilization of 16S rDNA sequences in phylogenetic analysis provides valuable insights into the classification and evolutionary relatedness of microorganisms, contributing to our understanding of their genetic diversity and potential functional characteristics.



The 16S rDNA gene sequences of a particular strain of the genus *Halomonas sp.,* a phylogenetic tree of that strain is shown in (Figure 1).

Determination of effect of NaCl with bacterial biomass :

The bacterial biomass was calculated at different salt concentration shows good biomass at 15% NaCl in 48hrs

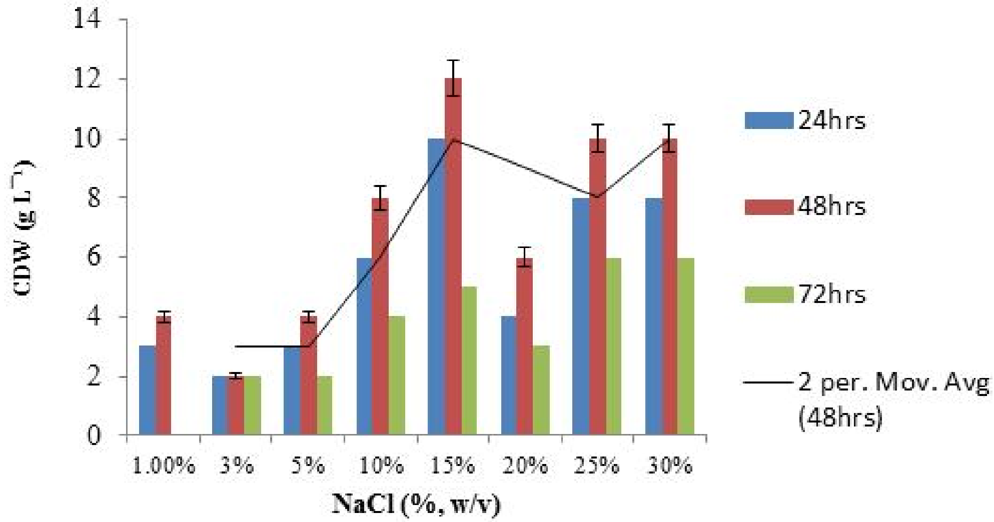


Fig. 3. Graph represents weighed Dry cell pellet with varying concentration of NaCl for hrs.

Extraction and analysis of ectoine :

At the 15% NaCl concentration, strain DSH-3 presented maximum CDW of 7.85 wt%, respectively. With rising NaCl concentration in the preliminary culture medium, ectoine accumulation increased. ectoine contents of 0.58 wt%. Furthermore, at 15% NaCl concentration, strain DSH-3 exhibited highest ectoine production.

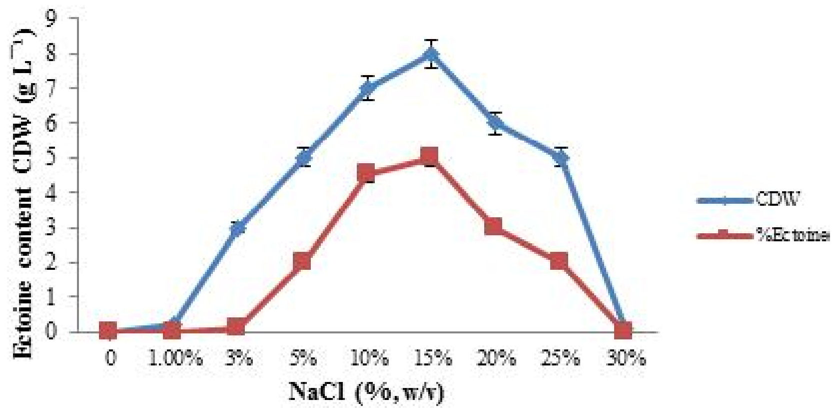


Fig. 4. Graph represent NaCl concentration's effects on the growth of cells and ectoine accumulation.

TLC analysis :

Thin-layer chromatography plate shows brown spot.

Fig. 5. TLC plate showing Brown-red spots with Rf value 0.7
S: Std Ectoine; E1: Sample DSH-3

HPLC anaylsis :

The HPLC peaks represent the detection of the ectoine.

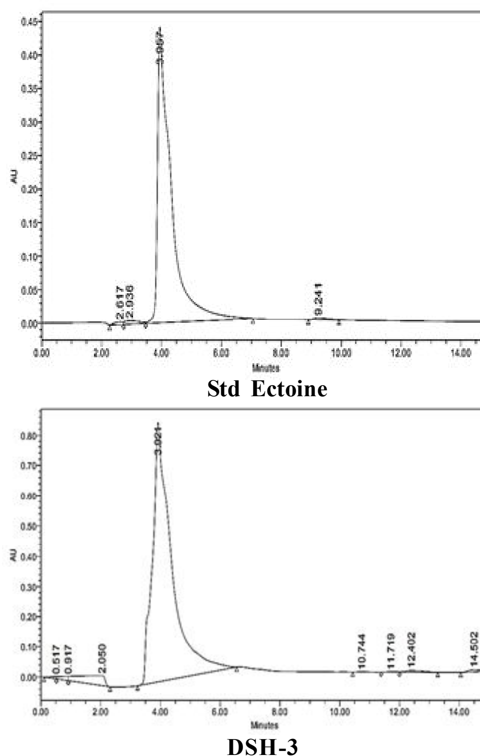


Fig. 6. HPLC peak shows the detection of ectoine in DSH-3 sample

HR-LCMS Orbitrap :

The peak represents the detection of ectoine at *m/z* 143

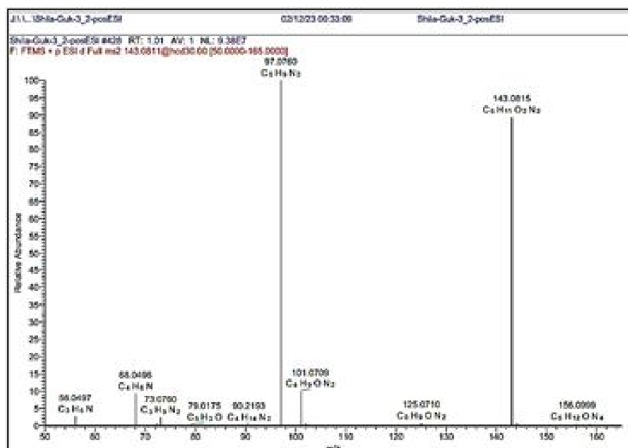


Fig. 7. HR-LCMS Orbitrap peak shows the detection of ectoine in DSH-3 sample

The current study investigates the occurrence of ectoine-producing bacteria within the Deccan trap region, specifically in soil samples collected from Hyderabad and Karnataka states. The primary objective of this research is to select for halophilic or high salt-tolerant bacteria using a cultivation approach involving a 15% NaCl medium. Following the isolation of various bacterial colonies, one particular strain exhibiting the capacity to synthesize ectoine is identified, indicating the presence of ectoine-producing bacteria in the region under investigation. The taxonomic classification of the ectoine-producing strain reveals its affiliation with the genus *Halomonas*, which comprises a diverse group of Gram-negative, rod-shaped bacteria commonly found in saline environments. The *Halomonas* genus is well-recognized for its moderate salt tolerance and has been extensively explored for numerous biotechno-logical applications. Notably, the selected strains in this study exhibit a remarkably high similarity (99.21%) to *H. daqingensis*, a moderately halophilic bacterium previously documented in hypersaline conditions in north-eastern China. This study's significance lies in being the first to report ectoine accumulation in *H. daqingensis* strain isolated from the Deccan trap region, thus underscoring the potential of these bacteria for ectoine production. Overall, this research contributes to our understanding of the adaptive strategies employed by microorganisms in high-salt environments while expanding our knowledge of microbial diversity and the potential applications of ectoine-producing strains within the *Halomonas* genus.

The current investigation showed that the bacterial strain obtained from the Deccan Trap region can be a feasible option for industrial production of ectoine. When exposed to hypoosmotic stress, the strain may produce significant levels of ectoine, release stored ectoine quickly, and sustain high survival rate. Studies are currently being conducted to optimise the growth medium for the isolated strain's ability to produce ectoine.

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Data Availability Statement: Not applicable

Conflicts of Interest: The authors declare no conflict of interest

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