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Research paper

Isolation and Characterization of Halophilic Bacteria from Coastal Areas of Odisha, India

Itishree Behera [a] *, **Umesh Chandra Naik** [a]

[a] *Environmental Microbiology Laboratory, Department of Botany, Ravenshaw University, Cuttack-753003, Odisha, India*

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ABSTRACT

The current study focuses on the screening and identification of halophilic bacteria from different habitats of Odisha. The halophilic bacteria were isolated and screened with NaCl concentration using 0%-24% (w/v). The selected halophiles were studied for their growth, colony morphology, multi-metal tolerance, sodium capture capacity and biochemical assay. The isolated bacteria were found to be moderately halophilic nature due to the presence of salt such as sodium, magnesium and bicarbonate in the collected samples. The result showed the utmost number of viable cells (0.022 to 1.75 cfu/mL) is due to the hypersaline nature of samples making the bacterial cells suitable to grow under 15%-18% (w/v) NaCl concentration. They were found to be gram-positive and organized in single rods and clusters. The presence of sodium (0.07-0.18 mg/L) were confirmed in the halophiles. The biochemical assay showed all strains are catalase, amylase, urease positive while showed negative results for indole test and cellulose activity. Thus, the findings suggest the existence of halophilic bacteria in the coastal areas of Odisha which can be further studied for their osmotolerant and osmoregulatory properties.

1. Introduction

Coastal areas are home to around 10 % of the global population (Ranasinghe, 2016) which are made up of fragile micro features where land, sea, river, atmosphere, and humans interact dynamically (Mishra et al., 2023). Coastal waters play a significant role in the global carbon and nitrogen cycle (Bauer et al., 2013). The main external sources of N and P to the coastal ecosystems are atmospheric deposition, river discharge, and nitrogen fixation (Howarth et al., 2002; Kumar et al., 2021). Coastal sediment is mainly dominated by terrigenous material through aeolian and alluvial process (Sathpathy and Panda, 2018). To meet the ever-growing demand for economic activities, the resilience of the coastal landforms is compromised due to the construction of onshore and offshore coastal infrastructures. For example, nearshore dredging to expand port activities disturbs the overall coastal ecosystem and erodes the coastline in the long run (Mohanty et al., 2015; Pramanik et al., 2016; Layek et al., 2019; Žilinskas et al., 2020). Most of the coastal areas of the world have been reported to be damaged from pollution, significantly affecting commercial coastal and marine fisheries. Therefore, control of aquatic pollution has been identified as an immediate need for sustained management and

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***Corresponding author:** Itishree Behera
Email: itishreebs16@gmail.com

conservation of the existing fisheries and aquatic resources (Islam and Tanaka, 2004).

Halophilic microorganisms are salt-loving, metabolically different and are potent candidates for bioremediation. They have the ability to grow under extreme saline conditions having potential mitigation properties against hazardous pollutants. The halophilic bacteria use the 'compatible solute adaptation' strategy to maintain osmotic balance by using compatible organic solutes such as polyols, glucosyl-glycerol, sucrose, trehalose, ectoine, and betaine. Halophilic bacteria have unique metabolic capabilities, production of stable enzymes under extremely hostile conditions, and unique biomaterials and/or secondary metabolites (Shukla et al., 2017; Leon et al., 2018; Weinisch et al., 2018; Corral et al., 2019; Matarredona et al., 2021; Van Thuoc et al., 2021; Baati et al., 2022). Under these circumstances, marine microorganisms that can withstand salt are more advantageous than traditional, non-extremophilic microorganisms because they can quickly adapt to a changing environment. (Mohapatra et al., 2017; Shukla et al., 2017; Diba et al., 2021; Matarredona et al., 2021). The physiological versatility of extremophiles especially halophiles and alkaliphiles and their enzymes (extremozymes) could conveniently be harnessed to remediate and detoxify heavy metals from the high alkaline saline environment (Varshney et al., 2023). Therefore, this study focuses on the characterization of the samples from different habitats of Odisha for studying the saline nature. Then, the bacteria were isolated and screened for their halophilic nature through different NaCl concentration. Further they are characterized for identifying the suitable halophilic bacteria.

2. Materials and Methods

2.1 Collection of sediment samples and Physicochemical analysis

Sediment samples were collected from different coastal areas of Odisha. Astaranga (19.929°, 86.283°) and Baulabandha, Chilika (Lat 19.801°, Long 85.325°) of Odisha. Sediment samples were collected in zipper plastic bags from each location and the composite samples were instantaneously moved to the laboratory for further characterization (Baati et al., 2010; Chen et al., 2010; Das et al., 2019). Temperature, pH, TDS, redox potential and electrical conductivity were measured as per the method described by Selvarajan et al., 2017 and Sharma et al., 2021. Sodium, potassium and calcium were measured with the help of flame photometry. Magnesium, chloride, carbonate, bicarbonate was measured using titration method (El Bilali et al., 2021 and Gharaibeh et al., 2021). Sulphate and nitrate were measured with spectrophotometric method of Mussa et al., 2009 and Dookie et al., 2022. All the Physico-chemical characteristics were performed in triplicates.

2.2 Isolation and Screening of halophiles

Under medium C (per liter): NaCl, 81g; $MgSO_4.7H_2O$, 9.7g; $MgCl_2.6H_2O$, 7g; $CaCl_2.2H_2O$, 3.6g; KCl, 2g; $NaHCO_3$, 0.06g; $(NH_4)_2SO_4$, 0.026g; Yeast extract, 50g; Agar, 12-15g), bacteria were seeming to grow luxuriantly at 35°C (Rohban *et al*., 2009), and the bacteria were isolated for 24 to 48 hours in triplicates. The selected strains were screened under NaCl (0% to 24% (w/v)), to know the nature of halophiles (slightly, moderately or extremely) (Das *et al*., 2019; Sharma *et al*., 2021; Rathakrishnan and Gopalan, 2022). Bacterial strains were screened for tolerance under heavy metals which includes Cd, Cr, Mn, Fe, Ni, As, Co, Cu, Zn and Pb. The heavy metal sources are As_2O_3, $Cr_2(SO_4)_3$, $CoSO_4.7H_2O$, $3CdSO_4.8H_2O$, $CuSO_4.5H_2O$, $Fe_2SO_4.7H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, Pb $(NO_3)_2$ and $NiSO_4.6H_2O$. Bacterial strains were seeded on agar plates for screening under the multi-metals (0 to 150 ppm) for incubation period of 24 hours at 35°C.

2.3 Characterization of Halophiles

The selected strains were inoculated in the halophilic medium C containing the NaCl concentration (0% to 24% (w/v)) and observed their growth by measuring their OD in spectrophotometer (Shimadzu, Japan, UV-2450). Colony morphology was observed under phase contrast microscope (Model-Nikon Eclipse Ci) at 100X using the gram stain method (Fitri et al., 2022). Potential isolates growing luxuriantly under 19% and 17% NaCl were screened for sodium content and soluble sugar content. For sodium capture capacity, after 24 hours of incubation, cells were harvested by centrifugation (Optima MAX-XP, Bekman, USA) (12,000 rpm) and the bacterial cell pellet was washed with sterilized distilled water to remove the traces of medium. Washed pellet was digested overnight (0.1 N HCl) at room temperature and again, centrifuged. The supernatant was taken for the estimation of uptake by bacterial cells. Sodium contents were measured by

Flame photometer as per the methodology given by the Damodaran et al., 2013 and Pérez-Inocencio et al., 2022.

The positive isolates were selected for biochemical assay. Amylase activity was determined by incubating the bacterial inoculates in starch agar plates containing (per liter): Peptone 10 g; KH_2PO_4 5 g; Agar 20 g and Starch 0.2% (w/v) for 3 days at 35°C. After incubation, starch degrading bacteria were identified by flooding the plates with Gram's iodine solution. The positive colonies demonstrated a region of clear zone of hydrolysis around the colonies when flooded with grams iodine solution. The negative colonies showed no zone of hydrolysis around them against a blue-black coloration on starch agar (Ashwini et al., 2011; Akulava et al., 2022). Cellulolytic activity was determined by seeding the bacterial isolates in medium containing (per liter): NaCl 5 g; Yeast extract 2 g; Peptone 5 g and agar 15 g supplemented with 0.5% carboxymethyl cellulose. The plates were incubated at 35°C for seven days. Then, flooded with Lugol's iodine and after 15 minutes, the cultures were checked for formation of clear halos around the colonies which indicate a positive result for cellulases (Selvarajan et al., 2017; Perez-Inocencio et al., 2022). Catalase and indole test were determined using 3% (v/v) hydrogen peroxide and Kovacs' reagent, respectively. The formation of bubbles resulting from production of oxygen gas indicates catalase positive while red colour formation indicates indole positive (Rezaeeyan et al., 2017; Das et al., 2019; Thompson et al., 2023). Urease activity was performed by incubating the bacterial isolates in the medium containing (per liter): NaCl 5 g; peptone 1 g; KH_2PO_4 2 g; glucose 1g; phenol red 0.012 g; agar 20 g and urea 20 g at pH 6.8 at 35°C for seven days. Throughout the incubation period, colour change was observed from pale yellow to pink red indicating the urease positive result (Dahlén et al., 2018 and Akulava et al., 2022).

3. Results and Discussion

3.1 Physicochemical analysis

Astaranga (AS) sample was found to be saline with EC value of 3.19 ds/m and pH of 5.5. Other physico-chemical parameters such as temperature (°C), redox potential (mv) and TDS (ppm) was found to be as 32.3, 47.5 and 3073, respectively. Baulabandha (BB) sample was found to be slight saline having EC value of 2.94 ds/m and pH of 2.84. Other parameters such as temperature, redox potential and TDS was found to be 34.5 °C, 233.5 mv and 1884 ppm, respectively (Table 1). The Cuatro Cienegas soils had a neutral pH, EC of 2.3e8 dS cm^{-1} classified as moderately saline. Whereas, the soils from Sayula and San Marcos lakes, had an alkaline pH, EC 15 to 65 dS m^{-1} classified as saline-sodic soil (Delgado-García et al., 2018). The salinity of the water at Puri, Digha and Haldia was tested and found to be 34 ppt, 35ppt and 33 ppt, respectively (Das et al., 2019). Magnesium concentration was highest in Astaranga (35006.67 ± 10744.49 Mg/L) and Baulabandha samples (32210 ± 11596.09 Mg/L) while calcium content was found to be lowest in Astaranga (0.25 ± 0Mg/L) and Baulabandha samples (0.35 ± 0 Mg/L), respectively. Similarly, nitrate was found to be lowest in Astaranga (0.22 ± 0.00 Mg/L) and Baulabandha samples (0.20 ± 0.00 Mg/L), respectively. Bicarbonate content was highest in Astaranga sample (385830 ± 531941.6 Mg/L) and Baulabandha sample (206913.3 ± 56093.17 Mg/L) followed by chloride concentration Astaranga water sample (2106 ± 0 Mg/L) and Baulabandha water sample (7121.4 ± 3377.49 Mg/L), correspondingly (Table 2).

3.2 Isolation and screening of halophilic bacteria

The highest CFU was recorded in Astaranga sample while the lowest was recorded in Baulabandha sample, correspondingly (Table 3). A total of 7 bacterial strains were isolated from the samples. As per the morphological study, 3 strains were from Astranga sample and 4 strains from Baulabandha sample. All the strains showed NaCl tolerance above 15% and 3 (AS'S-I, BB-II, BB'S-III) strains showed NaCl tolerance up to 19% making them moderately halophilic in nature (Table 4). Sumit Kumar *et al*., 2012 stated that 21 potent moderately halophilic bacteria (3-20%) identified as *Marinobacter*, *Virgibacillus*, *Halobacillus*, *Geomicrobium*, *Chromohalobacter*, *Oceanobacillus*, *Bacillus*, *Halomonas* and *Staphylococcus* isolated from Various Salt Lake, Rajashthan. Bacterial strains were seeded on agar plates for screening under the multi-metals (0 to150 ppm) for incubation period of 24 hours at 35°C (Table 4). Among the selected halophiles, all showed tolerance up to 120 ppm for multi-metal concentration and three strains (AS'S-I, BB'S-II and BB'S-III) showed tolerance at 130 ppm. Diba et al., 2021 reported that the isolated halophilic bacteria

(*Bacillus* sp. A21, *Oceanobacillus* sp. A22 and *Salinicoccus* A43) from Khara Salt Lake in Iran tolerated to 20% salt concentration and showed tolerance to 7.2 mM, 4.1 mM, and 6.7 mM lead and 3.6 mM, 3.7 mM, and 4.1 mM nickel, respectively. Moderately halophilic *Vigribacillus* sp. isolated from mangrove soil of Bhitarkanika, India showed high salt (25 wt.% NaCl) and Cr (VI) (1000 mg L−1) tolerance (Mishra *et al*., 2012). Similarly, Divakar et al., 2018 stated that *Bacillus pumilus* (accession no.MF472596) was found to tolerate against four toxic heavy metal ions (Cd^{2+}, Cu^{2+}, Fe^{3+} and Ba^{2+}) up to 1000 ppm each. Orji *et al*., 2021 stated that the bacterial isolates (*Pseudomonas putida* A4W, *Klebsiella* sp. USL2S and *Pseudomonas putida* USL5W) showed capacity to tolerate 50.0 mM Hg and Pb, 17.0 mM Ni, 12.50 mM Cd, 4.0 mM Zn, 16.0 mM and 4.0 mM Cu, respectively in solid media. Sahoo and Goli, 2020 stated that *Bacillus pumilus* out of 128 screened bacteria for tolerance against Pb, Cd, Ba, Cr, Fe, Cu, and F was found to be tolerateant against all of them.

3.3 Characterization of Halophilic bacteria

As per the cell growth, the maximum reach of growth was up to 18% NaCl concentration for AS'S-I and BB'S-III which was declined with increasing concentration (Fig. 1) observed through the heat map. When these strains examined under a phase contrast microscope, they found to be rod-shape and gram-positive bacteria (Fig. 2). Analysis of these isolates for sodium capture capacity at 19% NaCl concentration showed higher uptake of sodium content in comparison to untreated bacteria (Fig. 3). However, higher sodium content was seen by AS'S-I, and BB'S-II strain (0.023 to 0.159 mg/L,0.026 to 0.184 mg/L) than BB'S-III (0.015 to 0.076 mg/L), respectively which showed lower content of sodium. Pérez-Inocencio *et al*., 2022 reported that the isolates that showed sodium uptake content were 3 *Bacillus* species, 2 *Bacillus subtilis*, 2 *Oceanobacillus* species, *Staphylococcus epidermidis* SVHM1, *Marinococcus* sp. SVHM5, *Nocardiopsis* sp. SVHM6.2, 4 *Halomonas* species, 2 *Halomonas huangheensis*, ranging from 11 mEq (*Bacillus subtilis* SVHM10) to 38 mEq with higher production (*Halomonas huangheensis* SVHM6) after 24 h of incubation. Damodaran *et al*., 2013 reported that analysis of the sodium uptake pattern at different molar concentration of NaCl showed an increasing sodium uptake up to 1 M NaCl in all the isolates thereby significant decline in sodium content. Among them, *Bacillus pumilus* and *Bacillus subtilis* showed higher uptake of sodium (1.272 meq / L and 1.122 meq / L) at 1 M NaCl concentration, respectively. Yancey, 2005 stated that these small solutes used by cells of halophilic bacteria to maintain cell volume and act as antioxidants against the stressed condition. The positive isolates were selected for biochemical assay including amylase test, cellulose test, catalase test, indole test and urease test. The results of biochemical assay showed all strains are catalase, amylase and urease positive while showed negative results for indole test and cellulose activity (Table 5). Rengasamy and Thangaprakasam, 2018 reported the isolation of eight Streptomyces isolates (S1-S8) from mangrove regions of Muthupet, Tamilnadu which exhibited positive for amylase activity. Pérez-Inocencio et al., 2022 found that the halophilic bacteria isolated from the rhizosphere soil of *Sesuvium verrucosum* had promotional activity for amylases, proteases, lipases and cellulases. Ashwini et al., 2011 stated that *Bacillus sp. marini* isolated from marine environment of Andaman and Nicobar Islands, India showed amylolytic activity.

4. Conclusion

The outcome of the current findings suggests that they were moderately halophilic bacteria and suitable to grow between 15%-18% (w/v) NaCl concentration. The three isolates showed (AS'S-I, BB'S-II and BB'S-III) tolerance up to 130 ppm multi-metal concentration. The presence of sodium (0.07 – 0.18 mg/L) in potent bacteria maintains the osmotic balance required for existence. From, biochemical characterization, all strains are found to be catalase, amylase, urease positive while showed negative results for indole test and cellulose activity. Thus, the findings evidently point out the presence of halophilic bacteria in the coastal areas of Odisha which are relevant for further studies. Further investigation is needed to understand the potential mechanism of osmo-protection in these bacteria.

Table 1 Physico-chemical characterization

Sample	pH	Temperature (°C)	EC (ds/m)	Redox Potential (mv)	TDS (ppm)
AS	5.5 ± 0.70	32.3 ± 1.83	3.19 ± 0.09	47.5 ± 0.70	3073 ± 8.48
BB	2.84 ± 0.06	34.5 ± 0.70	2.94 ± 0.07	233.5 ± 4.94	1884 ± 16.97

AS - Astaranga, **BB** - Baulabandha

Table 2 Physico-chemical characterization

Sample	Na	K	Ca	Mg	
AS	9.067 ± 4.45	5.4 ± 0.9	0.25 ± 0	35006.67 ± 10744.49	
BB	395 ± 407.11	24.2 ± 15.6	0.35 ± 0	32210 ± 11596.09	
Sample	**Cl**	**SO_4^{2-}**	**NO_3**	**HCO_3^-**	**CO_3^{2-}**
AS	2106 ± 0	4.20 ± 0.06	0.22 ± 0.00	385830 ± 531941.6	526.66 ± 215.01
BB	7121.4 ± 3377.49	4.19 ± 0.06	0.20 ± 0.00	206913.3 ± 56093.17	3706.66 ± 184.75

AS - Astaranga, **BB** - Baulabandha

Table 3 Isolation of Bacteria

Sample	Dilution factor	No. of colonies	Colony forming unit (CFU/mL)
AS	10^0	108	5.40
BB	10^{-2}	44	1.75

AS - Astaranga, **BB** - Baulabandha

Table 4 Screening under NaCl Concentration (0 to 24% (w/v)) and multi-metal tolerance (0 to 150 ppm (w/v))

Bacterial strains	NaCl Concentration (%)	Multi-metal Tolerance (PPM)
AS'S-I	19%	130
AS'S-II	17%	120
AS'S-III	15%	120
BB'S-I	17%	120
BB'S-II	19%	130
BB'S-III	19%	130
BB'S-IV	17%	120

Table 5 Biochemical assay of halophilic bacteria

Bacterial strains	Amylase	Cellulose	Catalase	Indole	Urease
AS'S-I	+	-	+	-	+
BB'S-II	+	-	+	-	+
BB'S-III	+	-	+	-	+

+ = growth, **-** = No growth

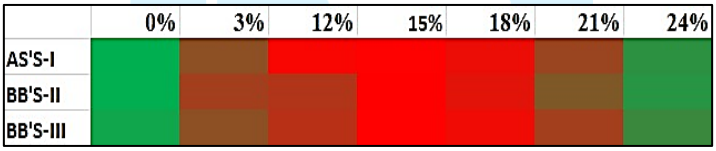
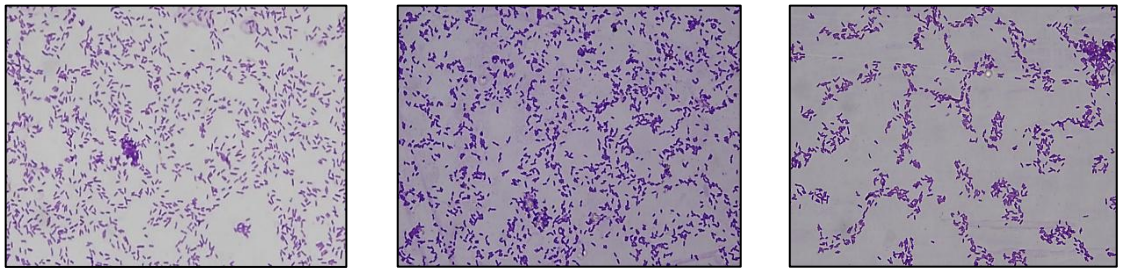


Fig. 1 Bacterial cell growth under NaCl concentration (0% to 24%)



(a) **(b)** **(c)**

Fig. 2 Colony morphology of halophiles. (a) AS'S-I (b) BB'S-II (c) BB'S-III

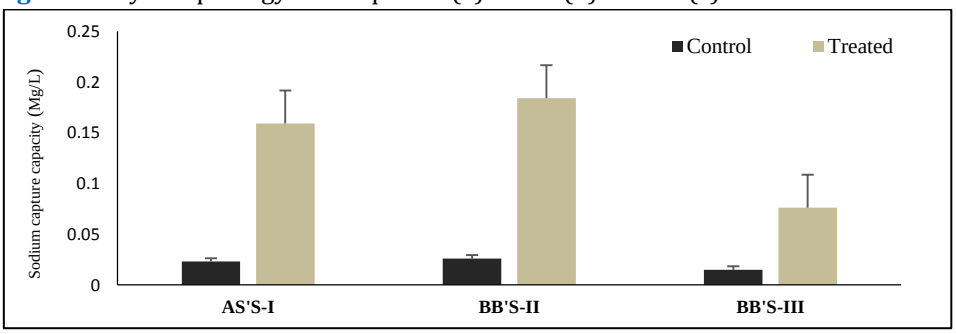


Fig. 3 Sodium capture capacity of the halophiles using flame photometry

Declaration

The authors declare that that the studies presented in the paper are the outcome of original research work and no part of this research has not been previously submitted for any publication or not accepted or is not under consideration for publication elsewhere. The manuscript has been prepared strictly in accordance with the "Instructions to authors" of the journal. The authors will not withdraw the manuscript after acceptance.

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