Isolation and characterization of Haloarchaea from low-salinity coastal sediments and waters of Goa

Halophilic archaea have been routinely isolated from marine salterns and hypersaline lakes with 3.5–4.5 M (20–30%) NaCl[1–3]. The salinity of sea water (2.5% NaCl concentration) is ten times less than that of the hypersaline environments and hence is expected to be non-conducive to the growth of extremely halophilic bacteria. Interestingly, three recent reports have indicated the presence of such extreme halophiles from sea waters in Spain, Japan and the United Kingdom[4–6]. So also existence of non-culturable archaea has been indicated in marine environments[7]. To investigate the occurrence of haloarchaea in the low-salinity coastal sediments and waters, we surveyed five different locations along the 100 km coastline of Goa, located on the mid west coast of India.

Water and sediment samples were collected separately from Palolem, Talpona, Vasco, Dona Paula, Miramar and Bainguinim, the coastal regions of Goa (Figure 1), during the post-monsoon months of November–December 1999.

Water samples, 1 l per site, were collected from the shoreline to a distance of 50 m up to a depth of 1 m. One kilogram of sediment per site was collected in a staggered mode over a total area of 10 m, pooled together in polythene bags and transported to the Haloarchaea laboratory. Salinity was determined in the laboratory using a salinometer (Tempo, India) and pH measurements were made using a single-electrode pH meter (Labindia, India). Sediment samples had a pH of 8.0–8.5 and a salinity of 5–7%, whereas the water samples recorded a pH between 7.1 and 7.8, and salinity of 3–7% (Table 1).

The occurrence of extremely haolphilic archaea in offshore marine (continental shelf) sediments from the west coast of India was determined using the method of most probable number (MPN), suitably modified for halophiles[8]. As extremophilic microorganisms/haloarchaea are known to require a minimum of 15% NaCl/crude salt for growth, MPN analysis for detection and enumeration of haloarchaea in sediments and water samples was carried out using nutrient-rich tryptone yeast extract (TYE) medium[8,9], containing 25% solar salt (NTYE). The MPN of haloarchaea was calculated according to McCrady's table[10]. It ranged from 25 to 155/g dry wt of sediment and 250 to 1800/100 ml of water sample (Table 1).

Simultaneously for obtaining extreme halophiles, aliquots of water/sediment samples from each site were pooled together and 1 ml of water or 1 g of sediment was inoculated into NTYE or glucose synthetic medium with 20% NaCl (NGSM)[11,12]. Flasks were incubated at 160 rpm and RT (26–30°C) on a rotary shaker (Remi) and then plated on the respective media containing agar. Small yellow and cream colonies appeared on NTYE agar and pinpoint white colonies on NGSM agar in 6 days, while pink, orange and reddish-orange colonies grew on further incubation for 20 to 30 days.

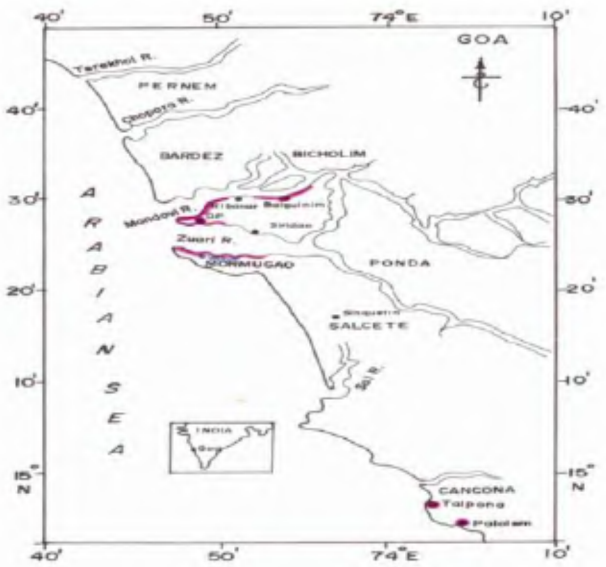

Figure 1. Sampling sites along the coastal regions of Goa.

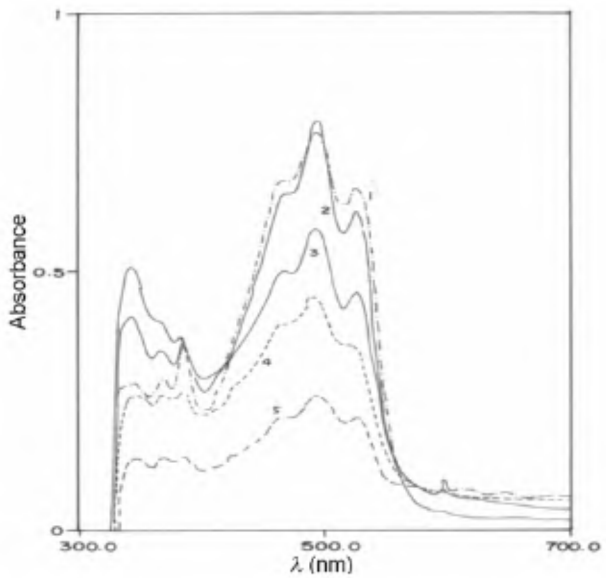

Figure 2. Pigment profile of the various extremely halophilic archaeal isolates. Pigments from GUBF5 (1), GUBF6 (2), GUBF9 (3), GUBF12 (4) and GUBF17 (5) were extracted by sonicating cells in acetone and recorded for absorbance[17].

Table 1. Physico chemical and microbiological analysis of coastal waters and sediments

Sampling site	Sample type	pH*	Salinity (%)*	MPN/ 10^2 ml/g	NTYE cfu/ml	Colony colour	NGSM cfu/ml	Colony colour
Palolem	Water	7.8	7.3	900	2×10^2	Cream	Nil	–
	Sediment	8.5	4.8	115	1.6×10^2	Cream	1.0×10^2	Cream
Talpona	Water	7.86	7.2	900	2.8×10^2	Cream	Nil	–
	Sediment	8.41	5.7	40–55	1.1×10^2	Yellow	Nil	–
Vasco beach	Water	8.2	3	1800+	2×10^2 2.2×10^2	Cream Yellow	1.2×10^2	Cream
Dona Paula Bay	Water	7.1	3	1800+	1.2×10^2 6	Cream White	1.4×10^1	White
	Sediment	6.8	2.9	40–55	1.3×10^1	Pink	Nil	–
Panjim Jetty	Water	8.01	6.8	1800+	1.6×10^1 1.2×10^1	Cream Orange	2.5×10^1	White
Panjim Patto	Water	7.3	0.72	1800+	1.9×10^1 1×10^1	Orange White	Nil	–
	Sediment	8.28	3.9	115	2	Orange	Nil	–
Capt. Port, Panjim	Water	7.5	2.9	1800	6	Orange red	1.2×10^2	White
	Sediment	6.8	2.9	25	8	Orange	1.2×10^1	Cream
Baiginim coast	Water	8	3	250	2.8×10^2 1×10^1	Orange White	Nil	–
	Sediment	7	2	25	4	Orange	6	Cream

*Salinity was determined using a salinometer (Tempo, India) and pH measurements were made using a single electrode pH meter (Labindia, India).

Table 2. Haloarchaea from coastal waters and sediments of Goa

Isolate	Econiche	Pigment	Gram character morphology	Tentative identification
GUBF5	Panjim Jetty	Orange	Negative peculiar arrangement	*Halobacterium* (ATCC BAA-648)
GUBF6	Panjim Jetty	Orange	Negative coccobacilli	*Halobacterium* (ATCC BAA-649)
GUBF9	Baiginim	Orange	Negative coccobacilli	*Haloarcula* (ATCC BAA-650)
GUBF10	Baiginim	Orange	Negative coccobacilli	*Halobacterium* (ATCC BAA-653)
GUBF11	Patto Panjim	Orange	Negative coccobacilli	*Halobacterium* (ATCC BAA-654)
GUBF12	Dona Paula Bay	Pink	Negative rods	*Halobacterium* (ATCC BAA-655)
GUBF14	Capt. Port, Panjim	Reddish-orange	Negative coccobacilli	*Halobacterium* (ATCC BAA-656)
GUBF15	Capt. Port, Panjim	Orange	Negative coccobacilli	*Haloarcula*
GUBF17	Baiginim	Orange	Negative coccobacilli	*Halobacterium*

*GUBF, Goa University, Braganca, Furtado.

Long incubation periods have been reported for maximizing the viable counts and diversity of the haloarchaeal groups[13]. Of these, the colourless or the yellow colonies obtained were bacteria tolerant to low concentration of salt and marginally tolerant to high concentration of salt, a characteristic of slight halophiles. These failed to survive on a medium containing 25% salt during subsequent subcultures. About 30% of such yellow colonies that continued to grow along with orange or reddish-orange colonies was eliminated when streaked on NTYE medium incorporated with a final concentration of 500 IU/ml penicillin[2]. Pure cultures growing on NTYE with penicillin were maintained on NTYE agar slopes, designated as *GUBF (Goa University, Braganca, Furtado), numbered as GUBF1 through GUBF14 and deposited at ATCC with accession numbers as listed in Table 2.

Interestingly, none of these cultures grew on TYE agar or NTYE agar with less than 15% NaCl or crude salt, unlike the isolates of Purdy *et al.*[6]. The inability of the isolates to grow at a concentration less than 15% NaCl or crude salt indicated the absolute requirement of the isolates for high concentration of NaCl, similar to the salt requirements exhibited by extremely halophilic bacteria. The ability of the cultures to grow in media with 500 IU penicillin possibly reflects the presence of a cell wall other than cross-linked peptidoglycan hence exhibiting resistance to penicillin[14]. Each of the isolates lysed on exposure to physiological saline/distilled water, thus indicating destablization of cell surface at a low salt concentration or in the absence of salt. Cells of haloarchaea, which require high concentration of NaCl in their environment to maintain the cell-membrane integrity, are known to undergo lysis when subjected to hypotonic shock[15,16].

Pigments from the cells of each of the isolates extracted with acetone[17,18] showed absorption maxima at 500, 470 and 370 nm, corresponding to bacterioruberin types of pigment, typical of haloarchaea (Figure 2)[17].

Whole cell acid methanolysis[19] of the cultures was done by taking 1 g of dry cells in boiling tubes containing 3 ml methanol, 3 ml toluene and 0.1 ml concentrated H_2SO_4, mixed and heated at 50°C for 12 h. A 1.5 ml aliquot of hexane was then added to this mixture, mixed thoroughly and allowed to separate into phases. The upper hexane layer was collected and 15 μl was spotted onto silica gel 60_{F254} (Merck, India) layer of 0.5 mm thickness on glass plates and developed in a pre-saturated chamber with the solvent system petroleum ether (b.p. 60–80°C) – diethyl ether (85 : 15 v/v). Lipids were visualized by spraying the chromatogram with 10% dodecamolybdophosphoric acid in absolute ethanol and heating at 100°C. The hexane extract cultures, separated by thin layer chroma-

tography, resolved into glycerol diether lipid moieties (GDEM) of *Rf* 0.2, which is not seen in the eubacterial cultures such as *Escherichia coli*. The presence of GDEM, which is absent in the eubacterial counterparts, and the presence of bacterioruberin type of pigments are the key chemotaxonomic markers for ascribing the isolates to haloarchaea.

The demonstration and isolation of archaea from coastal waters and sediments having salinity of 3–7%, an econiche in which they are not expected to survive, is a significant finding that reflects the physiological and ecological complexities of halophilic archaea. It further suggests that the extreme halophiles can survive at low salinities for long periods of time in a viable state.

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Visibility score for countries using SCOPUS Affiliation Identifier and the *h*-threshold approach

The *h*-index was first proposed by Hirsch[1] to quantify the impact of the academic research output (i.e. as seen from papers published rather than from say, patents registered or products or processes transferred to industry). 'A scientist has index *h* if *h* of his/her *N* papers have at least *h* citations each, and the other $(N - h)$ papers have fewer than *h* citations each', where *N* is the number of papers published over *n* years[1].

All such reductionist measures have their limitations, but it has been this writer's impression that of all such measures the *h*-index is the simplest but the most robust method of evaluating performance of an academic scientist. Indeed, this is now a feature of both the Web of Science and SCOPUS.

An interesting possibility that emerged from the definition of the *h*-index is that of using this to derive successive[2] or higher order indices[3]. The concept of meta *h*-indices also appears on the net (http://www.scopus.com/), where it has been suggested that 'a department has meta-*h*-index at least *h* iff at least *h* of its researchers have *h*-index at least *h*. Then a university has meta-meta-*h*-index at least *h* iff at least *h* of its departments have meta-*h*-index at least *h*, and a state has meta-meta-meta-*h* index *h* iff at least *h* of its universities have meta-meta-*h*-index at least *h*.' Recently, Egghe and Rao[4] recognized the possibility of applying the *h*-index methodology to non-citation data and using three indices based on papers (h_p), citations (h_c) and the successive *h*-index (h_2), and with the help of two-by-two Spearman rank correlation coefficients, demonstrated that these rankings are significantly related.

The SCOPUS Affiliation Identifier is arguably the world's first on-line tool to help identify and aggregate the research output portfolio of any organization or entity. Here I report a systematic study of the output of some leading countries doing science in terms of the number of institutions that have a *h*-type threshold of output, measured using the number of papers in the SCOPUS database (queried on 12 July 2008). The studies show that the leading country is the United States, having 1044 institutions with more than 1044 papers. The *h*-threshold is therefore 1044.