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Alkaliphiles in estuarine mangrove regions of Goa, (central west coast of India)

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Obligate alkaliphilic bacteria (28 strains) were isolated from various estuarine ecosystems of Goa. Most of these strains were found to be Gram positive, motile rods, capable of growth in aerobic condition upto *p*H 12, exhibiting optimum growth at *p*H 10.5. Isolates exhibited high buffering capacity confirming their alkaliphilic nature. Significantly high difference was noted in the buffering capacities of the alkaliphiles and the neutrophile indicating their ability to maintain their internal *p*H. The obligate alkaliphile A52 exhibited highest cytoplasmic buffering capacity of 8,500 nanomoles OH^- ions/*p*H unit/mg protein. The isolates also showed amylase (39%), protease (50%) and lipase (100%) activity under alkaline conditions. The present study depicts that such alkaliphilic bacteria also play an important role in the mineralization of organic matter under high *p*H conditions in natural ecosystems.

[**Key words:** Alkaliphiles, mangroves, buffering capacity, enzymes, biodiversity, Goa, bacteria, estuary]
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Introduction

Microorganisms and the archaea are found in the extreme conditions of temperatures, *p*H, pressures, salt and nutrient conditions. Alkaliphiles and haloalkaliphiles are one such diverse group of extremophilic microorganisms that thrive at very high *p*H and salt concentration. Organisms with *p*H optima for growth in excess of *p*H 8, usually between 9 and 10 *p*H are defined as alkaliphiles[1]. Obligate alkaliphiles are incapable of growth at neutral *p*H. Most of the organisms described to date as growing under high alkaline condition are prokaryotes, comprising heterogeneous collection of eubacteria with a few examples of archaebacteria. Alkaliphilic organisms (particularly prokaryotes) are widely distributed and can be found even in environments where overall *p*H may not be particularly alkaline[1]. Alkaliphiles grow well in *p*H range of 10-11 and are of ecological, industrial and basic bioenergetic interest[2]. A typical alkaliphile, which grows at an external *p*H of 10.5 or above does not allow its cytoplasmic *p*H to exceed a value of *p*H 9.5 even if the external *p*H is raised[3]. Alkaliphiles exist in a variety of unique environments that are hostile to other organisms and perhaps play a significant role in biotransformation occurring in such environments[1,4,5]. Several of these are entirely novel and have unique properties. Genes and genetic manipulations of industrially important enzymes of alkaliphiles have gained tremendous importance[6]. The marine and mangrove ecosystems offer a number of unique ecological niches for harboring such microorganisms and are reported to be rich in halophiles, alkaliphiles and haloalkaliphiles. The diverse organisms and their activities, specially their role in nutrient recycling has been documented[5]. We report here the isolation of obligate alkaliphiles from estuarine ecosystems of Goa, buffering capacity and the enzymic potentials of these alkaliphilic microorganisms.

Materials and Methods

Water and sediment samples were collected from various estuarine mangrove rich ecosystems of Goa in the area of Ribandar, Banastari, St. Cruz, Panjim, Merces during Oct 2000 and July 2002. The sampling sites were selected as they harboured rich mangrove forests, are affected by tidal fluxes and are water logged and also found to have econiches with alkaline *p*H. The media used for isolation, growth and maintenance of alkaliphilic bacteria was Polypeptone Yeast Extract Glucose (PPYG) Agar [7]. Solutions of 10% Glucose and 10% Na_2CO_3 were sterilized separately by autoclaving and the final *p*H of the medium was maintained at *p*H 10.5.

Water samples collected from each location were diluted ten fold in stabilized natural seawater (sterile) and plated on nutrient agar and PPYG (*p*H 10.5). Sediment samples collected from the subtidal regions of the estuarine ecosystem were suspended in stabilized natural seawater (sterile) at the ratio of 1:10

and incubated on the Orbitek shaker for 1 hr at 150 rpm. The suspension was allowed to settle and 0.1 ml of clear supernatant and the dilutions were spread plated on nutrient agar and PPYG (*p*H 10.5). The petri plates were incubated at room temperature and the colonies that appeared were counted. Predominant isolates obtained on PPYG (*p*H 10.5) were selected, purified and maintained on the same medium. Isolates growing on PPYG agar (*p*H 10.5), were replica-plated on PPYG agar with *p*H 7.0, 8.5, 10.5 and 12.0 to determine their *p*H tolerance and obtain obligate alkaliphiles. These obligate alkaliphiles (growing only at *p*H 10.5 and 12.0) were selected for further studies. The isolated obligate alkaliphiles were identified according to their morphological and biochemical characteristics based on Bergey's Manual[8]. The biochemical media used for identification was modified based on the alkaline conditions required for growth[7].

The ability of the isolates to hydrolyse polymers was determined using PPY agar *p*H 10.5, supplemented with starch, casein, tributyrin and cellulose instead of glucose. The cultures were streaked on the plates containing the substrates and incubated for 48 hrs. The hydrolysis of the substrates were detected by using standard methods[7].

Buffering capacities of selected isolates were determined by titration using 0.05 *M* KOH[9,10]. Cultures were grown in PPYG broth, incubated for 24 hrs on shaker and the cells harvested by centrifugation at 5000 rpm for 15 mins. The pellets were washed, resuspended in 10 ml of 0.2 *M* KCl solution and the protein content of this cell suspension was estimated using Folin Lowry's method[11]. Cell suspension corresponding to 5 mg of cell protein was taken in a beaker and the initial *p*H of the suspension was noted. The *p*H change at every addition of 10 µl of 0.05 *M* KOH was noted using calibrated *p*H analyser (Labindia). The whole cell buffering capacity (B_0) was measured as nanomoles of hydroxyl ions consumed to change 1 *p*H unit/mg protein. To determine the permeabilised cell buffering capacity (Bt), cell suspension in 0.2 *M* KCl corresponding to 5 mg of protein was treated with 10 ml of triton X-100, mixed, allowed to stand for 5 mins and centrifuged. The pellet obtained was washed with 0.2 *M* KCl to remove the permeabilizing agent, resuspended in 0.2 *M* KCl, titrated against 0.05 *M* KOH and change in *p*H noted. The permeabilised cell buffering capacity was noted as nanomoles of OH^- ions consumed to change 1 *p*H unit/mg protein. The cytoplasmic buffering capacity (Bi) was determined as a difference of Bo and Bt (Bi = Bo-Bt)[10]. Buffering capacity of a neutrophile was determined for comparison. Control titration was performed using the gram negative neutrophilic organism *Pseudomonas.*

Results and Discussion

Viable counts of general heterotrophic neutrophilic bacterial populations and alkaliphilic bacteria were enumerated from five different estuarine locations of Goa viz. Ribandar, Banastari, St. Cruz, Panjim and Merces (Table 1). The counts were much higher on nutrient agar as compared to PPYG medium, indicating the existence of neutrophilic and alkalotolerant bacteria. It has been reported that the ratio of alkaliphiles to neutrophiles[6] found in soil is about 1:10 to 1:100. Sediment samples had a higher count on nutrient agar as well as on PPYG (*p*H 10.5), as compared to the water samples. Highest counts of alkaliphilic bacteria were generally recorded from the Ribandar samples, which gave a viable count of 7×10^4 cfu/ml. It has been reported that the alkaliphiles depend on Na^+ ions for maintenance of internal *p*H[10]. It is noted that the concentration of Na^+ ions is very high in PPYG agar, the concentration best suited for organisms growing under alkaline conditions.

Predominant isolates (153) were picked up randomly from PPYG agar (*p*H 10.5) and replica plated on the same medium with *p*H 7.0, 8.5, 10.5 and 12.0. Isolates (28) growing only at *p*H 10.5 and 12.0 were considered to be obligate alkaliphiles and were selected for further studies.

Table 1—Total viable count of neutrophilic & alkaliphilic bacteria in samples taken from various mangrove ecosystems of Goa.

Sediment samples	Oct-2000 cfu/ml (× 102)		Jul-2001 cfu/ml (× 102)	
	N.A	PPYG	N.A	PPYG
Merces	7840	130	80	1
St. Cruz	2100	140	69	1
Panjim	6920	680	679	1
Banastari	5500	509	4	2
Ribandar	7500	700	210	4
Water samples	**cfu/ml (× 102)**		**cfu/ml**	
	N.A	PPYG	N.A	PPYG
Merces	130	3	100	2
St. Cruz	460	4	100	3
Panjim	410	2	241	2
Banastari	520	4	577	1
Ribandar	650	6	295	4

N.A - Nutrient agar; PPYG - Polypeptone yeast extract glucose agar; cfu - colony forming units

On the basis of morphological, biochemical and physiological characteristics, the cultures were identified as *Bacillus* (53%), *Corynebacterium* (21%), *Micrococcus* (7%), *Actinomyces* and *Flavobacterium* (Table 2). A majority of obligate alkaliphiles obtained during the study were found to be gram positive organisms confirming that they are better adapted to high *p*H as reported earlier [3,12-14].

Table 2—Identification status of alkaliphiles

Culture No.	Culture code	Tentative identification
A1a	RiMsX1a	*Corynebacterium*
A1b	RiMsX1b	*B. alcalophilus*
A3a	RiMsX3a	*B. laterosporus*
A3b	RiMsX3b	*Bacillus sp.*
A5	RiMsX5	*Actinomyces sp.*
A6	RiMsX6	*Actinomyces sp.*
A20	RiMsX20	*B. alcalophilus*
A27	RiMsX27	*B. alcalophilus*
A30	RiMsX30	*B. alcalophilus*
A37	PjMsX1	*B. alcalophilus*
A43	BnMsX7	*Micrococcus lylae*
A52	BnMsX12	*Micrococcus lylae*
A55	BnMsX15	*B. alcalophilus*
A59a	BnMsX19	*Bacillus sp.*
A59b	BnMsX19	*Bacillus sp.*
A61	BnMsX21	*B. schlegelii*
A62	BnMsX22	*B. schlegelii*
A64	BnMsX24	*B. stearothermophilus*
A65	BnMsX25	*Corynebacterium*
A66	BnMsX26	*Corynebacterium*
A67a	BnMsX27a	*Bacillus sp.*
A67b	BnMsX27b	*B. stearothermophilus*
A77	BnMsX28	*Corynebacterium*
A86	RiMsX86	*B. brevis*
A102	RiMsX102	*Corynebacterium*
A118	RiMsX118	*unidentified*
A129	RiMsX129	*unidentified*
A131	RiMsX131	*Flavobacterium*

Cytoplasmic buffering capacities and buffering by whole cells was examined in 12 alkaliphilic bacterial species. Acid base titrations were conducted on whole cells and cell permeabilised with triton X-100. Triton X-100 is the non-ionic detergent which specifically and selectively solubilises proteins of cytoplasmic membrane leaving the cell wall totally undisturbed[9].

The difference between buffering capacities of whole cells (B_0) and permeabilised cells (B_t) is interpreted as internal/cytoplasmic buffering capacity (B_i), which was found to be very high and stable for all the alkaliphiles selected during the study[10]. A consistent effect of permeabilisation treatment was noted to be a decrease in the buffering capacity for all tested cultures, indicating that certain components required for maintaining the buffering capacity are leached out on treatment with triton X-100[9].

Controlled titrations done with 0.2 *M* KCl indicate that KCl does not affect the changes in *p*H and hence is an ideal suspension medium for these studies. The amount of KOH required to bring about one *p*H unit change for whole cells and permeabilised cells of the 12 alkaliphilic isolates and a neutrophile was recorded. A significant difference was noted in the whole cell buffering capacity (B_0) of the neutrophile and the alkaliphiles. The amount of KOH required for the change of one *p*H unit was 150 nmoles/mg protein for the neutrophile while for the alkaliphiles it ranged from 2500 nmoles to 13,000 nmoles of OH ions (Fig. 1). Maximum whole cell buffering capacity was exhibited by the culture *Corynebacterium*.

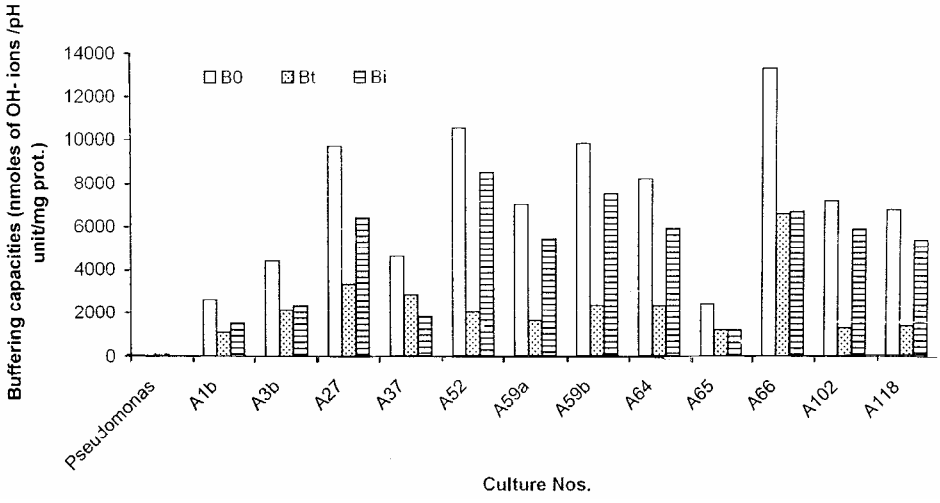

Fig. 1—Comparison of buffering capacities between the alkaliphilic cultures and the neutrophile. Bo-whole cell buffering capacity; Bt - permeabilised cell buffering capacity; Bi - cytoplasmic buffering capacity

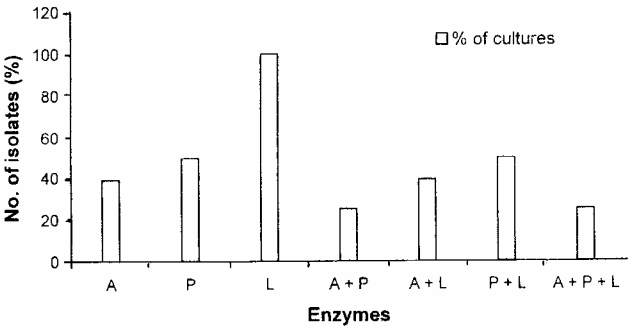

Fig. 2—Activity of enzymes exhibited by the isolates
A - Amylase; P - Protease; L - Lipase

Enzyme activities (Fig. 2) were checked using starch, skim milk, tributyrin and cellulose in the PPY medium as substrate and the production of amylase, protease, lipase and cellulase was noted respectively. It was noted that alkaliphiles producing amylase, protease and lipase were 39%, 50% and 100% respectively as compared to the cellulase production, which was however absent. Unique types of enzymes have been isolated from alkaliphiles, which have various industrial applications. It was observed that many of the isolates showed the production of more than one enzyme at alkaline *p*H as reported [15,16].

The present study has confirmed the presence of different genera of alkaliphilic organisms in estuarine mangrove ecosystems. The diversity of enzymes produced by these organisms such as amylase (39 %), protease (50%) and lipase (100%) activity under alkaline conditions indicates that such organisms also have a significant role to play in recycling organic matter in these ecosystems.

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