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ALKALIPHILIC AND HALOALKALIPHILIC PHOSPHATE SOLUBILIZING BACTERIA FROM COASTAL ECOSYSTEMS OF GOA

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Key words : *Coastal ecosystems, Alkaliphiles, Phosphate solubilization, Phosphatase, Chromohalobacter israelensis, Bacillus marisflavi.*

Abstract - The occurrence of alkaliphilic bacteria was studied in samples collected from diverse marine and coastal ecosystems of Goa representing different mangrove, khazan, sand dune and saltpan niches. Highest count of 2.6 x 10^7cfu/g of alkaliphiles was reported from Cavelossim khazan sediment sample on polypeptone yeast extract glucose agar. One forty one predominant alkaliphilic bacteria were selected and screened for inorganic and organic phosphate solubilization. Forty three alkaliphilic isolates were found to be inorganic phosphate solubilizers and 27 were organic phosphate solubilizers. Two potential phosphate solubilizing isolates, FA7 and WA3 were selected for identification. Alkaliphilic isolate FA7 was identified as *Bacillus marisflavi* obtained from mangrove Merces and haloalkaiphilic isolate WA3 identified as *Chromohalobacter israelensis* was isolated from Batim salt pan. The solubilization of phosphate at high pH of 10.0 by *Bacillus marisflavi* FA7 and at high pH and salt concentration by *Chromohalobacter israelensis* WA3 is reported for the first time.

INTRODUCTION

Among the 17 elements required for plant growth, phosphorus is known as the master key element. It plays an important role in plant's physiological and biochemical processes (Rodriguez & Fraga, 1999; Igual *et al*., 2001; Cordell & White, 2013). In most soils, phosphorus content is about 0.05% of which only 0.1% is available to plants (Jones & Oburger, 2011). The diverse soil phosphate forms can be categorized as soil solution phosphates (orthophosphate), insoluble inorganic and insoluble organic phosphates (Rodriguez *et al*., 2006; Khan *et al*., 2009; Khan *et al.*, 2014). Phosphorus is taken up mostly as the primary orthophosphate ion ($H_2PO_4^-$). Due to the large reactivity of phosphate ions with numerous soil constituents, it is the least mobile and available element in most soil conditions (Hinsinger, 2001; Mahdi *et al*., 2011). Therefore, it is a major limiting factor for plant growth.

Phosphate solubilizing bacteria and fungi can improve plant phosphorus nutrition by solubilizing inorganic and organic phosphates (Richardson, 2001). Environmental factors like high alkalinity and salinity of soil interfere with activity of such microbes and also increase the precipitation of available phosphorus (Nautiyal *et al*., 2000). Therefore, microorganisms which are able to thrive in such conditions and also capable of phosphate solubilization can be useful in increasing the fertility of alkaline and saline soils. Such bacteria may be found in ecosystems which are continuously in contact with different levels of salinity and alkalinity.

Natural and man-made ecosystems observed along the coast of Goa are sand dunes, mangrove plantation, salt pans and khazan lands. Each of these eco-niches has special structure, functions, biodiversity and productivity (Untawale, 2006). These ecosystems are conspicuously associated with the microbial flora responsible for several

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important biological processes (Uroz *et al*., 2009). Microorganisms in these eco-niches survive with exposure to varying levels of salinity, pH, temperature and moisture content due to the continuous influence of tidal cycle, freshwater influx and recurring seasonal changes (Vassilev *et al*., 2012).

Over the years, farmers have been successful in cultivating salt-tolerant rice varieties (Munns *et al*., 2006). But the productivity of these traditional varieties is comparatively low. Hence, there is a shift from these traditional varieties of paddy to high yielding varieties. High-yielding varieties require inorganic fertilizers and pesticides working in those conditions. Phosphate solubilizing bacteria promoting growth of plants in alkaline and saline environments may prove beneficial for crops grown in these areas (Sonak, 2006). Therefore, the focus of our study is to isolate and identify alkaliphilic and haloalkaliphilic phosphate solubilizing bacteria from coastal ecosystems of Goa.

MATERIALS AND METHODS

Collection of samples

Samples were collected in the pre-monsoon period during the months from January to May 2010. Sediment and water samples were collected from 13 mangrove habitats and 4 khazan lands (Figure 1, Table 1). Only sand and sediment sample was collected from 4 sand dunes and 2 man-made solar salterns. Samples were collected from a depth of 10 to 15 cm in sterile plastic bags and bottles, brought to the laboratory and stored at 4°C until further analysis.

Physico-chemical analysis of samples

The pH and salinity of both sediment and water samples were determined using pH meter and Argentometric titration method respectively (Clesceri *et al*., 1999). Phosphate content of only the sediment samples was determined using Bray's method (Bray, 1948).

Isolation of alkaliphiles

Alkaliphilic bacterial population was estimated by plate count method (Gee *et al*., 1980). The medium used for isolation of alkaliphilic bacteria was Polypeptone Yeast Extract Glucose Agar (PPYG) (Composition in g/L: Peptone: 5.0, Yeast extract: 1.5, Glucose: 5.0, Na_2HPO_4: 1.5, NaCl: 1.5, $MgCl_2$: 0.1, Na_2CO_3: 5.0, Agar: 15, pH:10.5). Solutions of 10% (w/v) glucose and 10% (w/v) Na_2CO_3 were sterilized separately by autoclaving. Final pH of the sterile medium was adjusted aseptically to pH 10.5 using 10% (w/v) Na_2CO_3 solution. Samples were serially diluted in physiological saline. Aliquot of 0.1mL of appropriate dilution was plated on PPYG. Plates were incubated at 30 °C and examined after 48 hours for bacterial colonies. Morphologically distinct colonies were selected, purified, and maintained on PPYG medium slants.

Characterization of alkaliphiles

Gram character of the alkaliphilic bacterial isolates was determined by Gram's staining and confirmed by KOH method (Buck, 1982). Further, isolates were spot inoculated on PPYG agar plates with pH 7, 8, 9, 10 and 11 to understand the alkaliphilic nature. Growth was observed and recorded after 24 hours of incubation. Based on the results, isolates were classified as alkalitolerant, facultative alkaliphiles and obligate alkaliphiles.

Screening of isolates for inorganic and organic phosphate solubilization:

Inorganic phosphate solubilization

Phosphate solubilizing activity of the alkaliphilic isolates obtained was evaluated by plate method on modified Pikovskaya's (PVK) medium using tri-calcium phosphate (LOBA Chemie) as a sole source of phosphorus (Composition in g/L: $Ca_3(PO_4)_3$: 5.0, Glucose: 10.0, $(NH_4)_2SO_4$: 0.5, KCl: 0.2, K2HPO4: 0.1, $MgSO_4$: 0.1, $MnSO_4$: 0.0001, $FeSO_4$: 0.0001, Yeast extract: 0.5 Agar: 20.0, pH: 10.0) (Pikovskaya, 1948). Bacterial isolates solubilizing inorganic phosphate show visible dissolution zones or halos around the colonies. PVK medium was modified by adjusting the pH to 10.0 for growth of alkaliphiles. Inoculation was carried out on PVK medium plates with freshly grown cultures of the isolates. Inoculated plates were incubated at 30°C and observed at 24 hours interval up to 5 days for phosphate solubilization. Halozone diameter and colony diameter were recorded to determine the solubilization Index (SI) (Afzal & Bano, 2008). SI was determined by dividing halozone diameter with colony diameter.

Effect of different pH and salt concentration on inorganic phosphate solubilizing isolates

Isolates showing inorganic phosphate solubili-

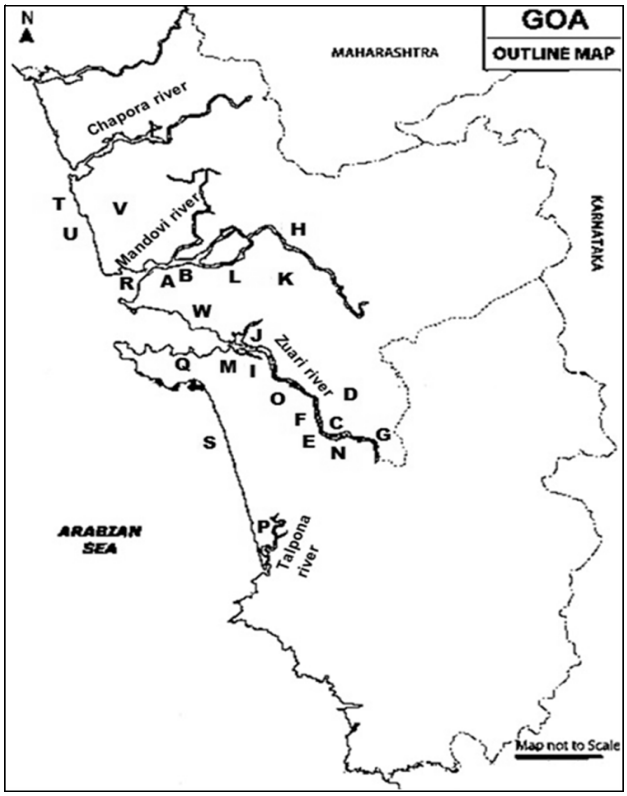


Fig. 1 Map of Goa showing location of sampling sites. Samples were collected from 23 sites representing four types of coastal ecosystems such as mangroves, khazan lands, sand dunes and salt pans (For details refer Table 1).

zation were spot inoculated on Pikovskaya's medium at different combinations of pH and salinity (pH 8 + 10% NaCl, pH 8 + 25% NaCl, pH 10 + 10% NaCl and pH 10 + 25% NaCl). Growth and phosphate solubilization capability of the isolates were noted after 72 hours of incubation.

Organic phosphate solubilization

Isolates were inoculated in mineral salts medium (MSM) with 1mM disodium-p-nitrophenyl phosphate (p-NPP) as the source of organic phosphate (Composition in g/L: $NaNO_3$: 2.0, $MgSO_4$: 0.2, $MnSO_4$: 0.02, $CaCl_2$: 0.02, $FeSO_4$: 0.02, Glucose: 5, pH: 10.0) (Sitdhipol *et al*., 2012). Bacterial isolates producing phosphatase enzyme show yellow colouration due to the liberation of para-nitrophenol from para-nitrophenylphosphate in the culture broth. Inoculated flasks were incubated at 30°C and observed at 24 hours interval up to 5 days for yellow colouration.The culture broths were centrifuged at 10000 rpm for 10 min. Absorbance of the supernatant was recorded at 405 nm.

Identification of potential phosphate solubilizing isolates

Morphological characterization

In addition to Gram staining, spore staining was performed after 48 hours of culture growth by Schaffer and Fulton's Method (Schaeffer & Fulton, 1933). Scanning electron microscopy (SEM) was performed for morphological characterization of the selected isolates. Samples for SEM were prepared by method described by Prior and Perkins (Prior & Perkins, 1974). After the sample preparation, the coverslip containing sample was placed on stubs. Stubs were then placed in sputter coater (JOEL JFC 1600). After sputtering, the stub was placed into the electron microscope sample chamber and observed with JOEL JSM-6360LV electron microscope.

Biochemical characterization

Biochemical tests were carried out to characterize the isolates (Starr *et al*., 1981). The tests were sugar fermentation (glucose, xylose, maltose, lactose, fructose, sucrose, trehalose, galactose, sorbitol and mannitol), Indole production, Methyl Red test (MR), Voges Proskaur test, Citrate utilization, enzyme production (urease, gelatinase, phosphatase, amylase, catalase, oxidase, ornithine decarboxylase and lysine decarboxylase), nitrate reduction and motility. Based on the results obtained, the isolates were tentatively identified using Bergey's Manual of Determinative Bacteriology, Volume III (Garrity *et al*., 2001). Buffering capacity of the isolates was determined using the method proposed by Krulwich *et al*., (1985).

Molecular characterization

Isolates FA7 and WA3 were grown in PPYG broth at 30°C for 24 hours. The culture broth was centrifuged and pellet was used for extraction of DNA. Genomic DNA was extracted by method described by Sambrook *et al*., (1989). Amplification of 16S rRNA gene was performed using universal primers (8F and 1429R) for 16S rRNA gene with a final reaction volume of 25 µL reaction (Gaonkar *et al*., 2012). Amplicons were detected by electrophoresis on 1.5% agarose gel staining with ethidium bromide. The amplified DNA was outsourced to Xcelris Genomics Pvt. Ltd. The 16S rRNA gene sequence obtained was compared with sequences in the GenBank using BLAST search program

Table 1. Physico-chemical analysis and viable count of alkaliphilic bacteria on PPYG agar isolated from sediment and water of samples from different coastal ecosystems of Goa.

	Site	Type	Physico-chemical analysis			Viable count of alkaliphiles in sediment (cfu/g) and water (cfu/mL)
			pH	Salinity (g/kg)(g/L)	Phosphate	
Mangrove Samples						
A.	Merces	Sediment	6.4	19.90	59.1	1.5×10^4
		Water	7.8	0.800	N.D.	1.8×10^4
B.	Ribander	Sediment	6.7	33.81	59.1	2.7×10^4
		Water	8.0	2.800	N.D.	5.5×10^4
C.	Durbhat	Sediment	7.3	18.14	89.6	3.2×10^5
		Water	7.1	0.400	N.D.	3.2×10^4
D.	Bandora	Water	7.4	0.500	N.D.	2.9×10^3
E.	Rasai	Sediment	6.5	3.290	47.3	9.4×10^5
		Water	7.4	0.082	N.D.	3.2×10^5
F.	Quellossim	Sediment	6.3	0.820	47.3	4.0×10^5
		Water	6.0	0.164	N.D.	2.0×10^5
G.	Borim	Sediment	7.3	19.79	48.7	6.1×10^5
		Water	7.1	0.500	N.D.	1.3×10^7
H.	Amona	Sediment	7.8	0.000	56.1	7.8×10^4
		Water	7.1	25.97	N.D.	5.1×10^4
I.	Cortalim Ferry	Sediment	7.8	0.000	53.22	1.0×10^5
	Point	Water	6.9	23.91	N.D.	3.2×10^4
J.	Madkai Ferry	Sediment	7.5	0.000	48.7	9.0×10^3
	Point	Water	5.0	28.20	N.D.	4.1×10^4
K.	Banastari	Sediment	6.9	1.650	60.6	5.5×10^4
		Water	7.0	28.45	N.D.	2.5×10^4
L.	Old Goa	Sediment	7.4	0.000	60.6	3.2×10^4
		Water	7.1	33.48	N.D.	2.2×10^4
M.	Cortalim	Sediment	7.6	23.09	51.84	6.3×10^5
		Water	6.9	32.74	N.D.	9.4×10^5
Khazan Land Samples						
N.	Loutulim	Sediment	10.0	4.948	79.2	2.3×10^6
		Water	6.8	0.330	N.D.	4.1×10^4
O.	Quellossim	Sediment	6.2	4.120	60.6	7.0×10^3
		Water	6.4	0.412	N.D.	4.6×10^5
P.	Cavellossim	Sediment	6.2	82.47	60.6	2.6×10^7
		Water	7.3	75.87	N.D.	2.8×10^6
Q.	Sancoale	Sediment	7.1	64.33	45.83	4.2×10^6
		Water	6.9	59.13	N.D.	1.6×10^5
Sand Dune Samples						
R.	Miramar	Sediment	7.4	32.90	67.2	1.4×10^4
S.	Colva	Sediment	7.5	1.640	60.6	5.1×10^4
T.	Vagator	Sediment	7.8	6.590	69.3	8.7×10^5
U.	Anjuna	Sediment	8.2	4.940	65.2	3.1×10^6
Salt Pan Samples						
V.	Arpora	Sediment	7.0	202.0	47.3	4.2×10^5
W.	Batim	Sediment	6.1	228.4	59.1	1.0×10^7

N.D. - Not Done

Table 2. Buffering capacity of alkaliphilic isolates (FA7 and WA3) and neutrophilic bacteria (*E. coli* and *B. subtilis*).

Isolate	Whole Cell Buffering Capacity		Triton X treated Cells		Decrease in OH^- ion consumption after treatment	Buffering Capacity		Cytoplasmic buffering Capacity
	µL of 0.05M KOH consumed	nmoles of OH^-	µL of 0.05M KOH consumed	nmoles of OH- ions		Whole cells (Bo)	Treated cells (Bt)	Bi=Bo-Bt
FA7	450	22500	320	16000	6500	4500	3200	1300
WA3	440	22000	210	10500	11500	4400	2100	2300
E. coli	15	750	11	550	200	150	110	40
B. subtilis	50	2500	20	1000	1500	500	200	300

Table 3. Morphological and biochemical characteristics of selected potential alkaliphilic phosphate solubilizing isolates FA7 and WA3.

Test	FA7	WA3
Gram character	Gram positive	Gram negative
Motility	Motile	Non-motile
Spore staining	Positive, Subterminal	Negative
Colony colour	Yellow	Cream
pH	8-11	7-10
Salt concentration (%)	0-5	1-25
Positive for production of	Catalase, Amylase, Phosphatase	Catalase, Nitratase, Lysine decarboxylase, Ornithine decarboxylase, MR
Negative for production of	Urease, Oxidase, Gelatinase, Indole	Phospatase, Urease, Amylase, Oxidase, Gelatinase, Indole
Acid produced from	Glucose, Maltose, Fructose, Mannitol, Sucrose, Xylose, Trehalose.	Glucose, Maltose, Fructose, Mannitol, Sucrose, Lactose, Galactose
Acid not produced from	Lactose, Sorbitol, Galactose	Sorbitol, Trehalose
Nitrate reduction	Negative	Positive
Citrate utilization	Negative	Positive
Tri-calcium phosphate solubilization	Positive	Positive

(Altschul *et al*., 1990) and aligned using the multiple alignment Clustal X program (Thompson *et al*., 1997). Phylogenetic tree was constructed using the 1000 Bootstrap Neighbour Joining method of Clustal X version 2.0. Final tree was drawn with MEGA 6.0 software. 16S rRNA gene sequence of isolates FA7 and WA3 was deposited in GenBank and accession numbers were obtained.

RESULTS

Physico-chemical analysis of samples

Twenty three sediment and 17 water samples were collected from different sites of Goa (Figure 1, Table 1) and analyzed for pH, salinity and phosphate content. The pH of the sediment samples ranged from 6.2 (Cavelossim Khazan sediment) to 10.0. (Lotulim mangrove sediment) and pH of water samples ranged from 5.0 (Madkai mangrove water) to 8.0 (Ribander mangrove water). Salinity of sediment samples was recorded and was found to be from 0.0 (Cortalim and Madkai ferry point sediment, Old Goa mangrove sediment) to 228.4g/kg (Batim saltpan sediment); whereas, salinity of water samples ranged from 0.082 (Rasai mangrove water) to 75.87g/kg (Cavelossim Khazan water). Least phosphate content of 47.3g/kg was found in sediment of Rasai mangrove and maximum value of 89.6g/kg was observed in Durbhat mangrove

sediment (Table 1).

Isolation and characterization of alkaliphilic bacteria

Viable count of heterotrophic alkaliphiles from mangrove sediment samples ranged from 7 x 10^3 - 2.6 x 10^7cfu/g and count recorded in water samples was from 2.96 x 10^3 to 1.3 x 10^7cfu/mL (Table 1). Highest count of alkaliphiles of 2.6 x 10^7cfu/g was reported from Cavelossimkhazan sediment sample and lowest of 7.0 x 10^3 cfu/g in Quellossim sediment sample. Sand dune samples gave the counts in the range of 1.4 x 10^4 to 3.16 x 10^6 cfu/g. Interestingly, salt pan sediment sample from Batim also gave the count of the order of 10^7cfu/g which was second highest among all samples. Higher counts of alkaliphiles were observed among sediment samples as compared to water samples. Isolates obtained were designated with alphanumeric code based on site of isolation.

A total of 141 alkaliphilic isolates were obtained on the basis of colony characteristics. A number of isolates were pigmented with colour ranging from yellow, orange, pink to brown. Based on colony characters, 16 diverse isolates were obtained from sediment of Batim salt pans. Among the isolates 75.56% were Gram negative and 24.44% were Gram positive. Gram staining of alkaliphiles also demonstrated wide variation in their morphology varying from rods, cocci, filamentous and pleomorphic forms.

Among these 141 isolates, 80 were alkalitolerant, 43 were facultative alkaliphiles and 18 obligate alkaliphiles. In this study, obligate and facultative alkaliphiles were detected in mangrove, khazan and sand dune ecosystems. However, all the isolates obtained from salt pan ecosystems were alkalitolerant.

Screening of alkaliphilic isolates for inorganic phosphate solubilization

Out of a total of 141 isolates, 43 isolates demonstrated inorganic phosphate solubilization at pH 10.0. Varying degree of phosphate solubilization was observed among the isolates as indicated by solubilization index (Figure 2). Among the 43 isolates showing positive result, 3 isolates gave the SI above 2.5. These isolates were FA7, WA3 and DA3. Only 6 isolates demonstrated the index below 1.5. Remaining isolates showed index between 1.5 and 2.5.

Effect of pH and salinity revealed that among 43 phosphate solubilizing isolates, twenty-eight isolates showed growth at pH 8 with 10% NaCl out of which three showed solubilization of tricalcium phosphate. On pH eight and 25% NaCl, five isolates showed growth and three showed solubilization. On pH 10 and 10% NaCl five isolates showed growth and three showed solubilization. On pH 10 with 25% NaCl, three isolates namely AA5, CA3 and WA3 showed growth and phosphate solubilization.

Screening of alkaliphilic isolates for organic phosphate solubilization

Twenty seven isolates showed organic phosphate solubilizing ability by production of phosphatase enzyme at pH 10. Among all the isolates, isolate FA7 followed by PA7 gave maximum absorbance (Figure 3). Figure 4 shows inorganic and organic phosphate solubilizing isolates obtained from each sample.

At the end of screening process, it was observed that among the 141 isolates, 30 isolates showed only inorganic phosphate solubilizing activity, 14 isolates only organic phosphate solubilizing activity and 13 isolates demonstrated both inorganic as well as organic phosphate solubilizing activity. Merces mangrove sample gave the highest number of phosphate solubilizing isolates.

Morphological, biochemical and molecular characterization of potential phosphate solubilizing isolates

Based on the results of phosphate solubilization, isolate FA7 and WA3 were selected for identification (Figure 5.a and 5.b). Isolate FA7 had both inorganic and organic phosphate solubilizing ability at pH 10. Isolate WA3 showed phosphate solubilizing activity at high salt concentration (25%) and high pH of 10.0. Morphological characterization and scanning electron micrographs revealed FA7 to be rod shaped Gram positive spore forming bacterium and WA3 to be Gram negative rod shaped bacterium (Figure 6.a and 6.b). The cytoplasmic buffering capacity of isolate WA3 was found to be 2300 nmoles of OH- ions which was higher than 1300 nmoles of OH- ions of isolate FA7 (Table 2). Buffering capacity of whole cells was higher than triton X treated cells. Cells permiabilised with triton X-100 were found to lose their buffering capacity. Isolate FA7 and WA3 demonstrated high buffering capacity as compared with neutrophilic controls *E. coli* and *Bacillus subtilis*. The details of

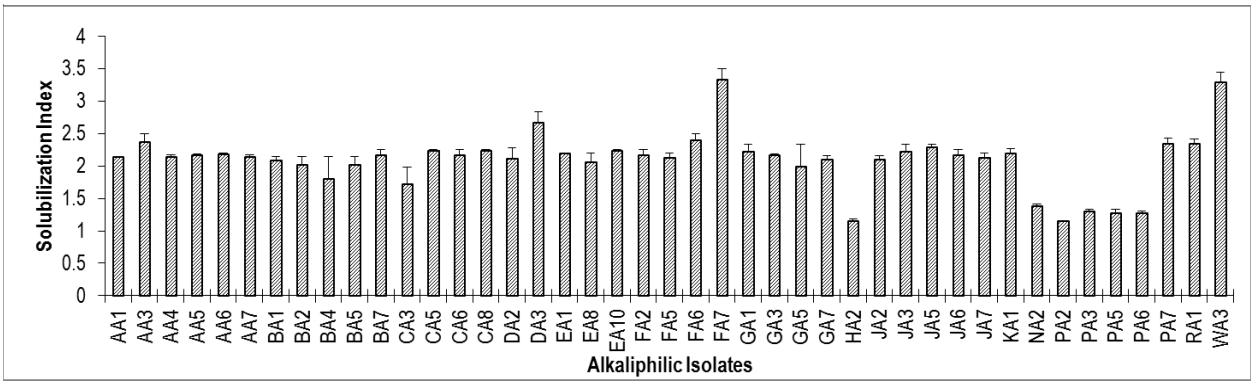


Fig. 2 Solubilization index of alkaliphilic isolates for solubilisation of tri-calcium phosphate on Pikovskaya's agar at pH 10.

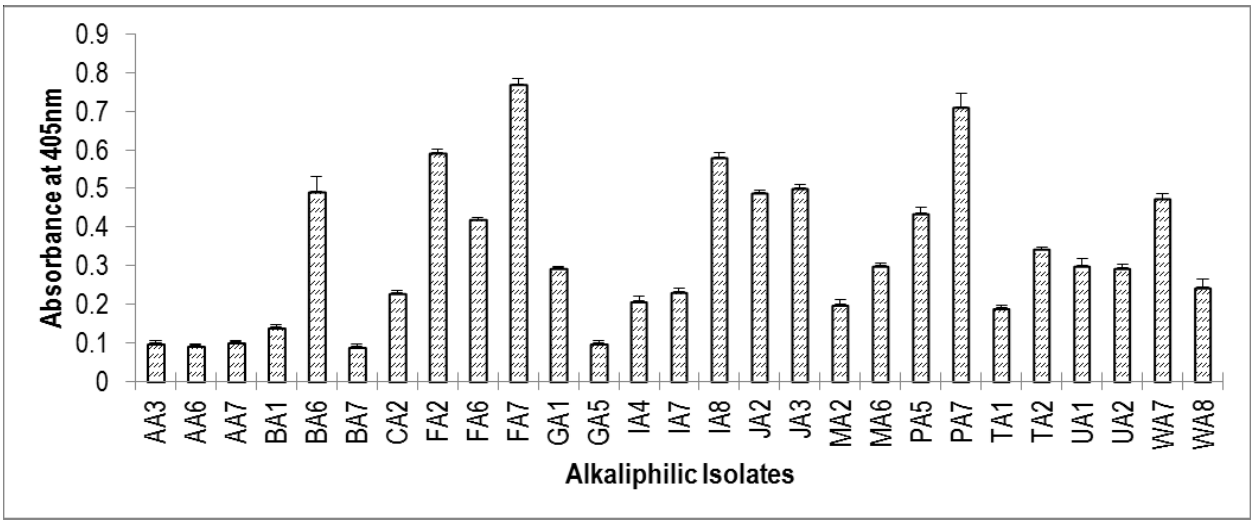


Fig. 3 Intensity of yellow colouration due to liberation of nitrophenol from p-NPP recorded at 405nm by phosphatase enzyme producing alkaliphilic isolates in MSM at pH 10.

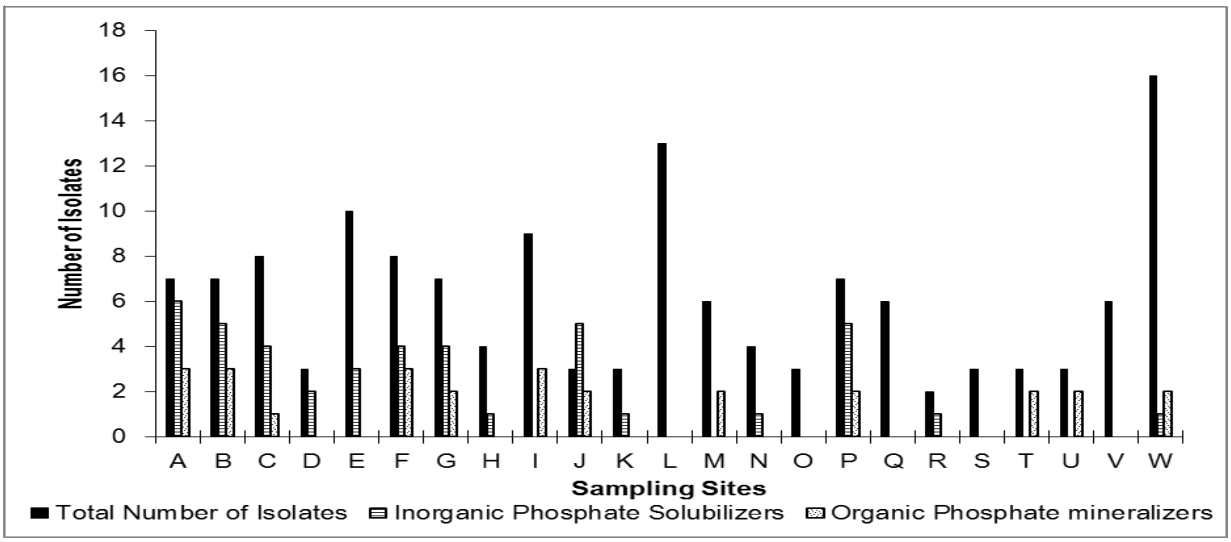
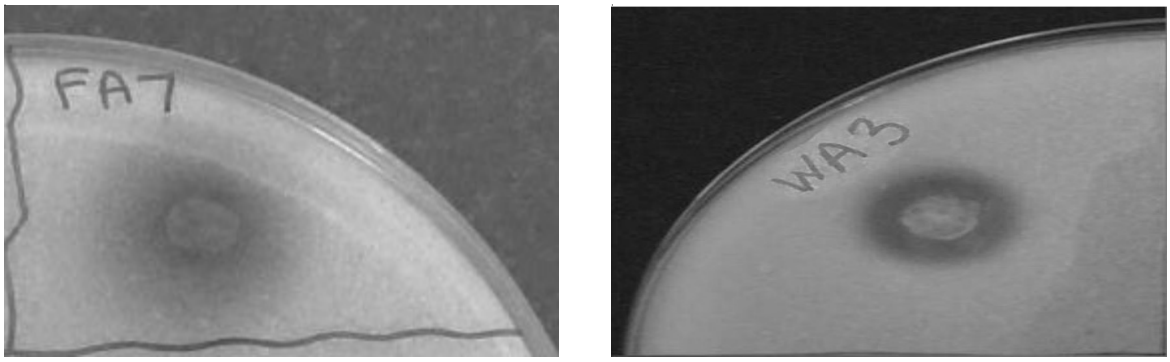
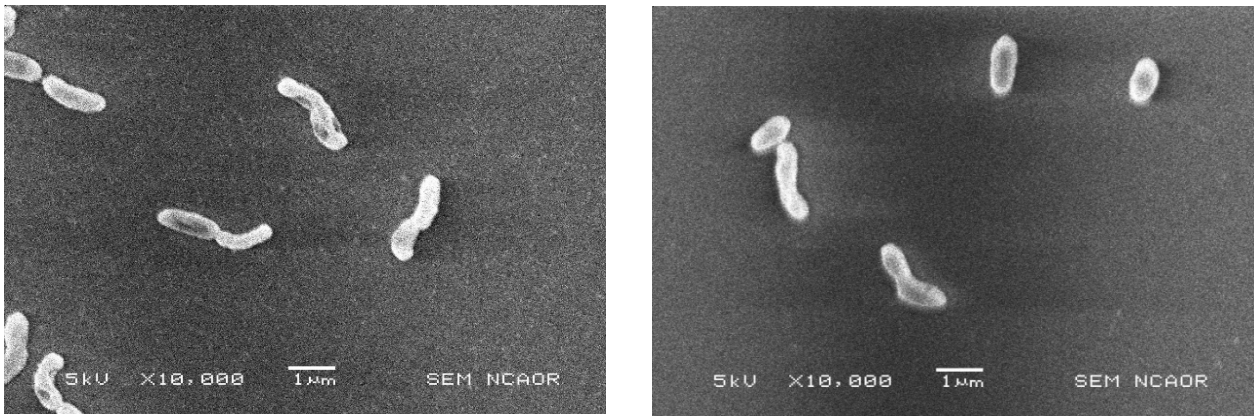


Fig. 4 Inorganic and organic phosphate solubilizing isolates obtained from each sample (For details refer Table 1).



(a) (b)

Fig. 5 Tri-calcium phosphate solubilisation on Pikovskaya's agar by selected potential alkaliphilic phosphate solubilizing isolates (a) FA7 and (b) WA3.



(a) (b)

Fig. 6 Scanning electron micrographs of selected potential phosphate solubilizing bacterial isolates (a) FA7 and (b) WA3.

biochemical characteristics are summarized in Table 3. Based on the results obtained, the cultures were tentatively identified using Bergey's Manual of Determinative Bacteriology, Volume III (Garrity *et al*., 2001). Alkaliphilic isolate FA7 belonged to family Bacillaceae and haloalkaliphilic isolate WA3 was found to belong to family Halomonadaceae. The isolates FA7 and WA3 were further identified by 16S rRNA gene sequencing as *Bacillus marisflavi* and *Chromohalobacter israelensis* respectively (Figure 6). 16S rRNA gene sequence of isolate FA7 and WA3 was deposited in Genbank with accession numbers KX098478 and KX098477 respectively.

DISCUSSION

Coastal ecosystems provide a unique ecological niche to diverse microbes which play various roles in environmental activities and nutrient cycling (Barea *et al*., 2005) (Desai *et al*., 2004; Appanna *et al*., 2007). In this study, alkaliphilic bacterial isolates were obtained from the various coastal ecosystems of Goa. Researchers have isolated facultative and obligate alkaliphilic bacteria for bio-prospecting from various coastal ecosystems of Goa, viz. mangrove, salt pan and sand dunes (Desai *et al*., 2004; Godhino *et al*., 2010; Gaonkar & Bhosle, 2013; Kamat & Kerkar, 2011; Surve *et al*., 2012; Borkar & Bhosle, 2015; Mishra *et al*., 2009). Desai *et al*., 2004, have isolated alkaliphiles from mangrove ecosystems of Goa and among their isolates 18.30% were obligate alkaliphiles and 81.69% were facultative alkaliphiles (Desai *et al*., 2004).

The diverse microbial community of coastal ecosystems plays an essential role in the environment by contributing to the release of key nutrients from primary minerals that are required not only for their own nutrition but also for that of plants. Phosphorous is one such most essential macronutrient for the growth of plants (Illmer &

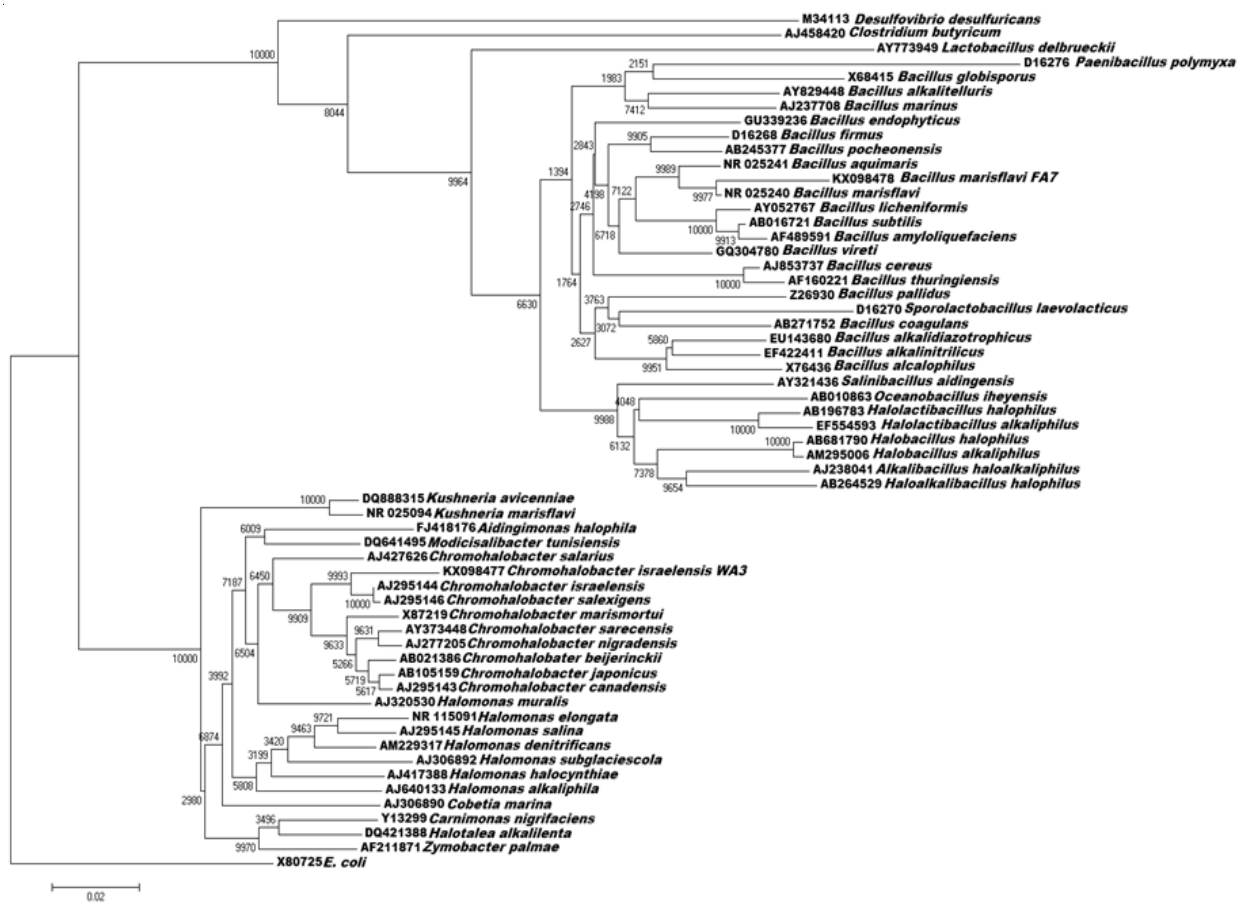


Fig. 7 Neighbour Joining phylogenetic tree based on 16S rRNA gene sequence of *Bacillus marisflavi* FA7 (KX098478) and *Chromohalobacter israelensis* WA3(KX098477) with closely related species of bacteria.

Schinner, 1995). Its fixation is a serious problem in alkaline and saline soils (Sharif *et al*., 2000; Mujeeb *et al*., 2010). Several studies have reported the occurrence of phosphate solubilizing bacteria in the coastal areas (Ayyakkannu & Chandramohan, 1971; Craven & Havasaka, 1982; Naik *et al*., 1982; Venkateswaram & Natarajan, 1984; De Souza *et al*., 2000; Vazquez *et al*., 2000; Gupta *et al*., 2007; Ravikumar *et al*., 2009; Dastager & Damare, 2013). Among the 141 predominant alkaliphilic bacteria obtained in this study, 30 isolates showed only inorganic phosphate solubilizing activity, 14 isolates only organic phosphate solubilizing activity and 13 isolates demonstrated both inorganic as well as organic phosphate solubilizing activity at pH 10.0. Significantly, alkaliphilic isolates also solubilized phosphate at combination of high pH (10.0) and salt concentration (25% (w/v)). Nautiyal *et al*., (2000), have isolated phosphate solubilizing bacteria from alkaline soils which solubilized phosphate at pH 12 and 10% NaCl (Nautiyal *et al*., 2000). Isolates screened by Mishra *et al*., (2009) in their study tolerated 10% NaCl; while, isolates of Nakbanpote *et al*., demonstrated phosphate solubilization at 8% (w/v) salt concentration (Mishra *et al*., 2009; Nakbanpote *et al*., 2014). Tolerance to high pH and salt concentration is important in the survival, multiplication and spread of bacterial strains in alkaline and saline soils (Nautiyal *et al*., 2000). Two potential isolates alkaliphilic FA7 and haloalkaliphilic WA3 were selected for identification. Isolate FA7 was identified as *Bacillus marisflavi* and isolate WA3 was identified as *Chromohalobacter israelensis*. To the best of our knowledge, this is the first report of haloalkaliphilic phosphate solubilizing bacterium belonging to the genus *Chromohalobacter* able to solubilize phosphate at high pH and high salt concentration. *Bacillus* species are among the most successful bacterial communities in solubilization

of phosphate (Illmer & Schinner, 1995). Organisms belonging to genera like *Pseudomonas, Rhizobium, Xanthomonas, Rhodococcus, Arthrobacter, Serratia, Chrysiobacterium, Delftia, Gordonia, Corynebacterium, Phyllobacterium, Acinitobacter, Gluconacetobacter, Enterobacter, Burkholderia, Micrococcus, Flavobacterium, Klebsiella* and *Bacillus* have been reported to solubilize inorganic and organic phosphate (Rodriguez & Fraga, 1999; Khan *et al*., 2009; Freitas *et al*., 1997; Seshadri *et al*., 2002; Collavino *et al*., 2010; Gulati *et al*., 2010).

Most phosphate solubilizing bacteria reported previously performed relatively low in alkalinity and salinity tolerance, being less appropriate for alkaline-saline soil based agriculture. Hence, it is urgently needed to develop alkaliphilic and haloalkaliphilic phosphate solubilizing bacteria for the agriculture in alkaline-saline soil.

CONCLUSION

Alkaliphilic bacteria isolated from coastal ecosystems of Goa, India, demonstrated phosphate solubilization at high pH (10.0) and high salt concentration (25%). Tolerance of phosphate solubilizing bacteria to high pH and salt concentration is of special interest to be used as biofertilizers for alkaline and saline soils. Hence, alkaliphile *Bacillus marisflavi* FA7 and haloalkaliphile *Chromohalobacter israelensis* WA3 have immense potential to be developed and exploited as biofertilizers for alkaline and saline soils.

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REFERENCES

Afzal, A. and Bano, A. 2008. Rhizobium and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum*). *International Journal of Agriculture and Biology.* 10 (1) : 85-88.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology.* 215 (3) : 403-410.

Appanna, V., Hamzehzarghani, H., Alagawadi, A., P.K. U. and B.C.S. 2007. Production of plant growth promoting substances by phosphate solubilizing bacteria isolated from versitols. *Journal of Plant Sciences.* 2 (3) : 326-333.

Ayyakkannu, K. and Chandramohan, D. 1971. The occurrence and distribution of phosphobacteria in the marine environment at Porto Novo. *Current Science.* 39 : 398-399.

Banik, S. and Dey, B.K. 1982. Available phosphate content of an alluvial soil as influenced by inoculation of some isolated phosphate solubilizing microorganisms. *Plant Soil.* 69 : 353-364.

Barea, J.-M., Pozo, M.J., Azcon, R. and Azcon-Aguilar, C. 2005. Microbial co-operation in the rhizosphere. *Journal of Experimental Botany*. 56 (417) : 1761-1778.

Borkar, S. and Bhosle, S. 2015. Alkaliphilic bacteria: Diversity, physiology and industrial applications. In: Borkar, S. *Bioprospects of Coastal Eubacteria*. Switzerland: Springer International Publishing. pp. 59-83.

Bray, R.H. 1948. Correlation of soil tests with crop response to added fertilizers and with fertilizer requirement. In: *Diagnostic Techniques for Soil and Crops*. Washington: American Potash Institute. pp. 53-86.

Buck, J.D. 1982. Non staining (KOH) method for determination of Gram reactions of marine bacteria. *Applied and Environmental Microbiology.* 44 (4) : 992-993.

Clesceri, L.S., Greenberg, A.E. and Eaton, A.D. 1999. *Standard Methods for the Examination of Water and Wastewater*. Washington: American Public Health Association.

Collavino, M.M., Sansberro, P.A., Mroginski, L.A. and Aguilar, M. 2010. Comparison of *in vitro* solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. *Biol Fertil Soils.* 46 : 727-738.

Cordell, D. and White, S. 2013. Sustainable phosphorus measures: Strategies and technologies for achieving phosphorus security. *Agronomy.* 3 : 86-116.

Craven, P. and Havasaka, K. 1982. Inorganic phosphate solubilization by rhizosphere bacteria in a *Zostera marina* community. *Canadian Journal of Microbiology.* 28 : 605-610.

Dastager, S.G. and Damare, S. 2013. Marine Actinobacteria showing phosphate solubilizing efficiency in Chorao island, Goa, India. *Current Microbiology*. 66 (5) : 421-427.

De Souza, M.-J.B.D., Nair, S. and Chandramohan, D.

2000. Phosphate solubilizing bacteria around Indian peninsula. *Indian Journal of Marine Sciences.* 29 : 48-51.

Desai, R.S., Krishnamurthy, N.K., Mavinkurve, S. and Bhosle, S. 2004. Alkaliphiles in estuarine mangrove regions of Goa, (central west coast of India). *Indian Journal of Marine Sciences.* 33 (2) : 177-180.

Freitas, J.R.d., Banerjee, M.R. and Germida, J.J. 1997. Phosphate solubilizing rhizobacteria enhance the growth and yield but not phosphorus upptake of Canola (*Brassica napus* L). *Biol Fertil Soils.* 24 : 358-364.

Gaonkar, T. and Bhosle, S. 2013. Effect of metals on a siderophore producing isolate and its implications on microbial assisted bioremediation of metal contaminated soils. *Chemosphere.* 93 (9) : 1835-1843.

Gaonkar, T., Nayak, P.K., Garg, S. and Bhosle, S. 2012. Siderophore producing bacteria from a sand dune ecosystem and the effect of sodium benzoate on siderophore production by a potential isolate. *The Scientific World Journal*, pp.1-2.

Garrity, G., Boone, D.R. and Castenholz, R.W. 2001. *Bergey's Manual of Systemic Bacteriology.* New York: Springer Science + Business Media.

Gee, J.M., Lund, B.M., Metcalf, G. and Peel, J.L. 1980. Properties of a new group of alkalophilic bacteria. *Journal of General Microbiology*. 9-17.

Godhino, A., Ramesh, R. and Bhosle, S. 2010. Bacteria from sand dunes of Goa promoting growth in eggplant. *World Journal of Agricultural Sciences.* 6 (5) : 555-564.

Gulati, A., Sharma, N., Vyas, P., Sood, S., Rahi, P. and Pathania, V. 2010. Organic acid production and plant growth promotion as a function of phosphate solubilization by *Acinetobacter rhizosphaerae* strain BIHB 723 isolated from the cold desserts of the trans-Himalayas. *Arch Microbiol.* 192 : 975-983.

Gupta, N., Das, S. and Basak, U.C. 2007. Useful extracellular activity of bacteria isolated from Bhitarkanika mangrove ecosystem of orissa coast. *Malaysian Journal of Microbiology.* 3 (2) : 15-18.

Hinsinger, P. 2001. Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant and Soil*. 237 : 173-195.

Igual, J.M., Valverde, A., Cervantes, E. and Velazquez, E. 2001. Phoshate solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. *Agronomie*. 21 : 561-568.

Illmer, S. and Schinner, F. 1995. Solubilization of inorganic calcium phosphates. *Soil Biol Biochem*. 27 : 257-263.

Jones, D.L. and Oburger, E. 2011. Solubilization of phosphorus by soil microorganisms. In: Bunemann, E.K. *Phosphorus in Action, Soil Biology*. Berlin Heidelberg: Springer-Verlag. pp.169-198.

Kamat, T. and Kerkar, S. 2011. Bacteria from saltpans: A potential resource of antibacterial metabolites. *Recent Research in Science and Technology.* 3 (9) : 46-52.

Khan, A.A., Ghulam, J., Akhtar, M.S., Naqvi, S.S. and Rasheed, M. 2009. Phosphorus solubilizing bacteria: Occurence, Mechanisms and their role in crop production. *J Agric Biol Sci.* 1 (1) : 48-58.

Khan, M.S., Zaidi, A. and Ahmad, E. 2014. Mechanism of phosphate solubilization and physiological functions of phosphate solubilizing micro-organisms. In: Khan, M.S. *Phosphate Solubilizing Microorganisms.* Switzerland: Springer International Publishing. pp. 31-62.

Krulwich, T.A., Agus, R., Schneir, M. and Guffanti, A.A. 1985. Buffering capacity of *Bacilli* that grow at different pH ranges. *J Bacteriol.* 162 : 768-772.

Mahdi, S.S., Hassan, G.I., Hussain, A. and Rasool, F. 2011. Phosphorus availability issue - its fixation and role of phosphate solubilizing bacteria in phosphate solubilization. *Research Journal of Agricultural Sciences.* 2 (1) : 174-179.

Mishra, R.R., Dangar, T.K., Rath, B. and Thatoi, H.N. 2009. Characterization and evaluation of stress and heavy metal tolerance of some predominant Gram negative halotolerant bacteria from mangrove soils of Bhitarkanika, Orissa, India. *African Journal of **B**iotechnology.* 8 (10) : 2224-2231.

Mujeeb, F., Akhtar, R.J. and Ahmad, R. 2010. Integration of organic and inorganic P sources for improving P use efficiency in different soils. *Soil & Environ.* 29 (2) : 122-127.

Munns, R., James, R.A. and Lauchli, A. 2006. Approaches to increasing the salt tolerance of wheat and other cereals. *Journal of Experimental Botany*. 57 (5) : 1025-1043.

Naik, V.M., D'Souza, J. and Araujo, A. 1982. Phosphorus solubilizing yeasts in estuarine environment. Indian J Mar Sci, 11 : 197-198.

Nakbanpote, W. 2014. Salt-tolerant and plant growth promoting bacteria isolated from Zn/Cd contaminated soil: identification and effect on rice under saline conditions. *Journal of Plant Interactions.* 9 (1) : 379-387.

Nautiyal, C.S., Bhadauria, S., Kumar, P., Lal, H., Mondal, R. and Verma, D. 2000. Stress induced phosphate solubilization in bacteria isolated from alkaline soils. *FEMS Microbiology Letters.* 182 : 291-296.

Pikovskaya, R.I. 1948. Mobilization of phosphorus in soil in connection with vital capacity of source microbial species. *Microbiologia.* 17 : 362-370.

Prior, R. and Perkins, R. 1974. Artifacts induced by preparation for scanning electron microscopy in *Proteus mirabilis* exposed to carbenicillin. *Canadian Journal of Microbiology*. 20 : 794-795.

Ravikumar, S., Inbaneson, S.J. and Seshserebiah, J.

2009. Cadmium induced effect on growth and physiology in halophilc phosphobacteria. *Journal of Environmental Biology*. 30 (5) : 673-676.

Richardson, A.E. 2001. Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Australian Journal of Plant Physiology*. 28 : 897-906.

Rodriguez, H. and Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*. 17 : 319-339.

Rodriguez, H., Fraga, R., Gonzalez, T. and Bashan, Y. 2006. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. *Plant and Soil.* 287 : 15-21.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Schaeffer, A.B. and Fulton, M. 1933. *A simplified Method of Staining Endospores. Science*, pp. 77-194.

Seshadri, S., Ignacimuthu, S. and Lakshminarsimhan, C. 2002. Variations in heterotrophic and phosphate solubilizing bacteria from Chennai, southeast coast of India. *Indian Journal of Marine Sciences*. 31 (1) : 69-72.

Sharif, M., Sarir, M.S. and Rabi, F. 2000. Biological and chemical transformation of phosphorus in some important soil series of NWFP. *Sarhad Journal of Agriculture.* 16 (6) : 587-592.

Sitdhipol, J., Niwasabutra, K., Wannissorn, B., Vsessanguan, W. and Tanasupawat, S. 2012. Characterization of alkaline phosphatase producing bacteria isolated from Thai fermented fish products. *International Journal of Biology*. 4 (3) : 44-55.

Sonak, S. 2006. *Multiple Dimensions of Global Environment Change.* New Delhi: TERI Press.

Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H.G. 1981. *The Prokaryotes*. Berlin Heidelberg: Springer Verlag.

Surve, V.V., Patil, M.U. and Dharmadekari, S.M. 2012. Moderately halophilic bacteria from solar salt pans of Ribander, Goa: A comparative study. *International Journal of Advanced Biotechnology and Research*. 3 (3) : 635-643.

Thompson, J.D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*. 25 (24) : 4876-4882.

Untawale, A. 2006. Change of coastal land use, its impact, and management options. In Sonak, S. *Multiple Dimensions of Global Environmental Change*. New Delhi: TERI Press.

Uroz, S., Calvaruso, C., Turpault, M.P. and Frey-Klett, P. 2009. Mineral weathering by bacteria: ecology, actors and mechanisms. *Trends in Microbiology.* 17 (8) : 378-387.

Vassilev, N., Eichler-Lobermann, B. and Vassileva, M. 2012. Stress-tolerant P-solubilizing micro-organisms. *Appl Microbiol Biotechnol.* 95 : 851-859.

Vazquez, P., Holguin, G., Puente, M. E., Lopez-Cortes, A. and Bashan, Y. 2000. Phosphate solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biol Fertil Soils*. 30 : 460-468.

Venkateswaram, K. and Natarajan, R. 1984. Role of phosphatase in mineralization of organic phosphorous in Porto Novo coastal waters. *Indian Journal of Marine Sciences*. 13 : 85-87.