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Studies on Non-symbiotic Diazotrophic Bacterial Population of the Saline Soils of Sunderbans

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Surface soils (0-15cm) from upland and mudflat land situations of five different locations of the Sunderbans, India were tested for their aerobic heterotrophic diazotrophic populations and dinitrogen fixation. Culturable diazotrophic populations in the upland soils, irrespective of locations and NaCl concentrations in the culture media, were statistically greater than that of the mudflat soils. The diazotrophic counts as well as nitrogen fixation of the soils were the highest with extraneous addition of 1 % NaCl in the media and then gradually decreased with further increase in salt concentration in the culture media. Out of the 12 isolates, 7 were Gram (-ve), 4 were spore formers and all could form capsules. The isolates could utilize glucose, sucrose, mannitol, citrate and starch. They were catalase and gelatinase positive but indole negative. The ARA of the isolates ranged from 14.87 to 174.47 nmole C_2H_4 produced ml^{-1} culture media without NaCl in 72 hours. The isolates could fix 3.61 to 5.44 mg N_2 g^{-1} sugar consumed in culture media without NaCl. The isolates from the mudflat soils showed greater degree of salt tolerance than the upland soils as determined by O.D of the cultures. 16S rDNA sequences of the 8 out of 12 bacterial isolates were similar to the genera: *Agrobacterium, Klebsiella, Pseudomonas, Bacillus* and *Vibrio.* Two isolates were identified as Bacterium VNS3-1-2 and Bacterium Antarctica 14 while the other two could not be unidentified.

Key words: Salinity, diazotrophs, dinitrogen fixation, Sunderbans, DNA sequencing.

The Sunderbans, located in the coastal region of West Bengal, India, are congregation of several islands intercrossed with creeks and canals linked to innumerable rivers that ultimately flow into the Bay of Bengal. The islands have mudflats and uplands. The mudflats are subjected to periodic inundation by tidal sea water, while the uplands are preserved from this inundation. The soils in this region are highly saline (Bandyopadhyay *et al*., 2003).

Saline habitats are nitrogen poor (Sprent and Sprent, 1990). Various groups of diazotrophic bacteria grow and fix atmospheric nitrogen, but their population and nitrogen fixing ability are affected by numerous environmental factors of which salt stress is important (Rai, 1991). Reports concerning diazotrophic population and dinitrogen fixation in the soils of the Sunderbans are few.

Agriculture in the Sunderbans region is severely constrained due to monoculture *vis a vis*

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poor yield. The farmers in this region are economically backward to afford costly input like fertilizers. Thus, there is a need to develop biofertilizers for exclusive use in this region.

The objective of this work was, (1) to determine the diazotrophic population and dinitrogen fixation in the upland and mudflat soils of the Sunderbans, (2) to isolate, characterize and identify the salinity adapted strains in the perspective of biofertilizer production for the Sunderbans region.

MATERIAL AND METHODS

Study site and soil sampling

Soils of this study were collected from the mudflats and uplands of five different islands of the Sundarbans region during March,2006. The mudflat soils are subjected to regular inundation by sea water at 12 hours interval and the adjacent upland soils are not inundated. The soil collection sites were: Sudhanyakhali (22°02'24''N and 88 °49'54''E), Bonicamp (21°49'48'' N and 88 °37'23''E), Buridhabri (22°04'34'' N and 89°01'44''E), Chamta (21°51'40'' N and 88 °54'50''E), Haldibari (21°43'33'' N and 88 °46'57''E). After collection of three replicate soil samples (0-15 cm depth) from each site, plant roots, visible faunas, stones, rubbles etc. were removed. About 1kg soil from each site was brought to the laboratory in labeled sterile containers. The samples were kept at 4°C before microbial analysis of the field moist soils. Physicochemical parameters of the soils were determined with the sieved (<2mm) air dried samples. The results are expressed on moisture free basis.

Determination of physico-chemical properties of soil

The pH of the soils was determined in 1:2.5 soil water suspension using glass electrodes. Electrical conductivity of the soil saturation extract (EC_e) was determined by measuring the electrical conductance of soil water saturation extract with the help of conductivity meter (United State Department of Agriculture [USDA], 1954). Organic carbon(OC) and total nitrogen (TN) of the soils were determined by the method as described by Nelson and Sommers (1975) and macro-Kjeldahl method as described by Sankaram (1966) respectively.

Enumeration of culturable aerobic non-symbiotic diazotrophic bacterial count of soil

It was carried out by soil-dilution plate technique using nitrogen free mineral salt-yeast extract-agar medium. The composition of the media in $gL^{-1}$of water was as follows: Sucrose 20g, K_2HPO_4 0.05g, KH_2PO_4 0.15g, $CaCl_2$ 0.01g, $MgSO_4.7H_2O$ 0.2g, $Na_2MoO_4.2H_2O$ 0.002g, $FeSO_4$ 0.01g, yeast extract 0.1g, agar 18g, pH adjusted at 7.2. To prepare salt containing media requisite quantity of NaCl was added to the medium to obtain final concentrations of 1%, 2% and 3% NaCl. Colony count was taken after 3 days of incubation at 28-30°C.

Determination of nitrogen fixation

One gram of each soil was added to duplicate flasks containing 50 mL nitrogen free mineral salt-yeast extract broth with or without added NaCl, and then sterilized. These served as soil blank. To the other duplicate flasks containing 50 mL sterilized medium, 1g of each soil sample was added aseptically. The flasks were incubated at 28-30°C for 7 days. Total nitrogen in the media was determined by macro-Kjeldahl method (Allen, 1957). Nitrogen fixation in soil, g^{-1} of sugar consumed, was determined by subtracting the value of nitrogen in the blank from the nitrogen in the sample.

Isolation and purification of free living heterotrophic diazotrophs

Isolation and purification of free living heterotrophic diazotrophs was carried out by enrichment culture technique in nitrogen free mineral salt-yeast extract-broth followed by streaking in the same agar plates to obtain single isolated colony. In this way, 12 pure cultures were obtained. The cultures were maintained in same agar slants.

Phenotypic and biochemical characterization of the isolates

Different phenotypic characteristics of the isolates like appearance on nitrogen free mineral salt-yeast extract- agar slants, cellular morphology by negative staining, Gram character, spore formation and capsule formation were determined by standard procedures. (Holtz, 1993).

Different biochemical characteristics of the isolates like sugar utilization pattern, starch hydrolyzing activity, catalase activity, NO_3^- utilization, IMViC analysis, gelatinase activity

and ability to grow on TSI agar slant were determined by standard procedures. (Holtz, 1993).

Determination of salinity tolerance of the isolates

The degrees of salt tolerance of the strains were determined by measuring the optical density of the broth cultures containing 0-3% NaCl. The O.D of the cultures was measured at 595 nm after 72 hours of incubation and salinity tolerance was expressed as percentage increase/ decrease in O.D of the cultures containing NaCl with respect to the culture that did not contain NaCl. Their growth was compared to that of a reference *Aztobacter vinelandii* strain isolated from non saline soil.

Acetylene reduction assay (ARA) of the isolates

The ARA of the isolates was measured by C_2H_2 reduction assay. The cultures were incubated in nitrogen free mineral salt-yeast extract-broth with or without extraneous addition of NaCl, for 72 hours at 29^{O}C in 7ml Becton Dickinson Vacutainer tubes stoppered with cotton plugs. After visible growth was observed, cotton plugs were aseptically exchanged with rubber stoppers and the headspace air was replaced with 10% (by volume) of high purity C_2H_2 gas by hypodermic syringe. The C_2H_4 production was measured after 24 and 72 hours incubation of the tubes in dark at 29^{O}C (Rao *et al*., 1983). Tubes without C_2H_2 served as control. For the determination of C_2H_4, 0.5 ml of the gas phase from each tube was injected into gas chromatograph fitted with flame ionization detector (FID). The nitrogenase activity was expressed in C_2H_4 formed ml^{-1}culture in 72 hours.

Determination of nitrogen fixing efficacy of the isolates

Nitrogen fixing efficacy of the isolates were estimated by growing the isolates in nitrogen free mineral salt-yeast extract-broth with or without extraneous addition of NaCl, for 7 days at 28-30^{O}C, followed by the determination of nitrogen in the cultures as well as blanks by macro Kjeldahl method (Allen., 1957) as compared to a blank where no inoculation was done. Nitrogen fixing efficacy was expressed as mg N fixed g^{-1} sugar consumed.

Isolation of genomic DNA

The DNA was prepared from all the 12 isolates by the sodium dodecyl sulfate proteinase K-cetyltrimethylammonium bromide (CTAB) method (Sambrook., 2001). All the DNA preparations were treated with RNaseA, and the DNA concentrations were estimated by visual examination of ethidium bromide-stained agarose gels as well as by spectrophotometric examination.

Amplification of 16S rDNA

Partial amplification of the 16S rRNA gene was performed with the thermal cycler ABI 2700 (ABI, Foster City, USA). The PCR of the genomic DNA of the 12 isolates were conducted in a final volume of 50 ml. The reaction mixture included 20–50 ng of isolated genomic DNA, 2U taq polymerase (Promega,USA), 1 x PCR buffer with 1.5 mM $MgCl_2$, 200µM each dNTP, and 10 pmol of each primer (IDT, USA). The primers were chosen to amplify partial 16S rDNA sequence. The forward primer 515F used was (5'–3') GTGCCAGCAGCCGCGGTAA and the reverse primer 1492R was (5'–3') TACGGYTA CCTTGTTACGACTT. Before amplification cycle DNA was denatured for 2 min at 94^{0}C and after amplification an extension step (7 min at 72^{0}C) was performed. The cycling parameters consisted of 28 cycles at: denaturation at 94^{0}C for 30s, primer annealing at 45^{0}C for 1 min, extension at 72^{0}C for 1 min. The samples were held at 4^{0}C until analysis by agarose gel electrophoresis. All the amplified PCR products were agarose-gel-eluted using Promega gel elution kit.

Sequencing of the 16SrDNA fragment and BLAST search

The amplified and gel-eluted PCR fragments of the rDNA were sequenced in ABI 3100 Genetic Analyzer with primers 515F and 1492R. Sequencing reaction was performed by using the Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. The partial 16S rDNA sequences of the isolated strains were compared with those available in the public databases. Identification to the species level was determined as a 16S rDNA sequence similarity of >99% with that of the prototype strain sequence in the GenBank.

Statistical analysis

Assigning the land situation and location as factors, analysis of variance (ANOVA) was carried out by completely randomized design (CRD). In another set of experiment, in addition

to these, salt concentration was also considered as another factor for ANOVA. The least significant difference (LSD) test (0.05P) was applied to evaluate the significant difference between the individual factors.

RESULTS AND DISCUSSION

Physico- chemical properties of soils

The soils were mostly silty clay loam in texture. The pH of the soils from different sites varied and those from the mudflats(7.6) were higher than those on uplands (6.8). The Ec_e(dSm^{-1}) of the upland soils soils was 7.08, while that of the mudflat soils was 13.78. The mean EC_e of soils from different locations, in decreasing order, could be ranked as: Sudhanyakhali (13.76), Bonicamp (11.09), Chamta (10.24), Haldibari (9.07) and Burirdhabri (7.99). The upland soils had higher organic carbon (7.07gkg^{-1}) than the mudflat soils (5.64gkg^{-1}). The mean organic carbon contents of different soils were: Sudhanyakhali (3.88), Bonicamp (5.00), Chamta (5.45), Haldibari (8.21) and Burirdhabri (9.21). The mean total nitrogen content of the upland soils (0.73gkg^{-1}) was also higher than the mudflat soils (0.52gkg^{-1}). Total N contents of different soils were: Sudhanyakhali (0.73), Bonicamp (0.45), Chamta (0.65), Haldibari (0.76) and Burirdhabri (0.84).

Non-symbiotic heterotrophic diazotrophic bacterial counts of soil

The soils varied significantly in this respect (Table 1). The highest value was observed in Burirdhabri soil (5.31), followed in decreasing order by Haldibari (4.88), Chamta (4.68), Bonicamp (4.69) and Sudhanyakhali (4.32) soils.

Table 1. Effect of added sodium chloride in the cultural medium on aerobic heterotrophic nitrogen fixing microbial population count in the Sunderban soils

Site	Log (colony forming units) in 72 hours							
	0% sodium chloride in LG medium		1% sodium chloride in LG medium		2% sodium chloride in LG medium		3% sodium chloride in LG medium	
	Land situation							
	Upland	Mudflat	Upland	Mudflat	Upland	Mudflat	Upland	Mudflat
SU*	5.86	3.67	4.02	4.9	3.85	4.76	3.42	4.09
BC	5.72	3.37	5.57	4.86	4.02	4.56	3.66	3.42
BR	7.87	3.65	7.06	4.62	5.72	4.11	5.66	3.77
CH	5.82	3.74	5.25	4.72	4.9	4.51	4.58	3.49
HL	6.94	3.44	6.05	4.51	5.46	3.67	5.36	3.65
Mean site		SU	BC	BR	CH	HL		
		4.32	4.39	5.31	4.68	4.88		
Mean land situation			Upland				Mudflat	
			5.34				4.29	
Mean salt concentration		0 %		1 %		2 %		3 %
		5.01		5.15		4.55		4.11
LSD (0.05P) Site (S)	0.164							
LSD (0.05P) Land Situation (L)	0.104							
LSD (0.05P) salt conc. (C)	0.147							
LxS	0.232							
LxC	0.328							
SxC	2.085							
LxSxC	2.214							

* SU = Sudhanyakhali ; BC = Bonicamp ; BR = Buridhabri ; CH = Chamta ; HL = Haldibari

Irrespective of locations and salt concentrations in the culture media, the upland soils(5.34) recorded higher count than the mudflat soils(4.29). The recovery of microorganisms from soils, irrespective of site and land situation, with the graded dose of NaCl in culture media was statistically different. The highest recovery of microorganisms was obtained at 1%NaCl (5.15), although it was not statistically different from that obtained at 0% NaCl. With further increase in NaCl in the culture media, the recovery of microorganisms gradually decreased. The variation in diazotrophic bacterial counts of both the upland and mudflat soils as well as in different sites seems to be related to the difference in Ece and organic carbon contents of the soils. El-Shinnawi and Frankenberger (1988) earlier reported that the salinity detrimentally influences diazotrophic populations. The type and diversity of the diazotrophs, capable of adapting to salinity stress in such soils might be the other reason for the variation (Laura, 1974; El-Shinnawi and Seifert, 1975). Highest recovery of microorganisms from saline soils of the Sunderbans occurred when media contained around 1% NaCl. Higher supplement of NaCl in the culture media seemed to be detrimental. This indicated that nitrogen-fixing microorganisms of the soils were basically salt tolerant, requiring certain amount of salts for their optimal growth. Russell(1989) opined that within the halotolerants, a distinction can be made between those, for which growth rate is decreased by the addition of any salt, and those for which growth

Table 2. Effect of added sodium chloride in the cultural medium on nitrogen fixation of the Sunderban soils

Site	Log (colony forming units) in 72 hours							
	0% sodium chloride in LG medium		1% sodium chloride in LG medium		2% sodium chloride in LG medium		3% sodium chloride in LG medium	
	Land situation							
	Upland	Mudflat	Upland	Mudflat	Upland	Mudflat	Upland	Mudflat
SU*	8.64	3.24	2.59	7.7	1.57	2.04	1.01	1.66
BC	3.42	3.51	2.16	10.33	1.01	1.94	0.46	0.46
BR	6.21	6.66	3.43	9.25	2.04	2.49	0.83	1.48
CH	2.3	8.13	1.11	9.24	1.11	6.75	0.38	2.49
HL	8.3	8.15	5.8	10.74	1.94	3.7	0.46	1.01

Mean site	SU	BC	BR	CH	HL
	3.56	2.91	4.05	3.94	5.01

Mean land situation	Upland	Mudflat
	2.73	5.04

	0 %	1 %	2 %	3 %
Mean salt concentration	5.85	6.23	2.455	1.02

LSD (0.05P) Site (S)	0.966
LSD (0.05P) Land Situation (L)	0.611
LSD (0.05P) salt conc. (C)	0.864
LxS	1.366
LxC	1.932
SxC	1.222
LxSxC	2.732

* SU = Sudhanyakhali ; BC = Bonicamp ; BR = Buridhabri ; CH = Chamta ; HL = Haldibari

rate reaches an optimum with the addition of some salt, but then declines at higher salinities. The N fixing microorganisms recovered from the soils of the Sunderban region seemed to be halotolerant belonging to the latter category.

Non-symbiotic nitrogen fixation in soil

There was significant variation in nitrogen fixation of soils collected from different sites (Table 2). The Haldibari soil showed significantly highest nitrogen fixation (5.01 mg) and the lowest value (2.91 mg) was recorded in Bonicamp. Nitrogen fixation in the soils of Buridhabri (4.05 mg), Chamta (3.94 mg) and Sudhanyakhali (3.56 mg) were statistically similar. Nitrogen fixation in the upland soils (2.73 mg), irrespective of site and salt concentrations in the culture media was much lower than those of the mudflat soils (5.04 mg). Nitrogen fixation of soils at 1% NaCl in the culture media was the highest (6.23 mg) but statistically similar when the culture media did not receive any added salt (5.85 mg). The nitrogen fixation sharply diminished when the concentration of NaCl was increased to 2 and 3% in the culture media.

The variation in dinitrogen fixation of soils from different locations seems to be related to the types of microorganisms and their capability of nitrogen fixation in such soils. The mudflat soils at all the concentrations of NaCl in the culture medium, recorded consistently higher nitrogen fixation than the upland soils, even though the former had lower count than the latter. The EC_e of the mudflat soils was higher but their organic carbon and total nitrogen contents were lower compared to the upland soils. Higher nitrogen fixation in the mudflat soils seemed to be related to lower nitrogen content of such soils and possibly with the higher nitrogen fixing efficiency of the diazotrophic microorganisms in the mudflat soils. Zahran (1997) stated that saline habitats have their own microbial population adapted to this environment. Apart from this, diazotrophic microorganisms are aquatic in nature. The nitrogenase enzyme system, one that is responsible for nitrogen fixation is oxygen labile. The mudflat condition maintains a better protective environment for nitrogenase system than that of the upland system.

Table 3. Phenotypic characteristics of the isolates

Strain Isolated and code	Colony Characteristics on nitrogen free mineral salt yeast extract agar slants	Morphology of cells by negative staining	Gram Character	Spore Formation	Capsule Formation
UPLAND					
BRU1	Gummy	Small coccoidal cells with clear zone around	Gram Negative	-	+
HBU1	Gummy	Small rod shaped organism, with clear zone around	Gram Negative	+	+
HBU2	Gummy	Small rod shaped organism, isolated	Gram Positive	-	+
CHU2	Gummy	Big coccoidal cells, in clusters	Gram Negative	-	+
BCU1	Gummy	Small coccoidal cells, isolated	Gram Negative	-	+
MUDFLAT					
HBM1	Dry, weak growers	Small coccoidal cells, in clusters	Gram Positive	+	+
BCM2	Dry	Small rods, scattered	Gram Negative	-	+
CHM1	Dry	Big coccoidal cells, in clusters	Gram Negative	-	+
CHM2	Dry	Big coccoidal cells, in clusters	Gram Positive	-	+
BRM1	Dry	Small rods, scattered	Gram Positive	+	+
BRM2	Dry	Big coccoidal cells, scattered	Gram Positive	+	+
SKM3	Gummy	Small coccoidal cells, isolated	Gram Negative	-	+

Table 4. Biochemical charactersitics of the isolates

Strain Isolated and Code	Sugar Utilization			Starch hydrolysis	NO_3^- utilization	Catalase	Gelatinase	Indole	Methyl red	VP	Citrate	Growth on TSI agar slant
	Glucose	Sucrose	Mannitol									
UPLAND												
BRU1	+++	+++	+++	+	++	+	+	-	-	-	+	Shows alkaline reaction
HBU1	+++	+++	+++	+	++	+	+	-	+	+	+	Shows acidic reaction
HBU2	+++	+++	+++	+	++	+	+	-	-	-	+	Shows alkaline reaction
CHU2	+++	+++	++	++	++	+	+	-	+	+	+	Shows acidic reaction
BCU1	+++	+++	+++	++	++	+	+	-	-	-	+	Shows alkaline reaction
MUDFLAT												
HBM1	+++	+++	++	++	++	+	+	-	-	-	+	Shows alkaline reaction
BCM2	+++	+++	++	+	++	+	+	-	-	-	+	Shows alkaline reaction
CHM1	+	++	++	+	++	+	+	-	-	-	+	Shows alkaline reaction
CHM2	+	++	++	+	++	+	+	-	-	-	+	Shows alkaline reaction
BRM1	+++	+++	++	++	++	+	+	-	+	+	+	Shows acidic reaction
BRM2	+++	+++	++	+	++	+	+	-	-	-	+	Shows alkaline reaction
SKM3	+++	+++	+++	+	++	+	+	-	-	-	+	Shows alkaline reaction

Table 5. Efficiency of nitrogen fixation and salinity tolerance of the isolates

Strain Isolated and code	ARA* (nmole of C_2H_4 formed ml^{-1} culture per 72 hours)		mg Nitrogen fixed g^{-1} sugar utilized		Culture O.D in nitrogen free mineral salt yeast extract broth containing extraneous addition of NaCl % increase(+)/decrease(-) over control			IdentificationBased on 16SrDNA sequence analysis
	0%NaCl N_2 free media	1%NaCl N_2 free media	0%NaCl N_2 free media	1%NaCl N_2 free media	1%NaCl N_2 free media	2%NaCl N_2 free media	3%NaCl N_2 free media	
UPLAND								
BRU1	174.47	55.65	5.44	4.76	-86	-89	-89	*Agrobacterium sp. ZY072*
HBU1	17.45	5.65	5.10	4.08	-59	-60	-68	*Klebsiella pneumonae strain CICC10072*
HBU2	46.01	23.20	4.76	3.40	-78	-84	-92	*Bacillus sp. HZBN165*
CHU2	22.85	12.09	4.42	4.08	-78	-83	-93	*Agrobacterium tumefaciens strain UP-3*
BCU1	14.87	2.43	4.76	3.40	-68	-69	-71	*Unidentified*
MUDFLAT								
HBM1	17.32	14.72	4.08	3.40	+52	+17	-7	*Bacterium VNS3-1-2*
BCM2	34.41	8.25	4.08	4.42	+46	+30	+6	*Pseudomonas pseudoalkaligenes*
CHM1	22.85	12.09	4.42	4.08	+30	+5	-15	*Pseudomonas sp HY-24*
CHM2	22.85	12.09	4.42	4.08	+36	+12	-47	*Vibrio sp ZX-PKU- 010*
BRM1	89.69	35.85	4.76	5.10	+22	-9	-36	*Bacterium Antarctica 14*
BRM2	17.58	4.55	5.10	4.08	+59	+12	-24	*Bacillus sp PN-13*
SKM3	18.32	5.54	3.61	2.70	+27	+13	-43	*Unidentified*
REFERENCE								
Azotobacter vinelandii	128	5.03	5.23	1.64	-95	-100	-100	

*Acetylene Reduction Assay

Phenotypic and biochemical characteristics of the isolates

Phenotypic characteristics of the 12 isolated pure cultures from different sites (Table 3) revealed that the colonies of some of them were gummy and some were dry. The morphology of the cells varied from small to big rods to big as well as small coccoidal cells. Out of the 12 isolates, 5 were Gram (+ve) and the rest Gram (-ve). Only 4 isolates were spore formers. All the isolates are capable of forming capsules.

Biochemical characteristics (Table 4) revealed that all the isolates could utilize glucose, sucrose and mannitol, with preference for the former two. All the isolates could hydrolyse starch, but the isolates HBM1, CHU2, BRM1and BCU2 showed greater degree of starch hydrolysis. All the isolates were capable of utilizing NO_3^- .They were catalase and gelatinase positive but indole negative. The isolates HBU1, CHU2 and BRMI were MR and VP positive. All the isolates were capable of utilizing citrate. Most of the isolates showed alkaline reaction on TSI agar plate excepting the isolates HBU1, CHU2 and BRM1.

Nitrogen fixation of the isolates

The nitrogen fixing efficacy of the isolates was determined by growing the isolates in culture media with 0% and 1% NaCl. 1% NaCl corresponds to 10dSm^{-1} Ec_e. It is well known that beyond 10dSm^{-1} Ec_e of soil, which corresponds to 1% NaCl, no agriculture is possible. The aim of this study was to identify the strains that fix nitrogen best at 1% NaCl concentration, so that such strains could be used as potent biofertilizer for agriculture in coastal areas. The isolates could fix nitrogen in the range of 3.61 to 5.44 mg of nitrogen without any extraneous addition of NaCl in the culture medium (Table 5). The same values at 1% NaCl were 2.70 to 5.10mg of nitrogen. The ARA of the isolates at 0%NaCl in the culture media ranged from 14.87 to 174.47 nmole of C_2H_4, and at 1% NaCl those were 2.43 to 55.65 nmole of C_2H_4.

The ARA activity of the isolates confirmed their potentiality for fixing nitrogen in media containing 0% and 1% NaCl . Reduction in ARA of the isolates grown in nitrogen free medium containing 1% NaCl may be the consequence of ionic component (Fernandes *et al*., 1993). It is also established that microorganisms exposed to salinity stress deviates cellular energy towards osmoregulation, especially Na^+ efflux and hence loss of nitrogenase activity by the isolates.

Salt tolerance of the isolates

The isolates from the upland soils showed negative growth as determined by the O.D, with the graded addition of NaCl (1,2 and 3%) in the culture medium (Table 5). In contrast, the isolates from the mudflat soils showed positive growth both in 1 and 2% NaCl in most cases, but negative growth at 3% NaCl in the culture media. The negative growth of the isolates in 3% NaCl in the culture media may be due to the fact that the organisms are more tolerant of salinity stress in soil than in culture media (El-Shinnawi and Frankenberger, 1988). The higher salt tolerance of the isolates from the mudflats compared to the isolates from the uplands seemed to be related to their exposure to high salinity in the mudflat soils, where intermittent inundation by sea water occurs. Identification of the strains on the basis of 16SrDNA sequences.

Based on the sequencing of 16SrDNA of the isolates and subsequent comparison with GenBank, the isolates were identified as 8 different strains of rarely reported nitrogen fixing bacterial species of Sunderbans upland and mudflat soils (Table 5). There were two isolates each of Agrobacterium (CHU2 and BRU1), Pseudomonas (BCM2 and CHM1) and Bacillus (BRM2 and HBU2). One strain each of Vibrio (CHM2) and Klebsiella (HBU1) were also identified. Agrobacterium (Kanvinde and Sastry, 1990), Pseudomonas (Chan, 1985), Bacillus (Ding *et al*., 2005), Vibrio (Shieh *et al.,* 1987) and Klebsiella (Govindarajan *et al*.,2007) were previously reported to exhibit diazotrophic activity. Two strains, Bacterium Antarctica 14 and (BRM1) and Bacterium VNS3-1-2(HBM1) were not previously reported as diazotrophs. The other two strains could not be identified.

CONCLUSION

The non symbiotic diazotrophic microorganisms of the studied soils from different locations of the Sunderbans were basically salt tolerant, requiring certain amount of salt for their growth. They could fix substantial amount of atmospheric nitrogen even under salt stress. The

16S rDNA studies of the isolates revealed certain genera of bacteria that are rarely reported as diazotrophs and two isolates, viz Bacterium VNS3-1-2 and Bacterium Antarctica 14 were not earlier reported as diazotrophs. Some of the isolates promised to be good candidates for use as N_2 fixing biofertilizer for coastal agriculture.

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