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Full Length Research Paper

Characterization and evaluation of stress and heavy metal tolerance of some predominant Gram negative halotolerant bacteria from mangrove soils of Bhitarkanika, Orissa, India

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In the present study both Gram positive and Gram negative bacteria were isolated using enrichment media from five different stations from mangroves soils of Bhitarkanika, Orissa, India. Among the bacterial populations studied, the Gram negative bacterial population was found to be more in all the stations. Out of several Gram negative bacterial isolates, six predominant and morphologically distinct isolates were selected and characterized. Phenotypical the isolates were identified as one each of *Pseudomonas aeruginosa* and *P. alcaligenes* and two strains each of *Methylococcus* sp. and *Desulfotomaculum* sp. The strain *P. aeruginosa* and one strain of *Methylococcus sp.* tolerated up to 13% NaCl and 10% sea salt, respectively. But the other four isolates tolerated 10% NaCl as well as sea salt. Most of the organisms were sensitive to several antibiotics tested except for the antifungal antibiotic Nystatin. The stress tolerant enzyme activities for catalase, peroxidase, polyphenol oxidase, ascorbate peroxidase, ascorbic acid oxidase were found to be variable among the strains. Evaluation of heavy metal tolerance towards the heavy metals; $ZnSO_4$, $CuSO_4$, $NiCl_2$, $CdNO_3$ and K_2CrO_4 showed that the isolates tolerated 600 - 1000 ppm K_2CrO_4 but only up to 10 - 20 ppm $CdNO_3$.

Key words: Biochemical characterization, gram negative bacteria, mangroves, stress enzyme, heavy metal, saline ecology.

INTRODUCTION

Mangroves are typically tropical fragile coastal ecosystems of inter-tidal zones of river deltas and back water areas. They are mostly moderately saline and fragile ecosystems. In spite of that they are highly productive and biologically diversified habitats. Because of richness in carbon and other nutrient content, the mangrove ecosystem harbors diverse microbial communities which can adapt to the saline condition of this ecosystem. The bacterial communities in saline environment would be halophilic and halotolerant bacteria (Zaharan et al., 1992) of various functionalities, that is, CO_2 fixation, nitrogen fixation, phosphate solubilization, cellulose degradation, methanogenesis, agarolysis, antibiotic production and enzymes etc (Holguin et al., 2001).

Complex interactions of various microbes of different biogeochemical processes maintain the nutritional status, ecological balance etc. of these soils. The nitrogen-fixing bacteria (*Azatobacter* sp.) populations were more in sediments of Pichavaram mangrove habitat than in marine backwaters and estuarine systems (Lakshmanaperumalsamy, 1987). Two halotolerant, nitrogen fixing *Rhizobium* spp. have also been isolated from root nodules of *Derrris scandens* and *Sesbania* spp. growing in the mangrove swamps of Sundarbans (Sengupta and Chaudhuri, 1990). The sulphate reducing, iron oxidizing/reducing, methanogens etc. have been isolated from the mangrove swamps of Goa (Saxena et al., 1998) and other places. The Gram negative bacteria viz. *Vibrio, Pseudomonas, Methylococcus, Acinetobacter* and *Altero-*

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monads spp. were predominant in saline habitats (Del Moral et al., 1987; Evans et al., 1972). Nevertheless, compared to the terrestrial microbes, information on the microbes of the marine environment are limited.

Many of these microbes possess unique capability to tolerate the hyper saline condition as well as various heavy metals and metalloids. Heavy metals are increasingly found in microbial habitats due to natural and industrial processes, for which microbes have evolved several mechanisms to tolerate the presence of heavy metals (Alm, 2003). Due to their high stress tolerance capacity these micro organisms are very useful for biotechnological applications in terms of bioremediation and biominerilization.

Therefore, there is a need to study the micro organisms of these soils. Bhitarkanika is one of the largest mangrove forests in India whose microbial diversity has not been studied thoroughly. Therefore, in the present study, an attempt has been made to isolate and identify the microbial communities especially the halotolerant Gram-negative bacteria from mangrove soils of Bhitarkanika, Orissa to understand the functionalities of the soils. Further, these strains were evaluated for their biotechnological potentials in terms of their stress enzyme activity and heavy metal tolerance potential.

MATERIALS AND METHODS

Site description and sample collection

Mangrove area of Bhitarkanika (20° 30' N to 20° 50' N latitude and 86° 30' E to 87° 6' E longitude) is situated in the Kendrapara district of Orissa, India. It extends about 139.39 Sq. Km. in the Brahamani and Baitarani deltaic region of Orissa. Four soil samples were collected from three different location of the Sanctuary, removing top layer of soil (about 1 cm). Soils were mixed thoroughly and put in sterile polythene packets with proper levels and preserved at 4°C.

Enumeration of Gram positive and Gram negative bacteria

The Gram negative bacterial populations were enumerated in nutrient agar media supplemented with 0.001% crystal violet following dilution plate technique. Then the plates were incubated at 37°C for 72 h and the violet colonies formed were counted. Similarly the Gram positive bacterial populations were enumerated in nutrient agar medium supplemented with 0.001% acroflavin. The colonies formed after 72 h incubation was counted.

Isolation of bacteria

One gram of soil was suspended in 9 ml sterile distilled water; diluted logarithmically up to 10^{-5} level. One ml suspension of each soil was inoculated separately in 20 ml nutrient broth medium containing10% NaCl. The flasks were grown on a shaker at 100 rpm at 30 ± 1°C for 72 h and 10 µl of each growth medium was plated separately on nutrient agar plates containing equivalent concentration of NaCl (Holt, 1984). The plates were incubated at 30 ± 1°C for 72 h. The bacteria grown on 10% NaCl (w/v) were isolated and used for the study.

Morpho-physiological and biochemical characters of the isolates

The characters of the organisms were studied following standard microbiological methods. Morphology, vegetative cell and spore characters were observed under a phase contrast microscope (100X objective) from 12 h old culture grown on rotary shaker at 100 rpm, 30 ± 1°C. The physiological and biochemical characters viz. indole production oxidase, catalase, urease hydrolysis, acid from glucose, mannitol, arabinose, xylose, citrate, and propionate utilization and tyrosine hydrolysis were studied. Assay of casein, gelatin, starch hydrolysis etc. were also checked.

Antibiotic resistance

Response of the organisms to different antibiotics was tested on nutrient agar medium. The plates were surface seeded with 2 µl of 10^6 bacterial suspension/ml. Different antibiotic discs with effective concentrations were placed over the plates. Inhibition of growth depicted by a clear zone formation around the discs indicated sensitive reaction; otherwise the organism was resistant to the antibiotic. Diameter of the inhibition zone was measured with an antibiotic zone scale.

NaCl and common salt tolerance

Growth of the organisms on nutrient agar medium supplemented with different concentration of NaCl and common table salt (locally available) was checked. Highly diluted suspensions of the organisms were spotted on the plates, incubated at 30°C for 72 h and growth was recorded.

Determination of minimum inhibitory concentration (MIC)

In order to determine MICs, the strains were grown in nutrient agar medium supplemented with five different heavy metals viz., zinc sulphate, copper sulphate, nickel chloride, cadmium nitrate and potassium chromate at increasing concentrations. The concentrations used were in increments of 20 to 100 ppm and thereafter, in increments of 200 ppm up to the final concentration of 1000 ppm. The pH of the NA medium was adjusted to 7.0 and growth of the micro organisms was measured by c.f.u. counting after 48 h of incubation at 30°C.

Extraction and assay of enzyme activity

The enzymes catalase (CAT) (EC 1.11.1.6), peroxidase (PO) (EC1.14.18.1), ascorbate peroxidase (APO) (EC1.11.1.11), polyphenol oxidase (PPO) (EC1.14.18.1) and ascorbic acid oxidase (AAO) (EC 1.10.3.3) were extracted from the bacteria according to Selander et al. (1986). The bacterial pellet was washed three times by centrifugation at 5,000 g for 20 min with 8.5% (w/v) NaCl and finally with Tris-EDTA buffer (10 mM Tris containing 1 mM EDTA, pH 6.8). The final pellet was stored for 12 h at 20°C, macerated with sterilized glass beads or wool in 4 ml Tris-EDTA buffer on a chilled mortar and pestle placed on an ice bath, centrifuged (15,000 g, 4 ± 0.1°C, 15 min) and the enzyme activity of the supernatant was assayed. Heat killed enzyme (98 ± 1°C, 5 min) was used for all control sets of assay and the enzyme protein was estimated as BSA equivalent with coomassie reagent (Bradford, 1976).

Catalase (CAT) activity was measured following decrease in absorbance for H_2O_2 at 230 nm (Kar and Mishra, 1976) and the units (U) (µmol H_2O_2 decomposed/mg protein/min) of activity were calculated considering ε 23.04/mM/cm for H_2O_2 at 230 nm. The

Table 1. Colony characters of six bacterial isolates from Bhitarkanika mangrove soils.

Bacteria	Form	Colour	Elevation	Margin	Size (mm)	Consistency
BSB1	Circular	Yellow	Convex	Entire	1.4-2.3	sticky
BSB2	Irregular	Brown	Pulvinate	Undulate	1.5 -2.5	Gummy
BSB3	Irregular	Brown	Umbonate	Undulate	2.5-4.0	Gummy
BSB4	Circular	White	Umbonate	Undulate	2.5-4.5	Gummy
BSB5	Irregular	Brown	Convex	Undulate	2.5-4.2	Sticky
BSB6	Punctiform	White	Umbonate	Umbonate	1.4-2.0	Gummy

peroxidase (PO) and polyphenol oxidase (PPO) activities were determined from the increase in absorbance at 430 nm (Kar and Mishra, 1976) and the enzyme activities were expressed as units (µ mol purpurogallin formed/mg protein/min) using ε 2.47/mM/cm at 230 nm for purpurogallin.

The ascorbate peroxidase (AP) activity was determined from the decrease of absorbance at 290 nm and the activity units (µ mol ascorbate decreased/mg protein/min) were assayed considering ε 2.8/mM/cm for ascorbate at 290 nm (Nakano and Asada, 1981). The ascorbic acid oxidase (AAO) activity was recorded following decrease in absorbance at 265 nm (Mahadevan and Sridhar, 1986) and the activity units (µ M ascorbic acid decreased /mg protein/min) were calculated from ε 14 mM/cm for ascorbic acid at 265 nm. All experiments were repeated three times.

RESULTS AND DISCUSSION

Population dynamics of gram positive and gram negative bacteria from mangrove soils of Bhitarkanika, Orissa, revealed that the gram negative bacterial population was more compared to gram positive bacteria in all five sampling stations (Figure 1). Similarly occurrence of more gram negative bacterial population in saline environment than the other groups was reported by (Zaharan et al., 1992). Colony characteristics of gram negative bacteria, isolated in nutrient agar media with 0.001% crystal violet and 7% NaCl were studied. From among several gram negative bacterial isolates, six morphologically distinct and predominantly occurring salt tolerant gram negative bacterial isolates were selected. These isolates were subjected to morphological, biochemical and physiological characterization with a view to identify them. The colony characters of the isolates were irregular, punctiform and circular, 2.5 - 4.5 mm diameter (Table 1). The isolates BSB 1 and BSB 5 were cocci in shape where as other isolates are rod shaped (Table 2).

The isolates BSB 4 and BSB 6 were pleomorphic. The organisms did not hydrolize cholesterol, tween 80, chitin and pectin but Tributyrin hydrolysis is positive (Table 3). Fermentation of carbon components by the organisms was not identical and most of them were non-fermentative (Table 4). All organisms except BSB 2 were catalase positive, but BSB 6 was indole negative. All the isolates studied were positive for nitrate reduction whereas negative for citrate utilization (Table 4). Based on Morpho-physiological and biochemical characters two isolates; BSB 2 and 3 were identified as *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*, respectively.

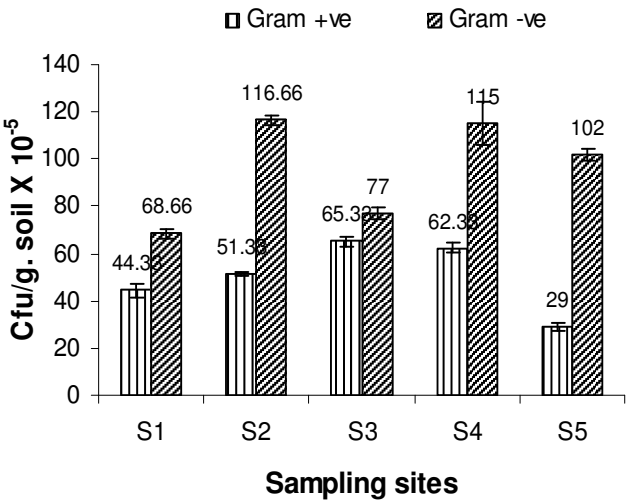

Figure 1. Population dynamics of Gram positive and Gram negative bacteria in five different station of Bhitarkanika, mangrove forests of Orissa.

Two isolates; BSB 1 and 4 belong to the genus *Methylococcus* spp. and two other isolates namely BSB 5 and 6 belong to the genus *Desulfotomaculum* spp. Among the Gram negative bacteria, the members of the genus *Pseudomonas, Vibrio, Methylococcus* are predominant in saline soils (Zaharan et al., 1992). Our finding is also corroborating with the previous findings.

Some of the isolates could not be assigned to species level which would be unique to the mangrove ecology of Bhitarkanika. The micro organisms isolated in the present study have also been reported from other mangrove environments of India. The bacterial isolated already been reported earlier include the methane oxidizers (*Methylomonas, Pseudomonas, methanomonas and methylococcus*) (Ravikumar, 1995), nitrogen fixers (*Azatobacter sp.*), sulphur oxidizers, iron oxidizers and iron reducers (Lokabharathi, 1991; Panchanadika, 1993).

Evaluation of antibiotic assay revealed that all isolates were sensitive to penicillin G, polymyxin B, norfloxacin, bacitracin, chloromphenicol, erythromycin, gentamycin, tetracycline, methicycline, ciprofloxacin, chlorotetracycline, kanamycin and vancomycin, except the antifungal antibiotics nystatin (Table 5). The observations proved that the bacteria had no intrinsic resistance to several

Table 2. Morphological characteristics of six bacterial isolates from Bhitarkanika mangrove soils.

Bacteria	Shape	Length (µm)*		Breadth (µm)*		Motility	Gram stain
		Range	Mean	Range	Mean		
BSB1	Cocci in chains	2-3	2.5	0.5 - 1	0.75	Motile	-
BSB2	Rods in chain	2-3	2.5	1.0 - 1.05	1.25	Motile	-
BSB3	Rod	3.5 - 4	3.75	0.75 - 1	0.87	Motile	-
BSB4	Rod	2.5 -3	2.75	0.45 – 0.75	0.6	Motile	-
BSB5	Cocci	1.0-1.5	1.05	0.25-0.45	0.5	Motile	-
BSB6	Rod	2.5-4.5	2.25	1.5-2.5	0.25	Motile	-

*Results are means of five observations.

Table 3. Extra cellular enzymatic activities of bacterial isolates from Bhitarkanika mangrove soils.

Test	BSB1	BSB2	BSB3	BSB4	BSB5	BSB6
Protease						
Gelatinase	+	+	+	-	+	+
Casein hydrolysis	+	+	+	-	+	-
Lipase						
Tributyrin hydrolysis	+	+	+	+	+	+
Tween 80 hydrolysis	-	-	-	-	-	-
Cholesterol hydrolysis	-	-	-	-	-	-
Lecithinase	-	+	-	-		
Chitin hydrolysis	-	-	-	-	-	-
Pectin hydrolysis	-	-	-	-	-	-

+ = Positive result. – = negative result.

Table 4. Identification scheme of the isolates up to species level based on morpho-physiological characters.

Character	BSB1	BSB2	BSB3	BSB4	BSB5	BSB6
Cell diameter >1 µm	+	+	+	+	+	+
Spores round	-	-	-	-	-	-
Sporangium swollen	-	-	-	-	-	-
Catalase	-	+	+	+	+	+
Anaerobic growth	-	+	-	+	-	-
VP test	-	-	-	-	-	-
Acid production:						
Melibiose	-	-	-	-	-	-
L-Arabinose	-	-	-	-	+	-
Fructose	-	-	-	+	+	+
Dextrose	-	-	-	+	+	-
Lactose	-	-	-	+	-	-
Sucrose	-	+	+	+	+	-
Salicin	-	+	+	-	+	-
Arginine hydrolysis	-	-	-	-	-	-
Hydrogen sulphide test	-	+	-	-	+	+
Starch hydrolysis	-	-	+	+	+	-
Citrate utilization	-	-	-	-	-	-
Egg yolk lecithinase	-	+	-	-	-	-
Nitrate reduction	+	+	+	+	+	+
Indole production	-	-	-	-	-	+

Table 5. Antibiotic assay of six Gram negative bacterial isolates from Bhitarkanika mangrove soils.

Antibiotic	BSB1		BSB2		BSB3		BSB4		BSB5		BSB6	
	S/R	C	S/R	C	S/R	C	S/R	C	S/R	C	S/R	C
Nystatin (10µg)	R	-	R	-	R	-	S	18	S	16	R	-
Penicillin G (10 U)	S	36	S	16	S	21	S	40	S	32	S	31
Polymyxin B (300 U)	S	15	S	13	S	16	R	-	S	15	S	17
Norfloxacin (10 µg)	S	18	S	25	S	18	S	20	S	21	S	24
Bacitracin (10 U)	S	32	S	11	S	10	S	39	S	15	S	25
Chloromphenicol(30 µg)	S	21	S	35	S	24	S	18	S	22	S	16
Erythromycin (15 µg)	S	14	S	6	S	14	S	39	S	25	S	12
Gentamycin (10 µg)	S	39	S	39	S	29	S	30	S	25	S	20
Tetracycline (30 µg)	S	24	S	30	S	27	S	18	S	22	S	22
Methicycline (15 µg)	S	21	S	24	S	27	S	18	S	22	S	16
Ciprofloxacin (30 µg)	S	32	S	40	S	36	S	23	S	20	S	24
Chlorotetracycline (30 µg)	S	34	S	37	S	38	S	20	S	16	S	22
Kanamycin (30 µg)	S	31	S	32	S	21	S	18	S	16	S	14
Vancomycin(30 µg)	S	20	S	19	S	7	S	20	S	24	S	16

C = Diameter of inhibition zone in mm; R = resistant; S = sensitive

Table 6. Salt tolerance and growth characteristics of bacterial isolates from Bhitarkanika mangrove soils.

Isolate No	NaCl/sea salt tolerance (%)	Optimum temperature(°C)	Optimum pH	Anaerobic growth in NB
BSB 1	13/10	40 - 45	7.5 - 8.5	+
BSB 2	13/10	45	9.0	-
BSB 3	10/10	40 - 45	8.5 - 9	+
BSB 4	10/10	40	9.0	+
BSB 5	10/10	40	8.5	-
BSB 6	10/10	40	8.5 - 9.0	-

groups of antibiotics. As they were not exposed to the antibiotics, therefore resistance has not been induced.

All the isolates tolerated 10% NaCl but *P. aeruginosa* and one species of *Methylococcus* (BSB 1) could tolerate NaCl up to 13% (w/v) where as all of them tolerated up to 10% sea salt (Table 6). High salt tolerance would be due to intrinsic adaptability of the organisms to coastal saline soils. Growth of the organisms without NaCl or salt also proved that they were halotolerant, and tolerance of 10% NaCl, grouped them as moderately halotolerant but not halophilic (Vreeland, 1987; Vreeland, 1984). Nevertheless, more growth in presence of NaCl/salt proved that they have preference to salinity which is not observed in moderate halotolerant but found in extreme halotolerant organism (Ventosa et al., 1998). The isolates changes their shape in saline condition which may be due to adaptation to osmotic stress (Chan et al., 1979), an exclusive character of moderately osmotolerant non-halophilic microbes (Rosenberg, 1983) which are known to have potency for commercial exploitation (Vreeland, 1993; Verma, 1993).

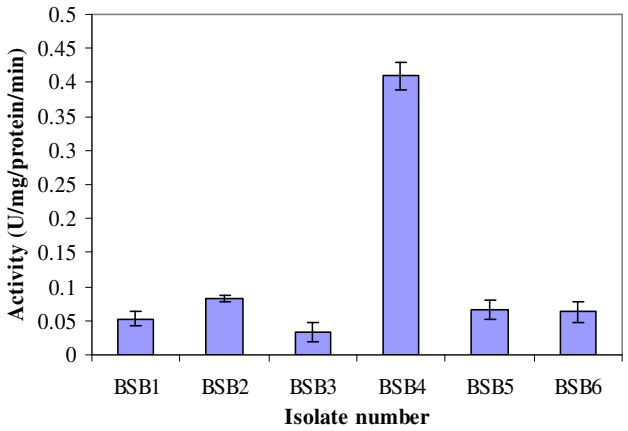

Figure 2. Catalase activity of the isolates.

The activities (U/mg protein/min) of the different oxidative enzymes of the organisms; catalase (CAT) (0.05 - 0.41) (Figure 2), peroxidase (PO) (0.06 - 0.98) (Figure 3),

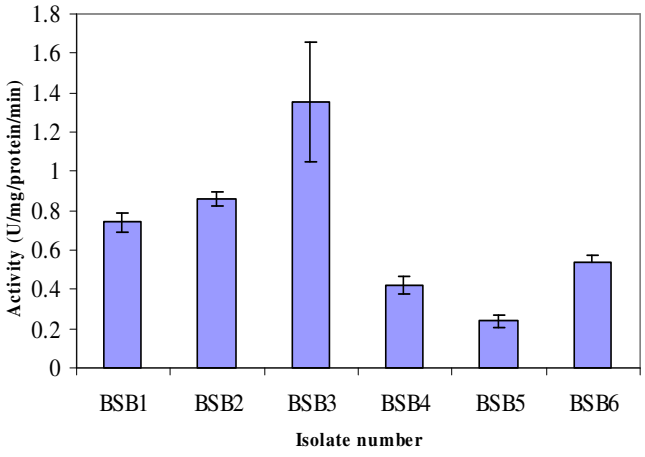

Figure 3. Peroxidase activity of the isolates.

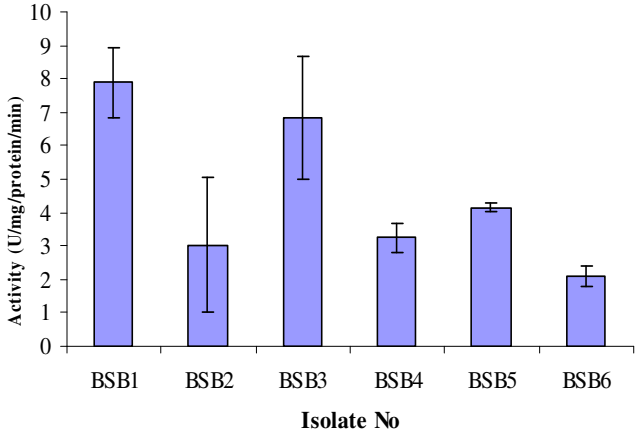

Figure 4. Polyphenol oxidase activity of the isolates.

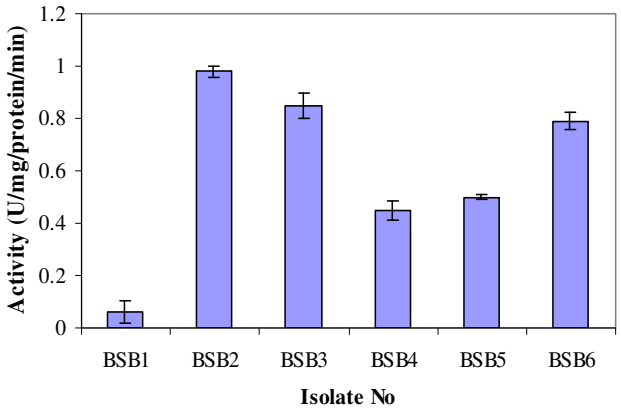

Figure 5. Ascorbate peroxidase activity of the isolates.

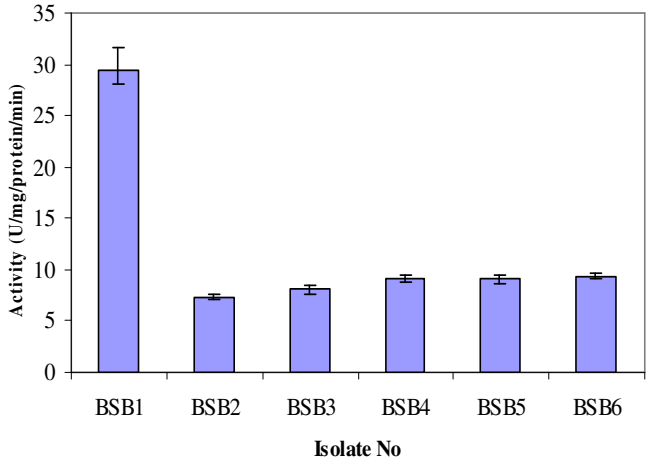

Figure 6. Ascorbic acid oxidase activity of the isolates.

polyphenol oxidase (PPO) (0.25 - 1.13) (Figure 4), ascorbate peroxidase (APO) (8.10 - 29.35) (BSB 2 has no activity) (Figure 5), and ascorbic acid oxidase (AAO) (0.02 - 39.43) (Figure 6) were variable and no correlation could be obtained with their level of stress tolerance. Among the enzymes, the AAO and APO activity was found more in BSB 1; similarly the organism BSB 1 tolerated up to 13% NaCl.

From the observation it is proved that the activity of two stress enzymes is more in presence of salt, which could be attributed to the salt stress mechanism of the isolates. Although BSB 2 tolerated up to same concentration of NaCl, but the enzyme activity is not found similar with that of BSB 1. Though BSB 1 and 2 are two different species, their mechanism of stress tolerance may also vary. It has been reported that under high salt condition microorganisms produced enzymes for synthesis of trihalose or glutamates or prolines to overcome the different stress condition (Dinnbier et al., 1988; Omori et al., 1992).

There are two strategies to cope with a saline environment (Ventosa et al., 1998). Halophilic Archaea maintain an osmotic balance of their cytoplasm with the hypersaline environment by accumulating high concentration of salt. This mechanism of osmoregulation requires special adaptations of the intracellular enzymes that have to function in the presence of salt. Probably different enzymes behaved differentially stress endurance of the organisms. Similarly, stress tolerance of many organisms; *Pseudomonas alcaligenes, E. coli, Pseudomonas* spp. etc. were also controlled differentially by different enzymes (Vreeland, 1987; Moat et al., 2002; Gort and Imlay, 1998; Das and Dangar; 2007). The organisms produce the necessary stress enzymes to withstand the multiple salt stress conditions such as ionic stress, oxygen stress, and osmotic stress prevalent in saline mangrove environment. However, detail studies are required to ascertain the mechanism of stress tolerance.

All the isolated strains (BSB 1 - 6) were tolerant towards five different heavy metals (Table 7). The MIC (minimum inhibitory concentration) values exhibited for

Table 7. Screening of heavy metals (MIC) of six gram negative isolates from Bhitarkanika mangrove soils.

Isolate	MIC (ppm)				
	$ZnSO_4$	$CuSO_4$	$NiCl_2$	$CdNO_3$	K_2CrO_4
BSB1	200	200	100	20	1000
BSB2	200	200	200	20	1000
BSB3	200	200	200	10	600
BSB4	200	200	200	20	600
BSB5	200	200	100	20	800
BSB6	200	200	200	20	800

$ZnSO_4$ and $CuSO_4$ were 200 ppm. Similar MIC values were also exhibited for $NiCl_2$, except BSB 1 and 5 which were tolerating up to 100 ppm. All the isolates showed a very low MIC value against $CdNO_3$ (20 ppm) except BSB 3 which is tolerating only up to 10 ppm concentration. Two strains viz., BSB 1 and 2 showed a very high MIC value against K_2CrO_4 (1000 ppm) and BSB 3 and 4 were tolerating up to concentration of 600 ppm, whereas BSB 5 and 6 were tolerating up to 800 ppm concentration (Table 6). In the present study some of the isolates exhibited relatively a high MIC values towards some of the tested heavy metals which may be due to their stress tolerance characters. Similarly bacteria isolated from saline environment showed high level of tolerance to heavy metals like chromium, arsenate, tellurite, selenite and selenate has been reported by Amoozegar (2005)

Bacteria existing in the saline environment have to cope with a number of stresses including ionic stresses (Galinski and Triiper, 1994). As the organisms were isolated from saline environment, and could tolerate up to high concentration of salt which is generally at toxic level may be one reason for their tolerance towards relatively high concentration of heavy metals which is also toxic in nature. Salt tolerant bacteria that are able to adapt such hostile mangrove environment may exhibit potential for various activities including tolerance and reducing ability towards toxic metal/metalloids.

Conclusion

Mangroves are saline coastal ecosystem and rich in nutrient content. They harbor a large number of microbial populations of unique nature. The present study reveals the predominance of gram negative bacteria in mangrove soils of Bhitarkanika, Orissa. Since mangrove environment is a unique environment with prevalence of multiple stress conditions such as salt stress, ionic stress, oxic and anoxic stresses etc., organisms surviving in such hostile conditions are unique organisms capable of tolerating such stress condition. Isolation and characterization of gram negative bacteria showed occurrence of *P. aeruginosa*, *P. alcaligenes*, *Methylococcus* spp. and *Desulfotomaculum* spp. Evaluation of their stress tolerance could reveal the biotechnological potentials of these organisms.

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