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Isolation and Characterization of Halophilic Bacteria from Sundarban Soil

Debarati Halder[1], Pardita Dutta[1], Arpita Mondal[1], Malini Basu[1]*

[1]Post Graduate Department of Microbiology, Barrackpore Rastraguru Surendranath College, Kolkata, India

***Address for Correspondence:** Dr. Malini basu, Assistant Professor, Department of Microbiology, Barrackpore Rastraguru Surendranath College, Kolkata, India

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ABSTRACT- Halophiles, the most predominant organisms found in the mangrove forest, include varied genera of halophilic bacteria in different environment such as salt lakes, saline soils and salted food. The majority of halophilic microorganisms studied so far produce compounds with great potential in industrial process and they have physiological properties which facilitate its use with commercial aims. In this study of Sunderban soil, focus has been made on the isolation of halophilic organisms and their characterisation. Soils were collected from four different places of Sunderban and were used for physiochemical and microbiological analysis. Qualitative screenings of the isolates were done and three among them was selected having moderately good growth which was further optimised in different growth media. Characterisation of the isolates were done and based on 16S rRNA gene sequencing phylogenetic tree were constructed. The isolates showed multiple heavy metal tolerance and antibiotic resistance. The organisms were further tested for EPS as well as various extracellular enzyme productions thereby exploring their usage for various biotechnological purposes.

Key-Words: Sundarban, Halophiles, EPS, NaCl

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INTRODUCTION

Mangroves are highly productive marine ecosystem where bacteria actively take part in bio-mineralization and biotransformation of minerals [1]. Halophilic and halo tolerant microorganisms are able to thrive and grow in saline and hypersaline environments [2]. These microorganisms are being the object of basic studies in relation to the origin of life in our planet and the molecular mechanisms of adaptation to saline and hyper-saline conditions. 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 minerals and other nutrient content, the mangrove ecosystem harbours 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 of various functionalities i.e sulphur oxidisation, phosphate solubilization, cellulose degradation, antibiotic and enzymes production etc [3].

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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. Due to their high stress tolerance capacity these micro organisms are very useful for biotechnological applications in terms of bioremediation and biomineralization. Therefore, there is a need to study the micro organisms of these soils. Bacterial growth is often accompanied by the production of extracellular polymeric substance (EPS), which have important ecological and physiological functions. Increasing interest is being generated in the study of these molecules because of their wide applications in food, pharmaceutical, petroleum and other industries.

In the present study, diverse bacterial genera having different degree of sodium chloride tolerance were isolated from the soils collected from Sunderban and were characterised in details. Attempts have been made to delineate their properties for biotechnological purposes.

MATERIALS AND METHODS

Study area of Sundarban delta

Sundarban Mangrove forest that is located geographically in between 21°31´N and 22°30´N and longitude 88°10´E and 89°51´E along the North East coast of Bay of Bengal, India. Four soil samples collected during June & December month of 2014 by Microbial biodiversity and Environmental toxicology laboratory, department of

Microbiology from predominant area namely Sudhanyakhali, Sajnekhali, Burirdabri, and Dobanki.

Soil sample collection

Four soil samples were collected from four different places namely, Sudhanyakhali, Sajnekhali, Burirdabri, and Dobanki, of the Sunderbans aseptically at a depth of 10 cm from top layer using a hand-held soil borer. The samples were collected in sterilized polythene containers and transported to the laboratory.

Chemicals used

Nutrient Broth (NB), Luria Broth (LB), Luria agar (LA), Starch agar, KCl, $CdCl_2$, $MnCl_2$, $K_2Cr_2O_7$, $CuCl_2$, $CoCl_2.6H_2O$, $NiCl_2.6H_2O$, $ZnCl_2$, $HgCl_2$, $Pb(NO_3)_2$, Crystal violet. All chemicals used were of analytical grade.

Physical characteristics of soil

The soil samples were dissolved in 1N KCl in the ratio of 1:2.5, and the mixture was allowed to shake for 1hr; the pH & temperature was estimated using digital pH meter. Soil EC was determined by suspending the air dried sample in the ratio of 1:2 after shaking the mixture overnight, it was filtered and the filtrate was analysed using digital conductivity meter (304, Systronics).

Isolation of bacteria

One gram of soil was suspended in 9 ml sterile distilled water; diluted logarithmically up to 10^{-5} level. By taking 1ml soil suspension from dilution 10^{-1}, 10^{-3}, 10^{-5} pour plates were made in NaCl (5%) supplemented nutrient agar and incubated at 37°C for 72 hrs. The colony forming units (CFU) were then enumerated. The selected colonies were transferred and maintained on nutrient agar slants containing NaCl (5%).

Qualitative screening

The isolates were tested for their sodium chloride (NaCl) tolerance by growth in nutrient agar plates containing various concentration of NaCl (5%, 10%, 15% and 20%). The NaCl incorporated plates were streaked with freshly grown culture of the isolates and incubated at 37°C for 2-5 days. Growth of the isolates was determined visually as positive or negative.

Growth performance in different media

The isolates showing moderate growth at 20% NaCl incorporated NA medium were tested for their performance in different types of media such as Luria Bertani Agar, S Agar, Sg Agar SLEV Agar [4] and Basal medium [5] for their optimum growth.

Relative growth of selected isolates

Degrees of tolerance of selected isolates were evaluated in Slev broth containing 5%, 10%, 15% and 20% NaCl. Growth of the isolates in Slev broth was determined by measuring the optical density at 600nm using the uninoculated broth as blank. Relative growths of the isolates were expressed as the percentage of those obtained in untreated control which was taken as 100%.

Characterization and identification of microorganism

The potent NaCl tolerant isolates were characterized both morphologically and biochemically (standard methods). 16S rRNA sequence homology was determined for tentative identification of the organism. Genomic DNA was extracted from the culture by conventional method and 16S rRNA amplification was carried out with 8F and 1492R universal primer sequences. The capillary sequencing was done by ABi 3500 Genetic Analyzer machine as per manufacture's information (GCC Biotech Kolkata).

Heavy metal tolerance

Heavy metal tolerance of the isolates were determined by streaking on Luria-Agar plates supplemented with varying concentrations (25, 50 100, 200 µg/ml for Cd, Mn, Cr, Cu, Co, Ni, Zn; while 5, 10, 20, 40 µg/ml for Hg and Pb) of metals.

Exopolysaccharide (EPS) production

Extracellular polymeric substances (EPS) produced by selected isolates were extracted from mid log phase culture (10hr old) using standard method [6]. EPS was extracted from cell pellets and the amounts of polysaccharide, protein, DNA, RNA were measured by standard methods.

Antibiotic sensitivity

Antibiotic sensitivity of the organism was determined by using antibiotic impregnated discs on nutrient agar incorporated plates (Octadiscs G VIII plus and G I minus, Himedia).

RESULTS AND DISCUSSION

Physical characteristics of soil

Mangroves are saline coastal ecosystem and rich in nutrient content. They harbour a large number of microbial populations of unique nature. Areas of sample collection are shown in Fig. 1. The pH and temperature of soil samples (S1, S2, S3 & S4) was around 7 and 22°C respectively. Electrical conductance of sample S1 was 0.58, S2 was 0.66, S3 was 0.49, S4 was 0.68 (Table 1).

Fig. 1: Map of Sunderban area showing the sample collection sites

Isolation of bacteria

Halophilic bacteria were isolated on nutrient agar plates supplemented with sodium chloride. The CFU/gm of soil for samples S1, S2, S3 and S4 were 18×10^{-3}, 5×10^{-6}, 22×10^{-3}, 26×10^{-3} respectively (Table 1).

Table 1: Physical and microbiological characteristics of soil samples from Sunderbans

Soil Sample	pH	Temperature(°C)	Conductance(mS)	CFU/g of soil
S1	7.94	22	0.58	18×10^{-3}
S2	8.39	22	0.66	5×10^{-6}
S3	8.27	22	0.49	22×10^{-3}
S4	8.23	22	0.68	26×10^{-3}

Screening for NaCl tolerance

Halophilic bacteria have been categorized as slightly, moderately and extremely halophilic on the basis of tolerance to different concentration of NaCl (Fig. 2).

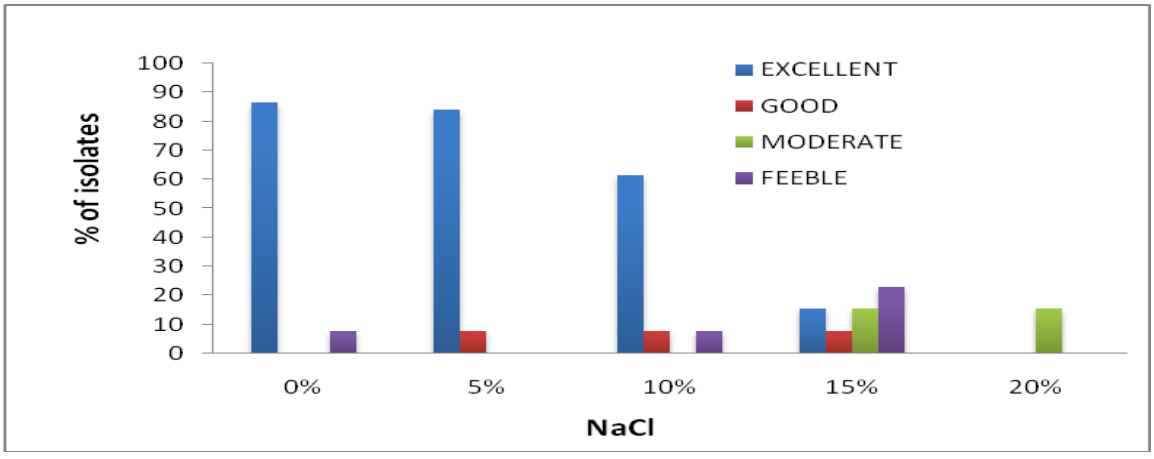


Fig. 2: Qualitative screening of isolates for NaCl tolerance

From the relative growth it is evident that the growth of the isolates has decreased gradually with the increase in concentration of NaCl. 40-50% relative growth was observed in medium supplemented with 15% NaCl; the growth was further reduced to around 30% at the maximum concentration of NaCl (20%) used. However as the organisms under study exhibited fair growth upto 20% NaCl they are categorised as moderate halophiles and were used for detailed study (Table 2).

Table 2: Relative growth of selected bacterial isolates

Isolate	Incubation period (hr)	Relative Growth, % NaCl				
		0%	5%	10%	15%	20%
S_2NaCl 08	24	100	97.05	79.41	50.00	29.41
	48	100	83.00	62.50	39.00	18.75
S_4NaCl 23	24	100	83.00	71.00	44.00	27.00
	48	100	83.82	73.29	47.05	30.88
S_4NaCl 24	24	100	94.00	68.00	48.00	32.00
	48	100	80.64	62.90	43.54	22.58

All values represent the average of triplicates

Characterization and identification of microorganism

Gram reaction confirmed the isolates S2 NaCl 08 and S4 NaCl 23 as gram positive short rods where as the isolate S4NaCl 24 as gram negative cocci (Fig. 3).

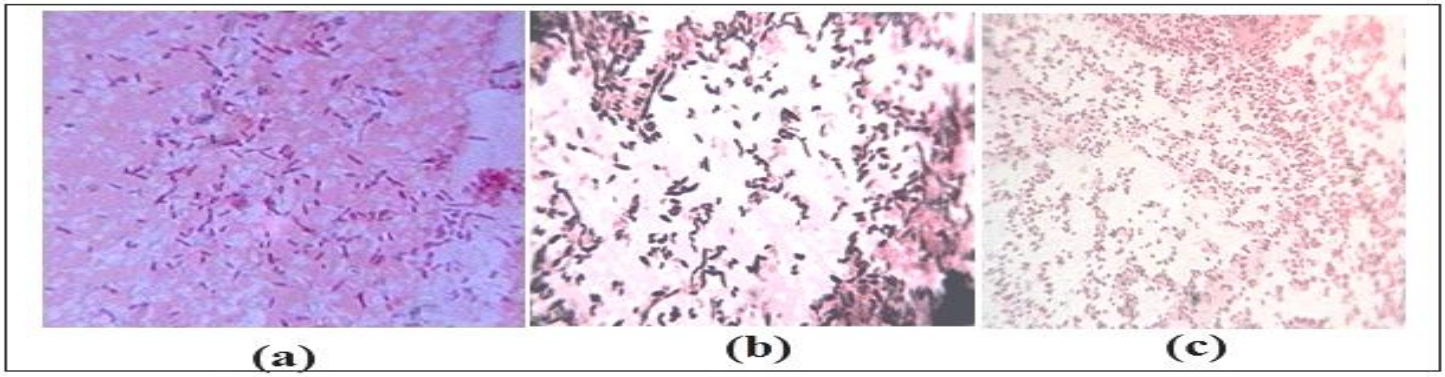
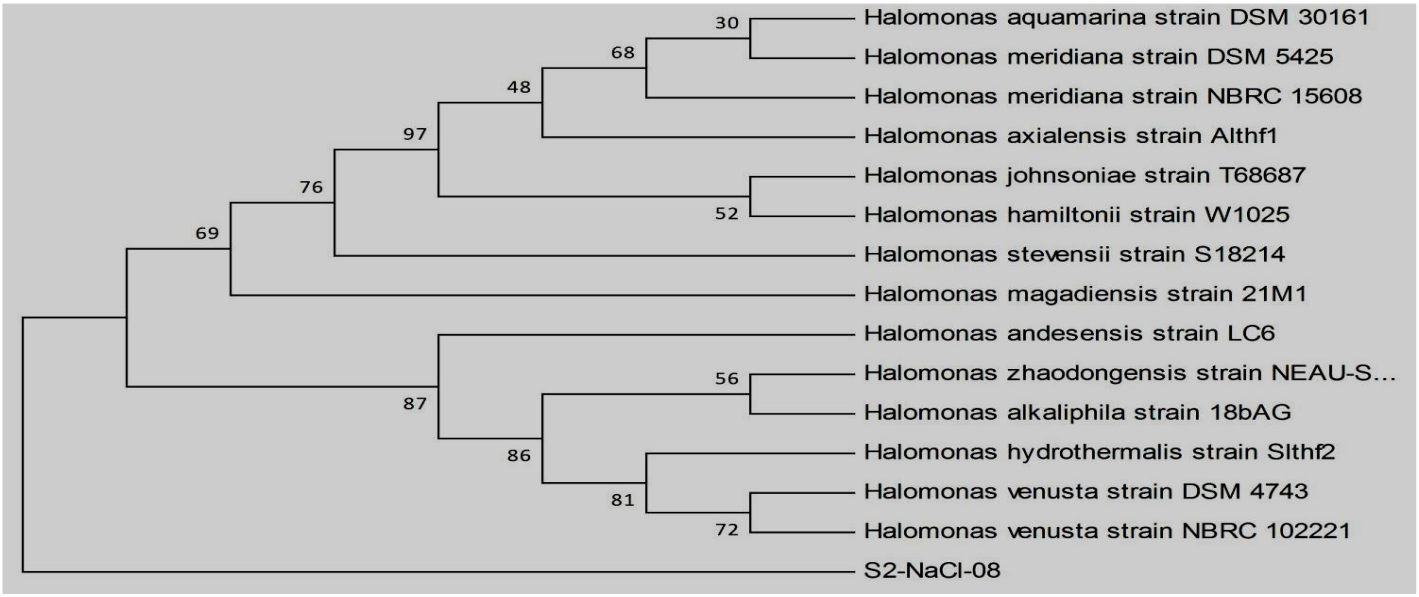
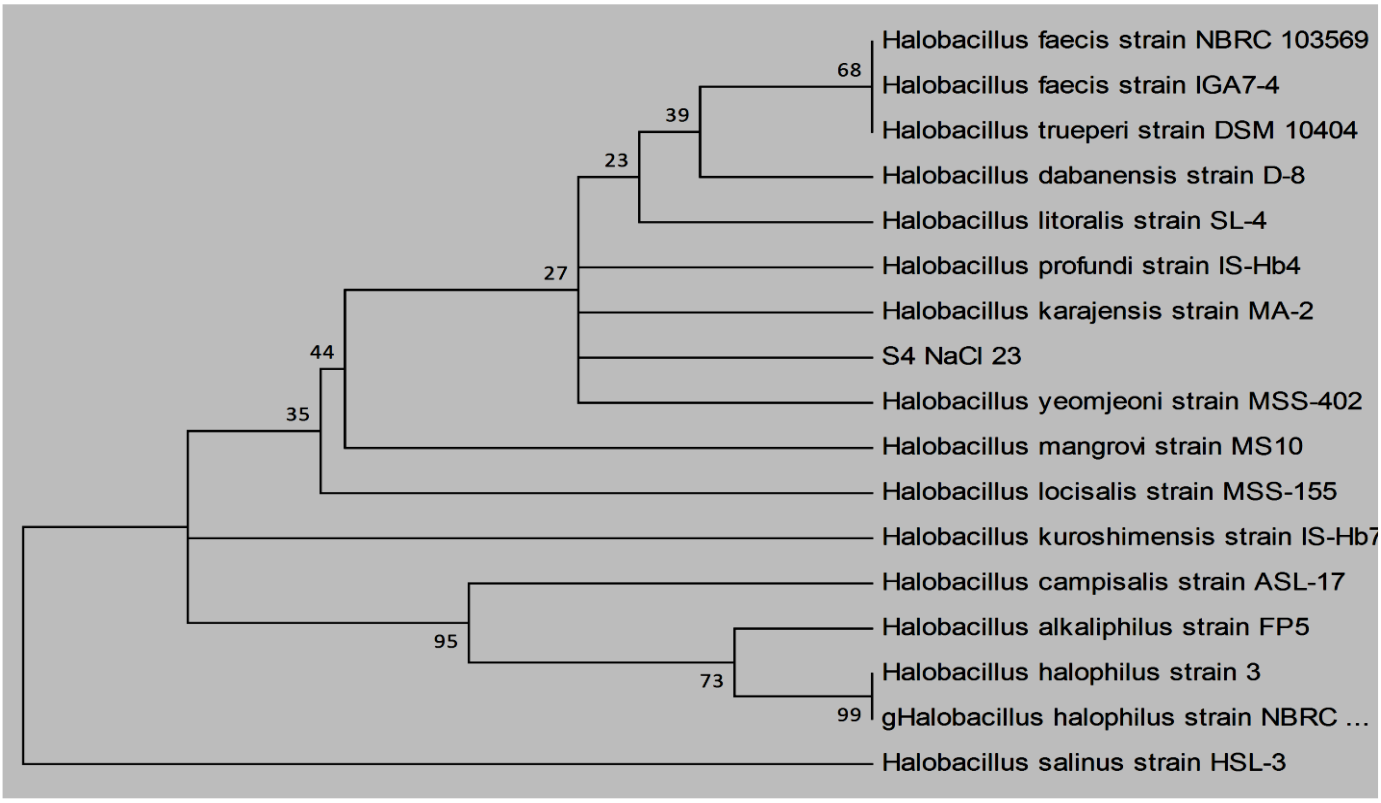


Fig. 3: Gram staining of selected isolates (a) S2 NaCl 08 (b) S4 NaCl 23 (c) S4 NaCl 24

Determination of phylogeny for the 16S bacterial identification data was done using the UPGMA method and tree reliability was tested by Bootstrap method for the Test of Phylogeny option in MEGA 4 software. Best probable similarity of S2NaCl 08 was shown with *Halomonas aquamarina* strain DSM 30161 and S4NaCl 23 with *Halobacillus trueperi* strain DSM 10404 (Fig. 4).



(a)



b)

Fig. 4: Phylogenetic analysis of (a) S2NaCl 08 and (b) S4NaCl 23

16S rRNA gene amplified with 8F (AGAGTTTGATCCTGGCTCAG) & 1492R (CGGTTACCTTGTTACGACTT). The capillary sequencing was done by ABi 3500 Genetic Analyzer machine as per manufacture's information. Sequence analysis was done by using BLAST search of NCBI. Finally based on the maximum identity scored Dendogram was constructed (GCC Biotech)

From standard biochemical tests (Table 3) it was observed that S4NaCl 23 was positive to citrate utilization, urease, glucose and adonitol production. S4NaCl 24 showed positive reaction for nitrate reduction, urease and adonitol production. S_2NaCl 08 also showed positive reaction for urease, phenylalanine deamination, glucose and sorbitol production. In case of cellulose degradation test, S4NaCl 24 showed a clear zone formation (positive) whereas a faint clearing zone (positive) was also seen in case of S4 NaCl 23, but no clear zone was observed against S2 NaCl 08 and hence was negative. It has been studied that halophilic bacteria are potent producers of many useful enzymes like amylase, protease, lipase, catalase, urease, phosphatase, cellulase etc. [7]. From the biochemical tests it has been observed that three isolates has confirmed the production of catalase as well as urease and two among the three has confirmed the production of cellulase. Similar enzyme producing halophiles have been isolated and characterised by several workers and have great significance in present day biotechnology [8].

Table 3: Biochemical characterisation of soil isolates

Sl. No.	Test	S_4 NaCl 23	S_4 NaCl 24	S2 NaCl 08
1.	Citrate utilization	+	–	–
2.	Lysine utilization	–	–	–
3.	Ornithine utilization	–	–	–
4.	Urease	+	+	+
5.	Phenylalanine deamination	–	–	
6.	Nitrate reduction	–	+	+ –
7.	H_2S production	–	–	+
8.	Glucose	+	–	+
9.	Adonitol	+	+	–
10.	Lactose	–	–	–
11.	Arabinose	–	–	–
12.	Sorbitol	–	–	+
13	Starch hydrolysis	–	–	–
14	Casein hydrolysis	–	–	–
15	Lipid hydrolysis	–	–	–
16	Catalase	+	+	+
17	Urease	+	+	+
18	Phosphate solubilising	–	–	–
19	Sulphur oxidizing	–	–	–
20	Cellulose degradation	+	+	–

N.B. +: Positive, -: Negative

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. Several workers also focussed on interaction of various heavy metals with halophilic bacteria, (9). In this study the halophilic bacteria were tested for their heavy metal tolerance which showed varying response (Fig. 5).

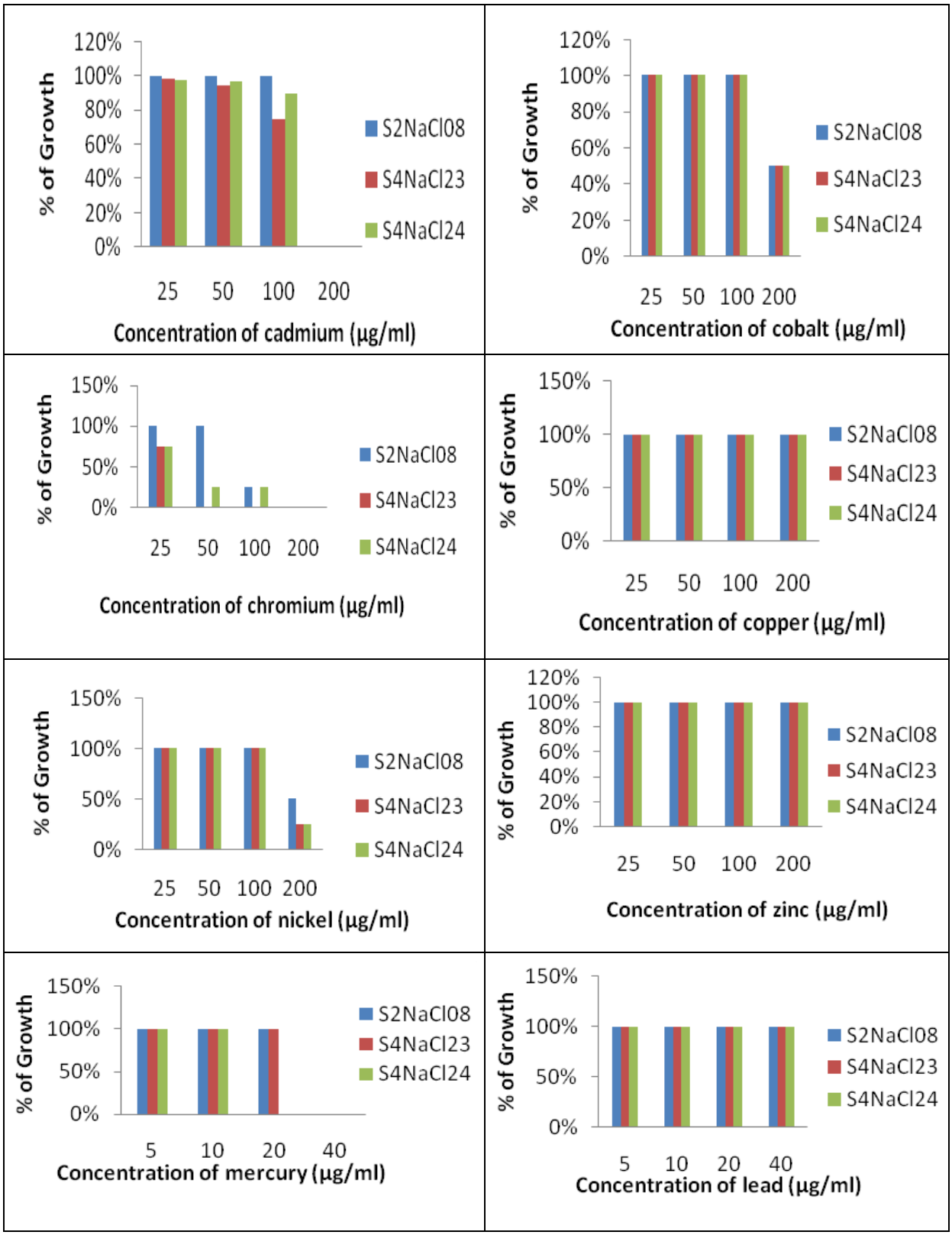


Fig. 5: Growth response of selected isolates to different heavy metals

The organisms showed 100% growth even at the highest concentration of the metal tested (in case of copper, zinc and lead) except S4NaCl24 which showed complete growth inhibition at 40µg/ml. The degree of resistance of the isolates could be summarized as Cd>Co>Ni>Cr. The isolates used in this study has exhibited promising results with respect to their heavy metal tolerance profile indicating that they may serve as potential bioremedial tools for removal of metal pollutants from contaminated sites.

Extracellular polymeric substance:

All the three isolates produced exopolymeric substances comprising of proteins and polysaccharide but no nucleic acids. EPS of isolate S2NaCl 08 contains maximum protein (0.3µg/mg) where as isolates S4NaCl 23 and S4NaCl 24 contain almost half the amount. S4NaCl 23 produced maximum polysaccharide (0.12µg/mg) unlike other two isolates Bacterial growth is often accompanied by the production of exopolysaccharides (EPS), which have important ecological and physiological functions. Increasing interest is being generated in the study of these molecules because of their wide applications in food, pharmaceutical, petroleum and other industries [10].The strains isolated have shown their ability to produce extracellular polymers (Fig 6) having attractive industrial application.

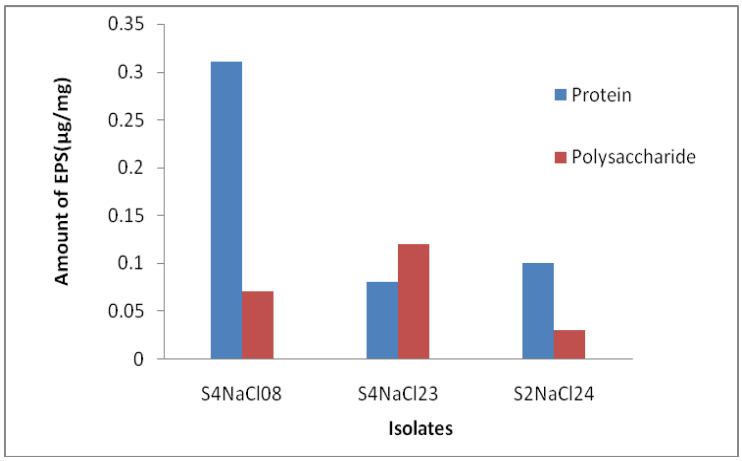
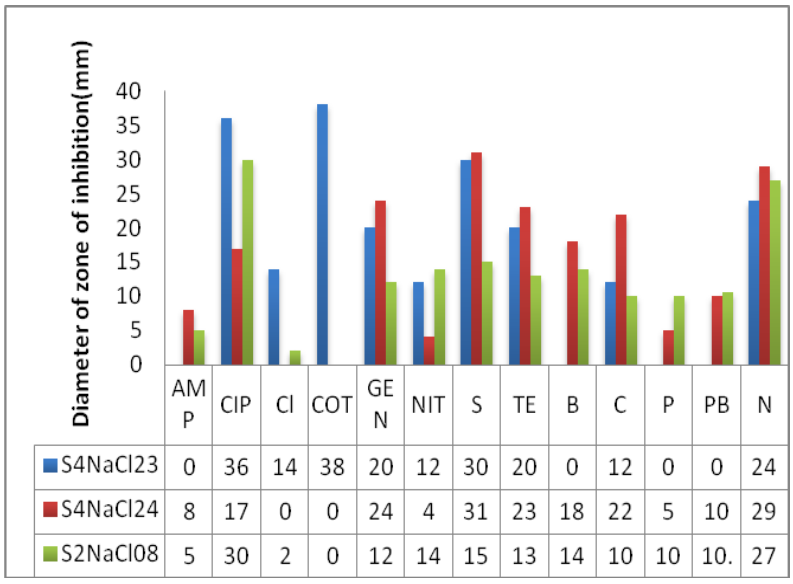


Fig. 6: Exopolysaccharide production of the selected isolates

Antibiotic sensitivity

Since heavy metal resistances often have a correlation with antibiotic resistance pattern all the three selected isolates were screened for their antibiotic resistance profile (Fig. 7)



	AMP	CIP	Cl	COT	GEN	NIT	S	TE	B	C	P	PB	N
S4NaCl23	0	36	14	38	20	12	30	20	0	12	0	0	24
S4NaCl24	8	17	0	0	24	4	31	23	18	22	5	10	29
S2NaCl08	5	30	2	0	12	14	15	13	14	10	10	10.	27

Fig. 7: Antibiotic sensitivity profile of the selected isolates

Resistant to [AMP 13mm or less, B-8mm or less, P-28mm or less, PB-11mm or less, CL-10mm or less, COT-10mm or less, NIT-14mm or less, TE-14mm or less, GEN-12mm], CIP-16-20mm(intermediate), NIT-15 16mm (intermediate).

S2NaCl 08 showed resistance against AMP, COT, P, PB, CL and sensitive to other antibiotics tested. S4NaCl 23 showed resistance to AMP, B, C, P, PB and intermediate to NIT whereas sensitive to rest of the antibiotics. S4NaCl 24 has shown resistance against AMP, COT, NIT, P, PB, Cl and intermediate to CIP whereas sensitive to the all other antibiotics tested.

CONCLUSIONS

The soil samples were collected from four different locations of Sunderban. Bacteria were isolated on NaCl incorporated nutrient agar for further microbiological analysis. Soil pH EC and temperature was also recorded. Based on qualitative assay three isolates having moderate to good growth were selected. Optimisation of suitable media for excellent and repetitive growth was done. Determination of relative growth of the selected bacterial isolates categorised them as moderate halophiles. Among the three selected isolates two of them were gram positive rods (S2NaCl 08, S4NaCl 23) and one was gram negative cocci (S4NaCl 24). 16S rDNA sequencing was done to assign the phylogeny. Antibiotic sensitivity profile and heavy metal tolerance of the isolates showed varying responses. All the three isolates produced EPS in varying amounts. With respect to various enzyme productions all the three isolates were found to be catalase positive and highly urease positive as well as cellulase positive except S2NaCl 08. Characterisation of halophilic bacteria hence will enable us to use them for various biotechnological purposes.

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