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Isolation of *Halomonas sp.* sm-sr10, an Extreme Halophilic, Hydrocarbon (engine-oil) Degrading and Mega Plasmid Harboring Bacteria, from Bay of Bengal, India

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Recently, studying on microorganisms of mangrove forests in Eastern India, an extremely halophilic bacterium was isolated and characterized. Designated as sm-sr10, this pleomorphic bacterium grows up to 4M NaCl added in medium and significantly degrade crude engine-oil. It is identified as *Halomonas* sp at 16S rDNA level (GenBank Acc. no HM446042**). The scanning electron micrography reveals small rod with a rough surface. This alkaliphilic bacterium is positive for protease and multi-drug resistant with possession of large plasmid (~50 kb). 16S rDNA sequence reveals sm-sr10 belongs to genus *Halomonas* and family *Halomonadaceae*. The phylogeny analysis suggests its similarity to *Halomonas shengliensis* SL014B-85 and *Halomonas* sp. MOLA 69 (99 %); *Halomonas* sp. Ad-1 and *Halomonas* sp. C-12 (98 %) and *Halomonas* sp. whb34 (97 %). It can significantly degrade (50% and 75% in 10 and 20 days respectively) engine-oil and appreciably nitrobenzene in BH media. Its respiration with nitrate as substitute to oxygen, an energetically more favored terminal electron acceptor may suggest the eutrophic status of such water body contaminated with industrial nitrogenous wastes. Present finding is important for the understanding of saline adaptations, stress and drug resistance of these types of halophiles which may be utilized for in situ bioremediation.**

Key words: Halophilic; 16S rDNA; Halomonas; large plasmid, engine-oil, bioremediation.

Halophiles inhabit soils, wetlands, fresh and marine waters, lakes and sediments (Echigo *et al*. 2005). Cellular growth is normally inhibited by extreme salinity but it is the environment which introduces life on earth and harbor taxonomically diverse bacterial groups (Buchalo *et al*. 1998). Moderately halophilic bacteria grow best in the range of 5-10 % and extremely halophilic bacteria grow optimally at > 10 % (w/v) NaCl concentrations (Yoon *et al*. 2001). Majority of these bacteria have developed different structure-function adaptations to survive under this conditions (Kennedy *et al*. 2010). Such adaptations are related to physical property of DNA and protein stability suggesting the convergent evolution of halophilic species resulting ultimate changes at metabolic level (Paul *et al*. 2008).

An extensive study on the molecular characterization by 16S gene of microorganisms of Sundarban at eastern part of India reveals that this area harbors *Proteobacteria, Flexibacteria* (CFB

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group), *Actinobacteria, Acidobacteria, Chloroflexi, Firmicutes, Planctomycetes and gammatimonadates* (Ghosh *et al*. 2010). One study indicated that dissolved oxygen, nutrients and turbidity are the limiting factors for the phytoplankton biomass in these types of land. Nevertheless, eutrophication as well as presence of toxic Dinoflagellates and Cyanophyceae favors the deterioration of the water quality. (Manna *et al*. 2010). Halotolerant bacterial species are also isolated from Gulf Sea, Red Sea, Mexican and Korean oil fields and several other places. In general, studies on halophilic bacteria are of great importance for their adaptative nature in saline system and their ability to degrade different aliphatic and aromatic hydrocarbons (González-Domenech *et al*. 2008; Oren 2010), toluene and other organic solvent (García *et al*. 2004) and crude petroleum (Mnif *et al*. 2009). A few reports demonstrate single or multi drug resistant *Halomonas* sp (Coronado *et al*. 1995) and some of those are demonstrated occurring plasmid (Fernandez-Castillo *et al*. 1992).

The present study describes the isolation and characterization of an extreme halophilc bacterium from the mangrove forests of Sundarban and its possible utility for bioremediation process in engine oil (long chain hydrocarbons, waste car engine oil, base oil or the c-alkane fraction of base oil) contaminated water bodies. Biotechnological use of halo-tolerant gene as to increase propensities in agricultural productivity in saline environment could also be a valid objective.

MATERIALS AND METHODS

Site description and sample collection

Water sample from one meter depth of sea level was collected from the halophytic mangrove forest of Sundarban of Bay of Bengal in eastern India. This is an estuarine and delta location which is composed of large natural water bodies and several small islands (Wahid *et al*. 2002). Average sea water temperature of the collection site varied between 26-31 °C and the pH remain between 7.31 – 7.9.

Isolation and enrichment of microorganisms

Direct plating was done on nutrient agar (NA) media with 50 % (v/v) autoclaved sea water for isolation of bacteria from water samples (Antunes *et al*. 2008). The plates were incubated at 37 °C for 72 h. Enrichment was done by culturing all single colonies in various liquid media i.e. glucose-yeast, tryptone-beef, glucose-mineral, glucose-peptone etc and inoculated at 30°C on a rotary shaking platform with 200 rpm. Optical densities were recorded at different time points. All morphologically distinct isolates were preserved on liquid media with 50 % glycerol at -20 °C.

Determination of salt tolerance, optimum pH and temperature

A number of isolated strains were checked for salt (NaCl-0.25 M to 5 M) tolerance and one extremely halophilic bacterium (sm-sr10) was selected for further investigation. The sm-sr10 was grown for 48 hours (30°C, 200 rpm) in tryptone-beef extract-yeast extract (TBY) broth supplemented with 2 M NaCl and varying pH (4-11) and at different temperatures (20, 30, 37 and 50 °C).

Morphological, physiological, and biochemical characterizations of strain sm-sr10

Gram character and cellular morphologies, motility, presence of endospore and capsule of sm-sr10 were determined by standard methods. Amylase activity on starch medium, protease activity against casein, urea hydrolysis ability, phenylalanine deaminase activity and activities of catalase and gelatinase were also done by routine microbiological procedure. Nitrate reductase activity was determined by growing the bacterium in nitrate broth for 4 days.

Scanning electron microscopy (SEM) of sm-sr10

The bacterial culture was centrifuged and the pellet was washed for three times with phosphate buffer saline (pH - 7.2). After addition of 0.25 % gluteraldehyde (in Na-phosphate, pH 7.2), cells were incubated followed by dehydration graded (30 to 100 %) ethanol. Cells were dried up to critical point and then attached to the stub. The sputter was coated with gold (2-3 min) by an IB2 ION COATER device. The coated sample was used for SEM (Model- S530 HITACHI, JAPAN) (Franson *et al*. 1984)

Antibiotic susceptibility

Twelve different antibiotic discs i.e. chloramphenicol, streptomycin, tetracycline, norfloxacin, refampicin, kanamycin, neomycin, ampicilin, nalidixic acid, methicillin, co-trimoxazole,

penicillin-G (HiMedia, Mumbai, India) were placed on Muller Hinton Agar (2M NaCl) plates that were previously surface-inoculated with 0.1 ml (10^7 CFU / ml) 24 h grown sm-sr10. After overnight incubation the bacterium was inferred for its susceptibility to antibiotics according to inhibition zone diameter. Streptomycin (highly resistant) was used with different concentrations with varying media NaCl concentration to elucidate the relation between salt and antibiotic tolerance.

Genomic DNA isolation and 16S rDNA analysis

Molecular characterization of sm-sr10 was done on the basis of 16 S rDNA sequences. In brief, DNA was isolated from the culture using QIAamp DNA Purification Kit from Qiagen (Zhang and Cahalan 2007). 16S rDNA gene was amplified in an Eppendorf Thermal Cycler from the DNA in the presence of 8F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' ACG GCT ACC TTG TTA CGA CTT 3') primers (Siqueira and Rôças 2005). The purified DNA was subjected to automated DNA sequencing. Consensus sequence of 1377 bp rDNA gene was generated. The 16 S rDNA gene sequence was used to carry out BLAST with the nr database of NCBI GenBank database. Based on maximum identity score first ten sequences were aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP (Ribosomal Database Project) and the phylogenetic tree was constructed using MEGA4 (Molecular Evolutionary Genetics Analysis) (Tamura *et al*. 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*. 2007).

Isolation of plasmid DNA

The plasmid DNA was isolated by alkaline hydrolysis method Birnboym and Doly (Maniatis *et al*. 1989) and Kado - Liu method (Kado and Liu 1981) with some modifications. The agarose gel electrophoresis was run (0.8 % for 3 h at 50 V) in Tris-borate buffer (Maniatis *et al*. 1989) and the l-phage DNA (49.2 kb, Bangalore Gennei, India) was used as standard.

Determination for Hydrocarbon Degradation ability of sm-sr10

Bushnell Hass (BH) media was taken for studying the microbial hydrocarbon degradation (Lee and Cho 2008). In the present protocol, broth culture (20 ml) was added with 1 ml any one of xylene, nitrobenzene, kerosene, engine oil (long chain hydrocarbons) and fructose (control) as the chief carbon source. For solid culture, before solidification, xylene, nitrobenzene or fructose was added to the media after dissolving in dimethyl sulfoxide (DMSO) for homogeneous mixing.

Following the initial result of growth rate of different hydrocarbon added broth culture, only engine oil group was used in further investigation for its extraction with cyclohexane and quantization by a spectrophotometer at 490 nm against a suitable standard graph plotted from cyclohexane extracted known amount of engine oil (Lau *et al*. 2010).

RESULTS

Physiological and biochemical characterization of sm-sr10

Salt tolerance test revealed that sm-sr10 can grow optimally at 2 M NaCl in 24 h though it can grow up to 4.5 M of NaCl in ≥ 48 h (Figure 2). And the optimum pH is 8-11 and temperature is 30°C for 24 h growth (Fig. 5). The bacterium shows Gram negative, motile, non-capsulated and non endospore forming with a highly pleomorphic character. In twenty hours, it is appeared round shaped and singly arranged at ≤ 2 M NaCl concentration. It gradually transformed to oval, rod or ramification and finally long chain arrangement at ≥ 3 M NaCl for ≥ 48 h growth condition (Fig. 1A). The strain can utilize glucose, fructose, mannitol, lactose, sucrose, and maltose as carbon source. It showed negative results for starch hydrolysis, indole, methyl red, citrate utilization, H_2S production, starch hydrolysis, gelatinase, Voges-Proskaur, cellulase, urease and positive result for casein hydrolysis, nitrate reduction, phenyl alanine deaminase test and catalase activity (Table 1).

Antibiotic susceptibility

Table 1 describe that sm-sr10 possess multi-drug resistance property (Fig. 4A). It also reveals that streptomycin sensitivity of sm-sr10 is not interfered by the salt concentration in the medium (Fig. 4B).

Scanning electron micrograph of sm-sr10

SEM data of 24 h culture of sm-sr10 suggest that the bacterium is oval to small rod

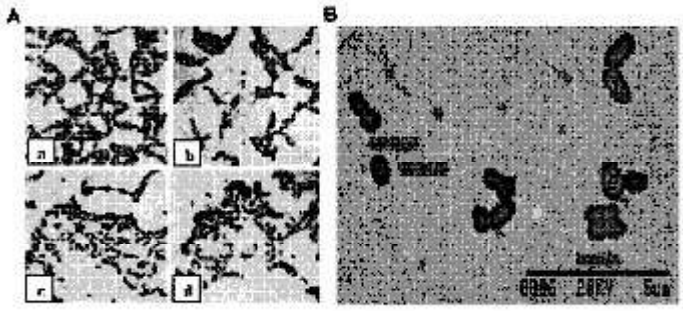


Fig. 1. (A) The sm-sr10 was cultured in tryptone-beef extract-yeast extract (TBY) media for 7 days with 1 M (a), 2 M (b), 3 M (c) and 4 M (d) of sodium chloride. Figure shows that bacterium is highly pleomorphic. (B) Picture of 24 h culture was taken with SEM (Model- S530 HITACHI, JAPAN) at 20 kv

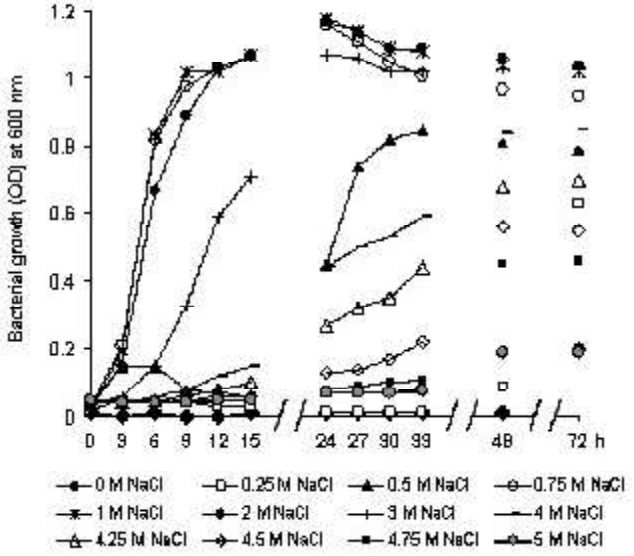


Fig. 2. Growth rate of sm-sr10 in tryptone-beef extract-yeast extract (TBY) media with varying amount of NaCl

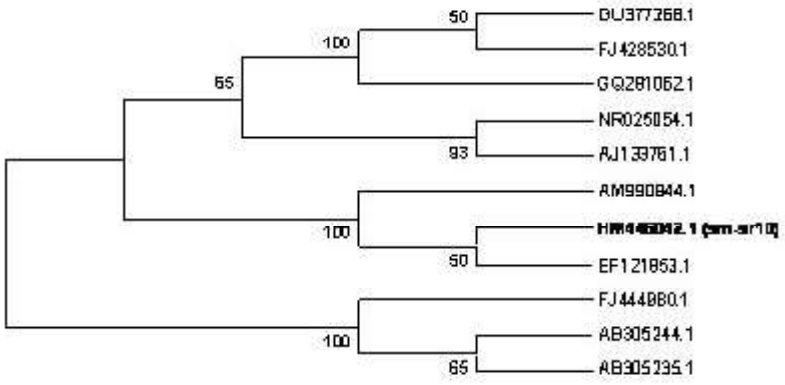


Fig. 3. Relative phylogenetic position (based on 16S rDNA alignment) of sm-sr10 with respect to first 10 best align. Phylogenetic tree was constructed using MEGA4. The bold letter denotes the GenBank accession number of sm-sr10

shaped with uneven and rough surface. Its length is 0.976 µM and diameter 0.671 µM at 20 kV. Some of the bacteria are shown to assemble with two or more in number (Fig. 1B).

16 S rDNA analysis and molecular characterization of sm-sr10

Phylogenetic analysis of 16S rDNA sequences revealed that sm-sr10 displayed close relationships to several bacterial species of *Halomonas*. BLAST searches revealed that the sequences of the isolate fell in class, γ-*Proteobacteria*, and family, halomonadaceae (Fig. 3). The isolated strain shows similarity to different strains of *Halomonas* sp. (*Halomonas shengliensis* strain SL014B-85, 99 %; *Halomonas* sp. MOLA69, 99 %; *Halomonas* sp.Ad-1, 98 %).

Presence of extra chromosomal DNA

Plasmid was isolated and detected by both the alkaline hydrolysis and Kado-Liu method. Single distinct band was noticed which correspond

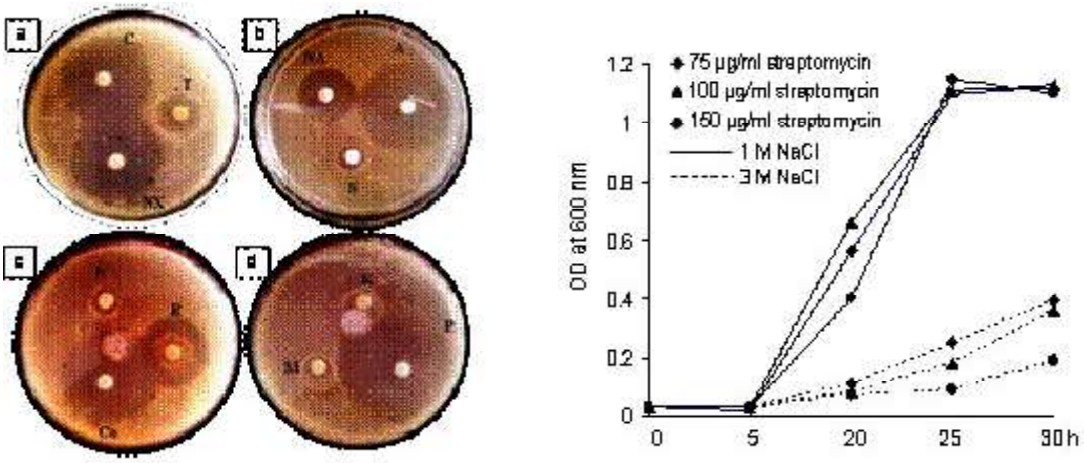


Plate a (C- chloramphenicol, T- tetracycline, NX- norfloxacin)
Plate b (A- ampicilin, S- streptomycin, NA- nalidixic acid)
Plate c (P- penicillin-G, K- kanamycin, M- methicillin)
Plate d (Co- co-trimoxazole, R- refampicin, N- neomycin)

Fig. 4. (A) Antibiotic sensitivity property of sm-sr10. Representing letters denote several antibiotics as follows. (B) Relation between salt dependence and streptomycin resistance

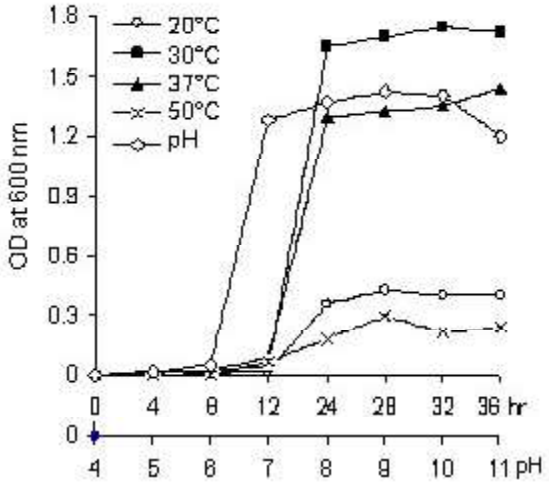


Fig. 5. Growth pattern of sm-sr10 in tryptone-beef extract-yeast extract (TBY) media with conditions of different temperature and pH

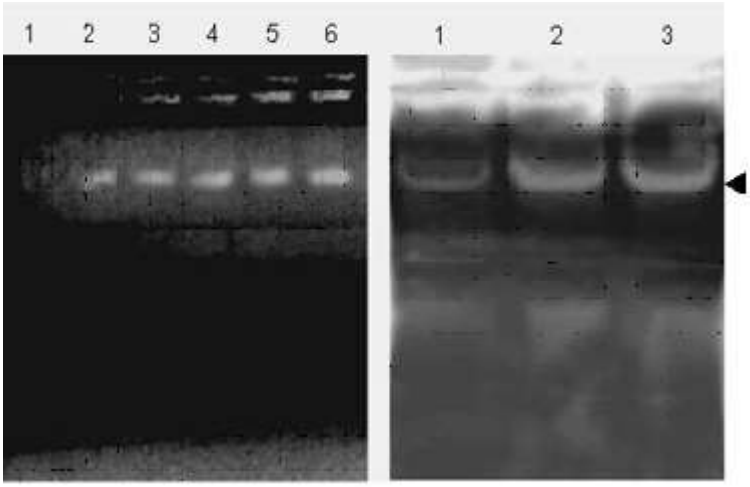
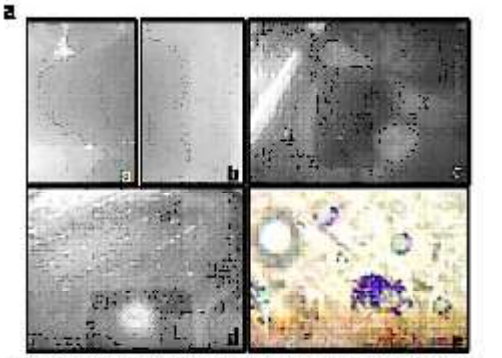


Fig. 6. Plasmid DNA isolated by alkaline hydrolysis method (Birnboym and Doly (Maniatis *et al*., 1989)) (a) or Kado-Liu method (Kado and Liu, 1981) (b) are presented in 0.8% agarose gel. (a) Lane-1, 2 hg; lane-2, 5 hg; lane-3, 10 hg; lane-4, 15 hg; lane-5, 20 g; lane-6, 30 hg of DNA. (b) lane-1, pure l-phage DNA (49.2 kb); lane-2 and 3, plasmid DNA isolated from sm-sr10 run in duplicate



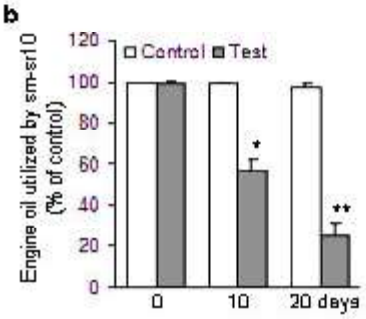


Fig.7. a. Culture and growth of sm-sr10 in BH-agar and liquid media. Gram's staining shows concentrated bacterial cells around the interface of water-hydrocarbon droplets. b. Degradation of engine oil by sm-sr10. Bar represents mean ± SEM (n = 4). Level of significance comparing with respective control; * *p<0.01*, ** *p<0.001.*

with 49.2 kb genome of »-phage (Figure 6).

Growth in hydrocarbon and its degradation by sm-sr10

Present results show a significant amount of bacterial growth in engine oil or fructose added media with concentration and time dependant manner. An appreciable amount of growth is also noticed in nitrobenzene but not in kerosene or xylene added media. The findings in plate culture are also in line with the results of broth culture. Small transparent and less number of colony is noticed in nitrobenzene plate whereas the engine oil plate was full of bacterial colony. Bacteria from both solid and liquid culture are stained and the staining from liquid culture shows significant number of bacteria present in the hydrocarbon (engine oil)-water interface. Cyclohexane extraction result shows a significant degradation of engine oil by the bacteria when compared to the control data (Figure 7).

DISCUSSION

Sundarban is the largest delta estuary in the world. Report is available on water quality, density of biomass, species diversity, phytoplankton abundance, eutrophication level and bacterial populations of this place (Manna *et al*. 2010). Studies reveal that the water is moderately polluted with different inorganic/

organic compounds, metals, pesticides, sewage released from industries situated at the riverside (Saha *et al*. 2006; Albaigés *et al*. 2010). The Nitrosomonas, Nitrobacter, Acinetobacter, Pseudomonas, Gordonia, Rhodococcus, Cobetia and few Halomonas species are shown to have several hydrocarbon degradation properties with varying degrees of efficiency (John and Okpokwasili 2012).

In a recent investigation, *proteobacteria* especially the *gammaproteobacteria* were found to be abundant in Sundarban sediment (Ghosh *et al*. 2010). In two earlier studies, *gammaproteobacteria* isolated from this region was found to be commercially important (Saha 2006). On the basis of the morphological, biochemical characteristics, and 16S rDNA analysis, the isolate sm-sr10 was identified as a new strain of *Halomonas* sp. sm-sr10 (GenBank Acc. No. HM446042.1). The sm-sr10 showed resistance to streptomycin without any dependence on media salinity. Certain halophiles are demonstrated with a high sensitivity to rifampicin regardless of salt concentration (Coronado *et al*. 1995). Others respond to several antibiotics relating to media salinities (Coronado *et al*. 1995).

The present bacterium sm-sr10 has the highest similarity (99 %) with *Halomonas shengliensis* strain SL014B-85 isolated from crude oil contaminated saline soil from China (Wang *et al*. 2007). Some physiological and biochemical features of sm-sr10 are similar to this isolate. Notwithstanding, in our study, we found sm-sr10 to be resistant to several antibiotics and carry large extra chromosomal DNA. Denitrifying character is an important taxonomic marker within the genus *Halomonas* (Li *et al*. 2008; González-Domenech *et al*. 2010). Several moderately halophilic species from *Halomonas* genera can use nitrate or nitrite (Martínez-Cánovas *et al*. 2004). In the present investigation sm-sr10 is found to have strong nitrate reduction ability. In some ecosystem, nitrogen is most likely the limiting nutrient (Seitzinger *et al*. 2006). In general, predisposition of denitrification is experienced at a depleted status of oxygen, a more energetically favorable electron acceptor, and bacteria respire with nitrate as a substitute terminal electron acceptor. Such report supports the present eutrophic status of Sundarban and occurrence of industrial nitrogenous waste (Manna *et al*. 2010). Denitrification processes and related micro-organism are commercially important to use in the treatment of industrial wastes (Constantin and Fick 1997). Report reveals that increased fertilization of soils with nitrogen causes a decrease in carbon sequestration (Oren *et al*. 2001).

In the present study, it is observed that both sides of the river of the studied area are rich in chemical, paper, tannery and rubber industry. So, effluent and industrial wastes are mixed in sea sore Sundarban. This is in the line of agreement with the results from isolates collected from Gulf and Persian Sea and several oilfields from Japan and China. Recent studies, demonstrates that streams and rivers receiving nitrogen inputs from urban and agricultural uses are a significant source of nitrous oxide to the atmosphere (Mulholland *et al*. 2008; Beaulieu *et al*. 2011). The sm-sr10 can efficiently utilize engine oil as sole carbon source and degrade it in time dependant manner which is comparable to the report Koma *et al* 2003 and Wang *et al* 2007. Staining of sm-sr10 from liquid culture added with engine oil showed presence of large number of bacteria present surrounding the interface of water oil droplets. Though this bacterium can also degrade nitrobenzene to some extent (data not shown) but more complex hydrocarbons will be tested for biodegradation ability in future experiments.

Our present study demonstrates the existence of a large (~50 kb) plasmid in sm-sr10. One early study demonstrates that several *Halomonas* species harbor large extra chromosomal DNA of 70 kb and 600 kb contributing the adaptability in extreme environment (Argandoña *et al*. 2003), resistance to antibiotics and metals (Choudhury *et al*. 1998). A 108 kb conjugative plasmid has been associated with streptomycin-resistant strains of *P. syringae pv.* (Burr *et al*. 1998). Phylogeny of the 16S rRNA gene sequences revealed that sm-sr10 is a member of the *Gammaproteobacteria* within family *Halomonadaceae* having a relationship with genus *Halomonas*, forming a cluster with *Halomonas aidingensis* Ad-1 (Liu *et al*. 2010), Halomonas sp. C-12 (Liu *et al*. 2010), Halomonas sp. MOLA69 (GenBank Acc. No AM990844.1) and *Halomonas* sp. YI8-47 (FJ428530.1), *Halomonas alimentaria*

YKJ-16 (Yoon *et al*. 2002).

In conclusion, present study has implication in the understanding of the adaptive surveillance mechanism of sm-sr10 in moderately polluted water of Sundarban. The factors responsible for genomic and proteomic stability under high salt conditions are important in protein engineering studies (Jaenicke 1991). Present work is also utilizable for eco-friendly degradation of contaminant like long-chain hydrocarbons (waste car engine oil, base oil or the c-alkane fraction of base oil). The isolated bacterial species can be used for in situ bioremediation of polluted sites as indigenous bacteria.

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