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Diversity of culturable sodium dodecyl sulfate (SDS) degrading bacteria isolated from detergent contaminated ponds situated in Varanasi city, India

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ABSTRACT

In the present investigation, an attempt has been made to isolate and identify SDS-degrading bacteria from different detergent contaminated ponds situated in Varanasi city, UP, India. Initial survey of ponds indicated that these ponds were contaminated with detergents. Employing enrichment technique in minimal medium (PBM) with SDS as a sole carbon source, a total of 24 isolates were recovered from 7 detergent contaminated ponds. Studies on rates of SDS degradation indicated that the rate of SDS degradation varied from 97.2% to 19.6% after 12h incubation under identical conditions. An estimation of alkyl sulfatase activity indicated that the activity varied from 0.168 ± 0.004 to 0.024 ± 0.005 μmol SDS/mg protein/min. Molecular characterization of these isolates was performed on the basis of ARDRA and ERIC PCR, which indicated that these isolates were broadly divided in 8 groups. Some selected isolates were identified on the basis of 16S rDNA sequencing. It was found that these isolates belonged to *Pseudomonas aeruginosa*, *Pseudomonas mendocina*, *Pseudomonas stutzeri*, *Pseudomonas alcaligenes*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas putida* and *Pseudomonas otitidis* respectively. Among these isolates *P. aeruginosa*, *P. putida* and *P. otitidis* have been previously shown to degrade and metabolize SDS, the rest of the isolates appear to be new.

1. Introduction

In India, man-made ponds have been used as an alternate source of drinking water. However, these ponds are also employed for washing of clothes and bathing purposes by washer men and local people (Prakash et al., 2009). Many ponds are situated in vicinity of temples and are used for bathing purposes for people who visit the temples for worshipping and also for disposal of wastes originating from the temples (Sharma et al., 2009). In certain ponds, sewage water and waste water from nearby cottage industries are also discharged. A detailed survey of few ponds located around the city was made by Tyagi et al. (2006), which revealed that most of the ponds are eutrophic. However, data related to amount of detergents present, if any in pond water is lacking. Furthermore, no attempt has been made to study detergent degrading bacteria. In a different study, Ghose et al. (2009), reported the presence of high amount of anionic detergents in surface and underground water in greater Kolkata, India.

Sodium dodecyl sulfate (SDS) is an anionic detergent widely used in household products and in Industry (Karsa, 1992). It has been reported that the presence of anionic detergent especially SDS in environment arises mainly from disposal of domestic and industrial wastes (Fendinger et al., 1994). SDS is shown to be toxic to health and survival of aquatic animals (Ribelles et al., 1995; Rosety et al., 2001; Rocha et al., 2007). It was realized that removal of detergent from environment was a necessity (Singer and Tjeerdema, 1993). Certain bacteria such as *Pseudomonas* sp., *Bacillus cereus*, *Acinetobacter calcoaceticus* and *Pantoea agglomerans* have been isolated which utilize SDS as a carbon source for growth (Payne and Feisal, 1963; Thomas and White, 1989; Singh et al., 1998; Abboud et al., 2007). However, little if any, work has been done on molecular diversity of SDS-degrading bacteria. Recent advances in studies pertaining to microbial ecology have led to the development of new approaches for the characterization of microbial communities (Zhou et al., 1997). Most common PCR-based genomic fingerprinting methods are Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Porteous et al., 1994) and Repetitive (Rep) PCR i.e. Enterobacterial Repetitive Intergenic Consensus (ERIC) elements (Versalovic et al., 1991; Hulton et al., 1991). Although, PCR based fingerprinting methods are suitable for characterization of bacterial communities, however, these techniques cannot be employed for the identification of bacteria (Ventura et al., 2001). 16S rDNA sequence analysis is currently the most widely used method for bacterial identification (Lawongsa et al., 2008). In the

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present study, an attempt has been made to survey various ponds situated in Varanasi city with a view to access the level of anionic detergents in pond water. The next approach was to isolate and screen the SDS-degrading bacteria present in pond water. Molecular diversity among various isolates was accessed by PCR based techniques such as ARDRA and ERIC-PCR. Identification of selected isolates was performed on the basis of 16S rDNA sequencing.

2. Materials and methods

2.1. Chemicals and culture media

Sodium dodecyl sulfate (SDS), methylene blue and other biochemicals were purchased from Sigma–Aldrich, MO, USA. All other inorganic chemicals were of analytical grade and obtained from SISCO Research Laboratories Pvt. Ltd. and HiMedia Pvt. Ltd., Mumbai, India. Luria-Bertani (LB) medium contained (g/l): tryptone 10.0, NaCl 5.0, yeast extract 5.0, pH 7.0. The Phosphate Buffered Medium (PBM) contained (g/l): K_2HPO_4 1.0, KH_2PO_4 1.0, NH_4Cl 1.0, $MgSO_4.7H_2O$ 0.20, NaCl 0.5 and $CaCl_2$ 0.02, pH 7.5. The medium also contained trace elements (1 ml of stock) having (g/l): $FeCl_3 \cdot 6H_2O$ 0.24, $CoCl_2 \cdot 6H_2O$ 0.04, $CuSO_4 \cdot 5H_2O$ 0.06, $MnCl_2 \cdot 4H_2O$ 0.03, $ZnSO_4 \cdot 7H_2O$ 0.31 and $Na_2MoO_4 \cdot 2H_2O$ 0.03. SDS (1 g/l) was added separately after autoclaving the medium.

2.2. Study sites

Detailed survey of various ponds located around Varanasi city, revealed that seven ponds namely, Assi (AS), Sundarpur (SP), Niralanagar (NN), Naipura (N), Kandwa (K), Pisachmochan (PM) and Jawaharnagar (JN) were extensively used for bathing and washing purposes and received either sewage water or industrial effluents or both. All these ponds were found to retain water throughout the year. However, the level of water decreases significantly during summer season.

2.3. Estimation of anionic detergent from pond water

Amount of anionic detergent in water samples was determined by the methylene blue active substance (MBAS) assay (Hayashi, 1975). Calibration curve was prepared by using standard solutions (based on weight) of pure SDS (Sigma–Aldrich, USA).

2.4. Enrichment and Isolation of SDS-degrading bacteria

For the enrichment of SDS-degrading bacteria, 1 ml of pond water was added to 100 ml sterilized PBM supplemented with SDS (1 g/l) in a culture flask and incubated at 30 °C and 120 rpm in a shaker (Orbitek LT, Scigenics Bioteck. Pvt. Ltd., Chennai). After 3–4 days of growth, 1 ml culture was transferred to fresh PBM supplemented with SDS (1 g/l). Repeated sub-culturing (at least 3–4 times) resulted in the enrichment of putative SDS-degrading bacteria. Spreading on solid PBM agar-plates containing SDS as a sole carbon source, resulted in the formation of minute colonies. Based on colony morphology different SDS-degrading bacteria were isolated.

2.5. Test for SDS degradation

Briefly, overnight grown cultures in LB medium were centrifuged at 8000 rpm for 5 min at room temperature, washed with PBM and suspended in 100 ml of PBM containing SDS. Initial OD (at 600 nm) of each isolate was adjusted to approximately 0.025. The cultures were incubated at 30 °C with shaking at 120 rpm. Samples from each flask were removed at desired intervals, and assayed for residual SDS and growth (Ellis et al., 2002).

2.6. Preparation of crude cell extracts

Crude cell extracts were prepared following the method of Ellis et al. (2002). Bacterial cells grown in PBM containing SDS as a sole source of carbon were harvested after mid logarithmic phase of growth by centrifugation at 10,000 rpm for 20 min at 4 °C in a Sorvall RC-5B superspeed refrigerated centrifuge (Du Pont Instruments, USA). Cell pellets were washed twice with 10 mM Tris–HCl by centrifugation and suspended in 10 mM Tris–HCl (pH 7.5). The cells were ruptured by sonication for a total duration of 3 min, consisting of intermittent sonication for 30 s on and 30 s off, operated at level 5 and a 25% duty cycle in a Branson Sonifier 450 (Branson Ultrasonics Corp., USA). To minimize heat inactivation of enzymes, samples were sonicated after placing eppendorf tubes in ice box. Cell debris was removed by centrifugation at 12,000 rpm for 10 min at 4 °C. The cell extracts were stored at −20 °C prior to assay for alkyl sulfatase activity.

2.7. Assay of alkyl sulfatase

Alkyl sulfatase activity in crude cell extracts was estimated as per the method of Ellis et al. (2002). Briefly, crude cell extract (10–50 μl) were incubated at 30 °C in 1 ml of 10 mM Tris–HCl (pH 7.5) containing SDS at a final concentration of 50 μg/ml. Samples (100 μl) were removed at fixed intervals and assayed for residual SDS by MBAS method as described earlier. One unit of enzyme activity was defined as the amount of enzyme which converted 1 μmol of SDS per minute under the assay conditions. The experiments were performed in triplicate and result was presented as mean ± standard deviation.

2.8. Biochemical characterization of SDS-degrading isolates

All isolates were characterized by conventional biochemical tests, i.e. Gram stain, motility, pigment production, growth, presence of enzymes such as catalase, oxidase, urease, nitrate reductase, gelatin and starch hydrolysis (Shaw and Latty, 1982). Utilization of carbohydrates, amino acids was tested in PBM as described by (Kämpfer et al., 1991; Molin and Ternstrom, 1982).

2.9. Isolation of genomic DNA

Genomic DNA of all the isolates was extracted by DNeasy Tissue Kit (Qiagen, Gmbh, Hilden, Germany) according to the instructions of manufacturer. The genomic DNA was electrophoresed on a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH-8.0) containing ethidium bromide (0.5 μg/ml) and visualized in a gel documentation unit (Bio-Rad Laboratories, USA).

2.10. Amplification of full length 16S rDNA

16S rDNA (1.5 kb) was amplified using universal primer pair For 5′-AGA GTT TGA TYM TGG CTC AG -3′, and Rev 5′-CTA CGG CTA CCT TGT TAC GA -3′. Amplification was performed in a final volume of 50 μl. The PCR reaction mix included; 1.5 U of *Taq* DNA polymerase (Bangalore Genei), 1× PCR buffer with 1.5 mM $MgCl_2$, 300 ng of each forward and reverse primers (Integrated DNA Technologies, USA), 125 μM of each dNTP's (Bangalore Genei, India) and 50 ng template DNA. Thermal cycle for the amplification was set as; 3 min at 95 °C, 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C followed by 5 min at 72 °C and storage at 4 °C. Amplification was performed in a PTC-100 Thermal Cyclar (MJ Research, Inc., Walton, USA). The samples were analyzed by agarose (1.5%) gel electrophoresis and visualized on gel documentation unit (Bio-Rad Laborotories, USA).

2.11. Amplification of 16S rDNA (426 bp)

426 bp long 16S rDNA partial fragment was amplified using universal primer pair; For 5′-ACT GGC GGA CGG GTC AGT AA -3′, and Rev 5′-CGT ATT ACC GCG GCT GCT GG -3′. Amplification was performed in a final volume of 50 μl. The PCR reaction mix included 1.5 U of *Taq* DNA polymerase (Bangalore Genei), 1× PCR assay buffer with 1.5 mM $MgCl_2$, 300 ng of each forward and reverse primers (Integrated DNA Technologies, USA), 125 μM of each dNTP's (Bangalore Genei) and 50 ng template DNA. Thermal cycle for the amplification was set as; 3 min at 95 °C, 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min 10 s at 72 °C followed by 5 min at 72 °C and storage at 4 °C. The amplified products were analyzed by agarose (3%) gel electrophoresis.

2.12. Amplified Ribosomal DNA Restriction Analysis (ARDRA)

16S rDNA gene (1.5 kb) amplified by PCR was subjected to restriction digestion using *Taq*1 and *Rsa*1 restriction enzymes, as per the instructions of the manufacturer (New England Biolabs Ltd, UK). Restriction digestion was done in a final volume of 25 μl containing 1× restriction enzyme buffer, 0.125 μl (1.25U) restriction enzyme and 15 μl PCR product. After mixing the reaction mixture carefully, samples were incubated for 3 h in a water bath preset at 37 °C. Reaction was terminated by heat inactivation at 70 °C for 20 min. The samples were analyzed by agarose (3%) gel electrophoresis and visualized on gel documentation unit (Bio-Rad Laborotories, USA).

2.13. ERIC (Enterobacterial Repetitive Intergeneric Consensus) PCR

ERIC-PCR was carried out in 40 μl reaction mixture containing, 1× *Taq* DNA polymerase assay buffer, 125 μM each of dNTPs, 250 ng of each primer (ERIC 1-5′- ATG TAA GCT CCT GGG GAT TCA C-3′ and ERIC 2-5′- AAG TAA GTG ACT GGG GTG AGC G-3′), 1U *Taq* DNA polymerase (Bangalore Genie, India) and 50 ng of template DNA. Amplification of inter-rep elements was done using thermal program set as initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1 min 30s and a final extension at 72 °C for 5 min. The reaction mixture was stored at 4 °C until use. The amplified products were analyzed by agarose (3%) gel electrophoresis.

2.14. Similarity analysis and clustering

Data of biochemical characterization of SDS-degrading isolates, restriction pattern of 16S rDNA and ERIC-PCR pattern of all the SDS-degrading isolates was used for the construction of dendogram with the help of $NTSYS_{pc}$ software 2.11a (Exeter Software, USA) (Rohlf, 1997). Every positive test of biochemical data was scored as 1 and negative test was scored as 0. Similarly, the bands arising from restriction digestion and those arising from amplification as in ERIC-PCR were scored for each isolate as 1 (band present) or 0 (band absent). A similarity matrix was generated from the pooled binary data using SIMQUAL module of the $NTSYS_{pc}$ software. Dice coefficient was used to derive similarity among isolates.

The similarity matrix thus generated was used for cluster analysis by unweighted pair group method of arithmetic average (UPGMA) using sequential, agglomerative, hierarchial, nested clustering module of $NTSYS_{pc}$ Software. The output data were graphically presented as a phenetic tree.

2.15. 16S rDNA sequencing and identification of bacteria

Amplified full length (1.5 kb) and partial (426 bp) 16S rDNA fragments were purified by Invitrogen kit (Invitrogen Corp., USA) following the instructions of manufacturer. The PCR product was eluted in Milli Q water and purity and concentration of DNA were checked by reading OD at 230, 260 and 280 nm. The sequencing PCR reaction mix (DNA sequencing kit, Applied Biosystems, USA) included 8.0 μL Bigdye Terminator V3.0 cycle sequencing ready reaction mixture with ApliTaq, 10 ng purified PCR product and 3.2 pmole forward primer (5′-ACT GGC GGA CGG GTC AGT AA -3′). Total volume was adjusted to 20 μl by adding deionised water. The thermal cycle conditions were set as; initial denaturation at 98 °C for 1 min, 25 cycles of denaturation at 96 °C for 10 s, annealing at 55 °C for 5s and extension at 60 °C for 4 min and hold at 4 °C. Ethanol precipitation was done for the removal of unused BigDye Terminator present in the reaction mixture. After drying the samples, 20 μl of TSR (template suppression reagent) was added, mixed well and heated for 2 min at 95 °C. Subsequently, the samples were chilled on ice and after vortexing thoroughly, centrifuged briefly in a microcentrifuge. Samples were hold in ice until loading. Sequencing was performed in an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems, USA). PCR and direct sequencing were performed at least twice to determine and confirm the DNA sequences for each isolate. 16S rDNA sequencing with partial 16S rDNA fragment (426 bp) was performed in four isolates namely SDS1, SDS2, K1, and JN2. In 15 selected isolates, full length (1.5 kb) 16S rDNA fragment was directly sequenced. The nucleotide sequences obtained from partial 16S rDNA fragment were in the range of 201–397 bp and sequences obtained from full length 16S rDNA fragment were in the range of 795–956 bp.

2.16. Nucleotide sequence accession numbers

Full length (1.5 kb) 16S rDNA fragments were directly sequenced using forward primer. The nucleotide sequences obtained from full length 16S rDNA fragment were in the range of 795–956 bp. All the sequences were matched against nucleotide sequences present in Gen Bank using BLASTn program (Altschul et al., 1990). The nucleotide sequences of partial 16S rDNA of all the isolates were submitted to NCBI and accession numbers for all the strains have been obtained (Table 3).

2.17. Construction of phylogenetic trees based on 16S rDNA sequences

16S rDNA sequences of selected 15 SDS-degrading isolates and 37 representative sequences of reference strains from NCB1 database were used to construct phylogenetic tree. The sequences belonging to isolates SDS1, SDS2, K1, and JN2 were very small as compared to rest of the 15 isolates, so, these sequences were not included in the phylogenetic tree. Multiple alignment of sequences were carried out using ClustalW2 (version 2.0.10). Neighbor joining tree was constructed in MEGA 4.0 (Saitou and Nie, 1987) using bootstrapping at 1000 bootstraps trials with the two- parameters model of Kimura.

3. Results

3.1. Concentration of anionic detergent in pond water

Initial survey showed that due to human activities, the ponds were highly contaminated. It was desirable to study amount of anionic detergent in pond water. From the data of Table 1, it is evident that amount of anionic detergent was at higher level in all the ponds studied. The level of anionic detergent varied from 5.5 to 9.5 mg/l. The highest level was observed in pond situated in Assi (9.5 mg/l) and lowest amount was observed in pond situated in Naipura (5.5 mg/l).

Table 1
Enrichment of SDS-degrading bacteria from different SDS contaminated ponds situated in Varanasi city, their alkyl sulfatase activity and % degradation after 12 h incubation.

Name of the pond	Amount of anionic detergent in Pond water(mg/l)	Isolates	Alkyl sulfatase specific activity (μmol SDS/mg protein/min)	% degradation after 12 h of incubation
Assi	9.5 ± 0.3	SDS1	0.142 ± 0.004	89
		SDS2	0.144 ± 0.005	88
		SDS3	0.168 ± 0.004	97.2
Naipura	5.5 ± 0.2	N1	0.136 ± 0.003	80.2
		N2	0.088 ± 0.006	78.3
		N3	0.085 ± 0.007	60.2
Kandwa	8.9 ± 0.4	K1	0.069 ± 0.003	50
		K2	0.067 ± 0.002	54
		K3	0.066 ± 0.003	49
		K4	0.059 ± 0.003	38.2
Pisachmochan	7.4 ± 0.3	PM1	0.146 ± 0.004	90
		PM2	0.124 ± 0.003	87.1
		PM3	0.129 ± 0.005	83
		PM4	0.088 ± 0.007	47.2
Niralanagar	9.2 ± 0.2	NN1	0.154 ± 0.003	89
		NN2	0.085 ± 0.005	74.3
		NN3	0.084 ± 0.007	62
		NN4	0.160 ± 0.004	91
Sundarpur	8.2 ± 0.4	SP1	0.046 ± 0.005	47
		SP2	0.083 ± 0.006	73.7
		SP3	0.087 ± 0.004	65
Jawaharnagar	7.0 ± 0.3	JN1	0.103 ± 0.006	72.6
		JN2	0.068 ± 0.004	45.5
		JN3	0.024 ± 0.005	19.6

3.2. Enrichment of SDS-degrading bacteria

Employing enrichment technique, 24 distinct isolates of bacteria were recovered from 7 ponds. It is evident from the data of Table 1, that 3–4 morphotypes were present in water of each pond. Various morphotypes were selected on the basis of distinct morphology, color, colony shape, appearance and pigment production. All these isolates were grown repeatedly in PBM supplemented with SDS, so as to confirm their potential to utilize SDS as the sole source of carbon. These isolates were routinely maintained on PBM agar-plates containing SDS (1 g/l) as a sole source of carbon.

3.3. Kinetics of SDS degradation

A typical representation of SDS degradation along with growth of selected isolates is shown in Figs. 1 and 2. For comparison of SDS degradation rates, percent degradation of SDS after 12 h of incubation under identical conditions was calculated. It is evident from the data that the rate of SDS degradation varied significantly in different isolates (Table 1). The highest rate of SDS degradation was noted in isolates SDS3, followed by NN4 and PM1. In isolate SDS3, 97.2% SDS was degraded after 12 h of incubation. The lowest rate of SDS degradation was observed in isolate JN3 where 19.6% SDS was degraded under identical conditions. In isolates, where rate of degradation was slow, a long initial lag phase was observed. Here it was observed that the cells formed macroscopic aggregates. However, these macroscopic aggregates gradually disappeared as SDS degradation proceeded.

3.4. Alkyl sulfatase activity

Once it became apparent that all the isolates indeed metabolize SDS and the rate of degradation varies significantly among different isolates, it was essential to study the activity of alkyl sulfatase, the key enzyme involved in the SDS biodegradation. Alkyl sulfatase activity in all the isolates was measured during mid-exponential phase of growth. Data of Table 1 represent alkyl sulfatase specific activity of all the isolates employed in this study. The specific activity of enzyme ranged from 0.024 ± 0.005 to 0.168 ± 0.004 μmol SDS/mg protein/min. The highest activity was noted in SDS3 (0.168 ± 0.004) followed by NN4 (0.160 ± 0.004), lowest activity was present in isolate JN3 (0.024 ± 0.005). It is also evident from the finding that isolates having higher alkyl sulfatase activity were capable to degrade SDS much faster (Table 1).

3.5. Biochemical characterization of SDS-degrading bacteria

Initial characterization of these isolates was performed on the basis of certain biochemical tests which are routinely employed of characterization/identification of bacteria. These tests indicated that all the isolates were gram negative, motile and showed positive tests for catalase. Few isolates showed production of blue/green fluorescent pigments, whereas few isolates showed production of brown and yellow pigments (Table 2). However, other tests revealed considerable variation among the isolates.

A dendrogram was constructed based on the results of the biochemical data (Fig. 3). It was observed that all the 24 isolates formed 9 broad groups with various sub groups. Group 1 contained isolates SDS1, SDS2, SDS3 and N1 in 2 sub groups, Group 2 contained isolates PM1, PM2, PM3 and SP1 in 2 sub groups, Group 3 contained isolates NN1 and NN4, Group 4 contained isolates N3, NN3, and SP3 in 2 sub groups, Group 5 contained isolates K1, K2, K3 and K4 in 2 sub groups, Group 6 contained isolates PM4 and JN2, Group 7 contained isolate JN1, Group 8 contained JN3, Group 9 contained isolates N2, NN2 and SP2.

3.6. Diversity based on ARDRA

A typical representation of ARDRA pattern after full digestion with *Rsa*1 and *Taq*1 restriction enzymes of 24 SDS-degrading isolates is shown in Fig. 4 and Fig. 5. It is evident from the gel photograph that digestion with *Rsa*1 and *Taq* 1 yielded 5 to 8 bands. On the basis of restriction profile generated by two restriction enzymes namely *Rsa*1 and *Taq*1, all the isolates could be placed in 10 groups. Group 1 contained 4 isolates; SDS1, SDS2, SDS3, N1, group 2 contained NN4 and SP3, group 3 contained NN1, JN1, JN2 and PM4, group 4 contained NN3, group 5 contained PM1, PM2, PM3, group 6 contained N2 and NN2, group 7 contained N3 and SP2, group 8 contained K1, K2, K3, and K4, group 9 contained JN3 and group 10 contained SP1 (Fig. 6).

3.7. Diversity based on ERIC PCR

Fig. 7 shows a typical representation of ERIC–PCR profile of 24 SDS-degrading isolates. Banding pattern of different fragments clearly reflects diversity among isolates. Dendrogram constructed on the basis of ERIC-PCR profile placed all the isolates in 9 groups (Fig. 8). Isolates SDS1, SDS2, SDS3 and N1 formed group 1, isolates PM1, PM2, PM3 and SP1 formed the group 2, isolates K1, K2, K3 and K4 formed group 3, isolates JN1, JN2, PM4 formed group 4, isolate NN1 and NN4 formed the group 5, isolate N3 formed the group 6, NN3 and SP3 formed the group 7, isolate N2, NN2 and SP2 formed group 8 and isolate JN3 formed the group 9.

3.8. Identification based of 16S rDNA sequencing

Data presented in Table 3 shows identification of selected SDS-degrading isolates on the basis of partial 16S rDNA sequencing. Results clearly show matching with the results of ARDRA and ERIC-PCR. A phylogenetic tree was constructed with our sequences and

Table 2
Biochemical characterization of SDS-degrading bacteria.

Isolates												
Characteristic	SDS1	SDS2	SDS3	N1	N2	N3	PM1	PM2	PM3	PM4	JN1	JN2
Grams test	−	−	−	−	−	−	−	−	−	−	−	−
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Fluorescent diffusible pigments	+	+	+	+	−	−	−	−	−	−	−	−
Non- diffusible, non fluorescent pigments	−	−	−	−	−	−	−	−	−	−	−	−
Diffuasible non- fluorescent pigments	+ blue-green	+ blue-green	+ blue-green	+ blue-green	−	−	+ brown	+ brown	+ brown	−	−	−
Urease test	−	−	−	−	+	+	−	−	−	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	−	+	+	+	+	+	+
Nitrate reductase	+	+	+	+	+	+	−	−	−	+	+	+
Gelatin hydrolysis	+	−	+	−	−	−	−	−	−	−	−	−
Starch hydrolysis	+	+	+	+	−	−	−	+	−	+	−	−
Malonate	+	+	+	+	−	+	+	+	+	−	−	−
Growth at 5% NaCl	+	+	+	+	+	+	+	+	+	−	−	−
Utilization of												
Glucose	+	+	+	+	−	+	+	+	+	−	−	−
Sucrose	−	−	−	−	−	+	−	−	−	−	−	−
Fructose	+	−	+	−	−	+	+	+	+	−	+	−
Malic acid	+	+	+	+	+	−	+	+	+	+	+	+
D-Ribose	+	+	+	+	−	+	−	−	−	−	−	−
Maltose	−	−	−	−	−	−	−	−	−	−	−	−
Glycerol	+	+	+	+	−	−	+	+	+	+	+	+
Gycine	+	−	+	−	−	+	+	+	+	−	−	−
L-Leucine	+	−	+	−	−	+	+	+	+	+	+	+
L-Valine	+	−	+	−	−	+	+	+	+	+	−	+
L-Serine	+	+	+	+	+	+	+	+	+	−	−	+
L-Histidine	+	+	+	+	+	+	+	+	+	+	−	+
L-Aspartate	+	+	+	+	−	+	+	+	+	−	+	+
L-Glutamate	+	+	+	+	−	+	+	+	+	+	+	+
Sarcocine	+	+	+	+	−	+	+	+	+	−	−	−
Ethanolamine	+	+	+	+	+	+	−	−	−	−	−	−
Adipate	+	+	+	+	−	−	−	−	−	+	+	+
Isolates												
Characteristic	JN3	K1	K2	K3	K4	NN1	NN2	NN3	NN4	SP1	SP2	SP3
Grams test	−	−	−	−	−	−	−	−	−	−	−	−
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Fluorescent diffusible pigments	−	−	−	−	−	−	−	−	−	−	−	−
Non- diffusible, non fluorescent pigments	−	−	−	−	−	−	−	−	−	−	−	−
Diffuasible non- fluorescent pigments	−	+ yellow	+ yellow	+ yellow	+ yellow	−	−	−	−	+ brown	−	−
Urease test	−	+	+	+	+	+	+	+	+	−	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	−	+	+	+	−
Nitrate reductase	+	+	+	+	+	+	+	+	−	−	+	+
Gelatin hydrolysis	−	−	−	−	−	+	−	−	+	−	−	−
Starch hydrolysis	−	+	+	+	+	+	−	−	+	−	−	−
Malonate	+	+	+	+	+	+	−	+	+	+	−	+
Growth at 5% NaCl	+	+	+	+	+	−	+	−	−	+	+	−
Utilization of												
Glucose	+	+	+	+	+	+	−	+	+	+	−	+
Sucrose	−	+	+	+	+	−	−	+	−	−	−	+
Fructose	−	+	+	+	+	−	+	+	−	+	−	+
Malic acid	+	−	−	−	−	−	+	−	−	+	+	−
D-Ribose	−	−	−	−	−	−	−	+	−	−	−	+
Maltose	−	−	−	−	−	−	−	−	−	−	−	−
Glycogen	−	+	+	+	+	+	−	−	+	+	−	−
Gycine	−	+	+	−	−	+	−	+	+	+	−	+
L-Leucine	+	+	+	+	+	+	−	+	+	+	−	+
L-Valine	+	+	+	+	+	+	−	+	+	+	−	+
L-Serine	−	−	−	−	−	+	+	+	+	+	−	+
L-Histidine	+	−	−	−	−	+	+	+	+	+	+	+
L-Aspartate	+	+	−	+	+	+	−	+	+	+	−	+
L-Glutamate	+	+	+	+	+	+	+	+	+	+	+	+
Sarcocine	−	−	−	−	−	−	−	+	−	+	−	+
Ethanolamine	−	−	−	−	−	−	+	+	−	−	+	+
Adipate	+	−	−	−	−	−	−	−	−	−	−	−

Table 3
Identification of SDS-degrading isolates on the basis of 16S rDNA sequencing.

Isolates	Identification	Accession no.	Percentage similarity	Length (bp)
SDS1	*P. aeruginosa*	EF197938	99%	332
SDS2	*P. aeruginosa*	EF471354	99%	201
SDS3	*P. aeruginosa*	EF197939	98%	850
N1	*P. aeruginosa*	HM627519	99%	894
N2	*P. pseudoalcaligenes*	HM854825	99%	944
K2	*P. stutzeri*	GQ328718	99%	234
K3	*P. stutzeri*	HM756165	96%	914
K4	*P. stutzeri*	HM756166	99%	944
PM1	*P. mendocina*	HM627520	99%	956
PM2	*P. mendocina*	HM627521	99%	924
PM3	*P. mendocina*	HM627522	99%	899
PM4	*P. alcaligenes*	HM854826	98%	946
NN1	*P. otitidis*	HM756163	97%	883
NN2	*P. pseudoalcaligenes*	HM756167	96%	824
NN4	*P. otitidis*	HM756164	98%	873
SP2	*P. pseudoalcaligenes*	HQ005306	99%	944
SP3	*P. putida*	HQ005307	99%	795
JN1	*P. alcaligenes*	HM756168	98%	887
JN2	P. alcaligenes	GQ328720	99%	397

with representative sequences of reference strains from NCBI database. The phylogenetic tree is shown in Fig. 9. It is evident from phylogenetic tree that isolate SDS3 (Accession number EF197939) showed 98% similarity with *Pseudomonas aeruginosa* strain R1 (Accession number GU220062.1) and N1 (Accession number HM627519) showed 99% similarity with *P. aeruginosa* strain SZH16 (Accession number GU384267.1), K3 (Accession number HM756165) showed 96% similarity with *Pseudomonas stutzeri* strain BRP83 (Accession number GU396287.1) and K4 (Accession number HM756166) showed 99% similarity with *P. stutzeri* strain FM22(Accession number DQ289075.1). Isolate PM1 (Accession number HM627520) showed 99% similarity with *Pseudomonas mendocina* strain 8-40B28 (Accession number EU927412.1), isolates PM2 (Accession number HM627521) and PM3 (Accession number

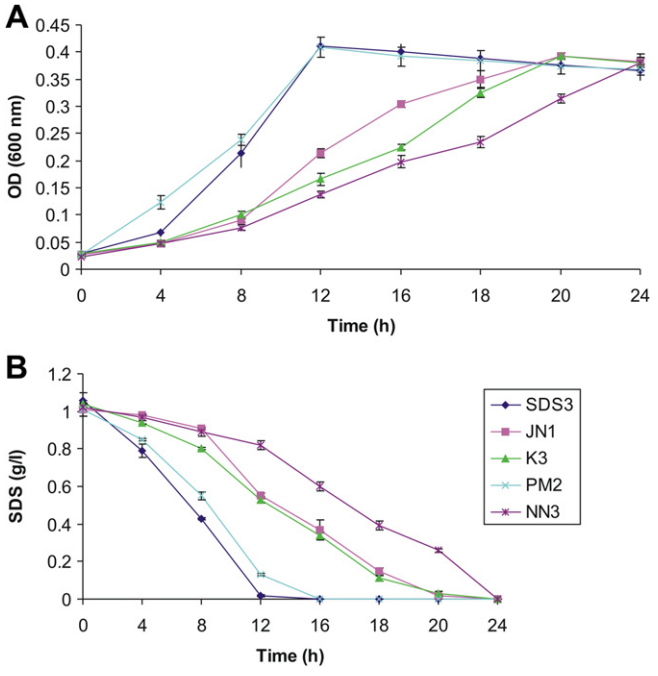

Fig. 1. Time course study of degradation of SDS in selected isolates in PBM supplemented with SDS (1 g/l).

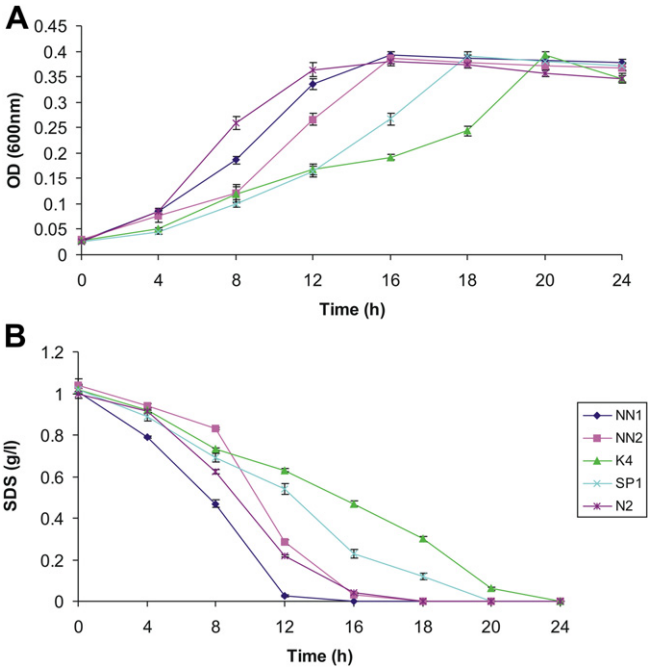

Fig. 2. Time course study of degradation of SDS in selected isolates in PBM supplemented with SDS (1 g/l).

HM627522) showed 99% similarity with *P. mendocina* strain DS0601-FX (Accession number FJ840535.1). Isolate JN1 (Accession number HM756168) and PM4 (Accession number HM854826) showed 98% similarity with *Pseudomonas alcaligenes* strain S2 (Accession number AY651922.1). Isolate N2 (Accession number HM854825) showed 99% similarity with *Pseudomonas pseudoalcaligenes* strain JM8 (Accession number FJ472860.1), NN2 (Accession number HM756167) and SP2 (Accession number HQ005306) showed 96% similarity with *P. pseudoalcaligenes* strain JM5 (Accession number FJ472857.1). Isolates SP3 (Accession number HQ005307) showed 98% similarity with *Pseudomonas putida* strain AKMP7 (Accession number GU396282.1). Isolate NN1 (Accession number HM756163) showed 97% similarity with *Pseudomonas otitidis* strain RW1 (Accession number GU204968.1) and NN4 (Accession number HM756164) showed 98% similarity with *P. otitidis* strain TNAU 45 (Accession number GQ339108).

In addition to this, SDS1 (Accession number EF197938) showed 99% similarity with *P. aeruginosa* strain T-1 (Accession number JN000304), SDS2 (Accession number EF471354) showed 99% similarity with *P. aeruginosa* strain RCTy5 (Accession number JN002387), K2 (Accession number GQ328718) showed 99% similarity with *P. stutzeri* ATCC 17588 (Accession number CP002881) and JN2 (Accession number GQ328720) showed 99% similarity with *P. alcaligenes* strain JXZ-6 (Accession number JF496555) respectively.

4. Discussion

Due to their excellent cleansing ability, long-chain aliphatic sulfate esters such as sodium dodecyl sulfate (SDS) are commonly used in surfactant formulations and are frequently discharged into wastewater (Fendinger et al., 1994). Therefore, removal of detergents is required from the environment to avoid pollution. This will make their application safer and widespread (Zeng et al., 2007). There are several reports of isolation and characterization of SDS-degrading bacteria from different parts of the world

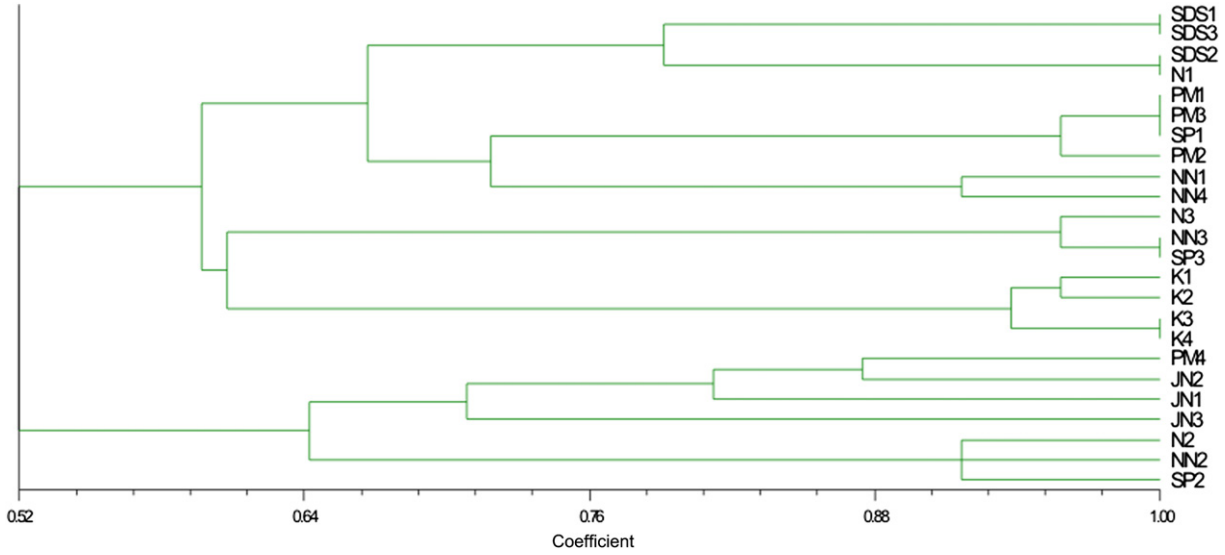

Fig. 3. Dendrogram based on Biochemical characterization data.

(Abboud et al., 2007; Hosseini and Malekzadeh, 2007; Shukor et al., 2009) but unfortunately, little if any report of isolation of SDS-degrading bacteria from India has been made (Singh et al., 1998). To address this problem, we have made an attempt to isolate SDS-degrading bacteria from detergent polluted ponds situated in Varanasi city, India. These ponds were used extensively for washing of clothes and also for bathing purposes. In this study, seven ponds situated in various parts of Varanasi city were screened. Amount of anionic detergent present in pond water was evaluated. As anticipated, the concentration of anionic detergent in pond water was moderately high in all the ponds, the reason being extensive use of detergents and the fact that ponds

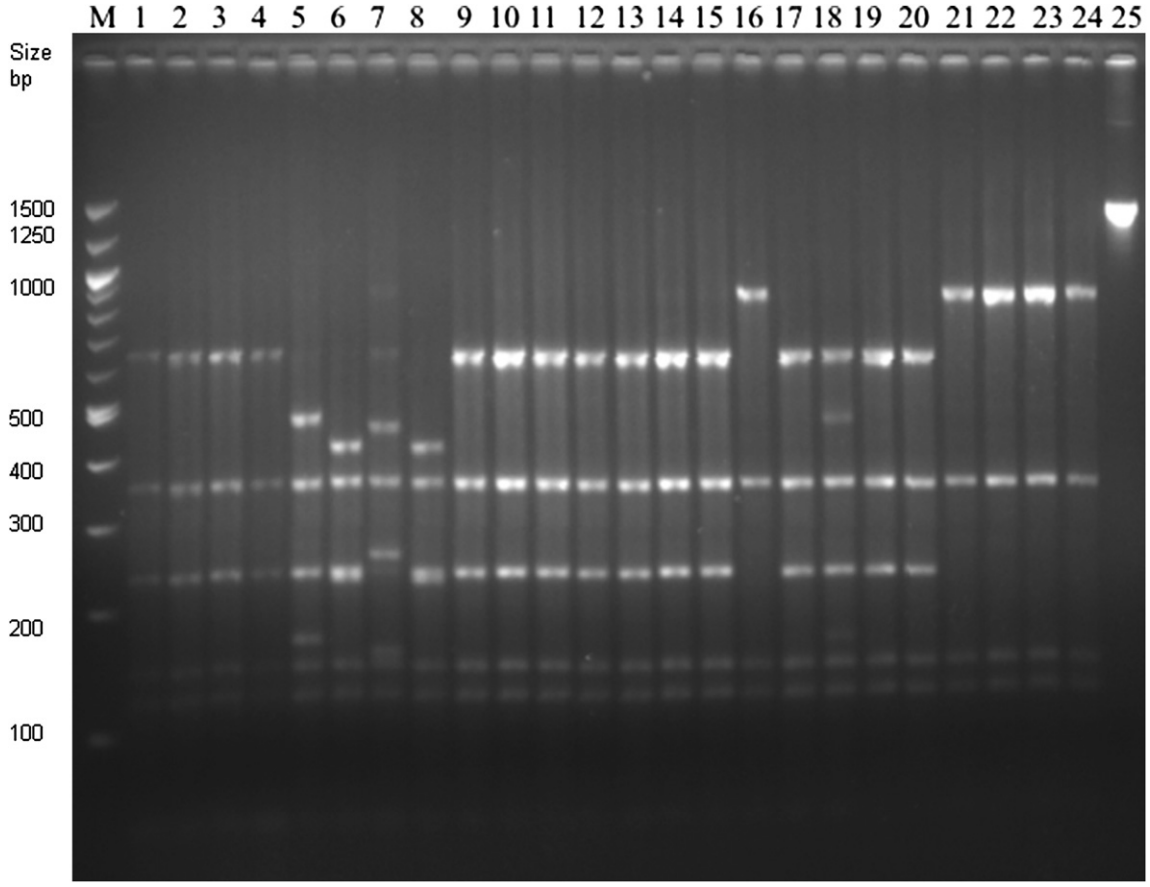

Fig. 4. Restriction digestion of 1.5 kb 16S rDNA gene fragment of SDS-degrading isolates by *Rsa*1. Lane M- 100bp Marker,1-SDS1, 2-SDS2, 3-SDS3, 4-N1, 5-N2, 6-N3, 7-SP1, 8-SP2,9-SP3, 10- PM1, 11-PM2, 12-PM3, 13-PM4, 14-JN1, 15-JN2, 16-JN3, 17–NN1, 18-NN2 19-NN3, 20-NN4, 21-K1, 22-K2, 23-K3, 24-K4, 25- Undigested product.

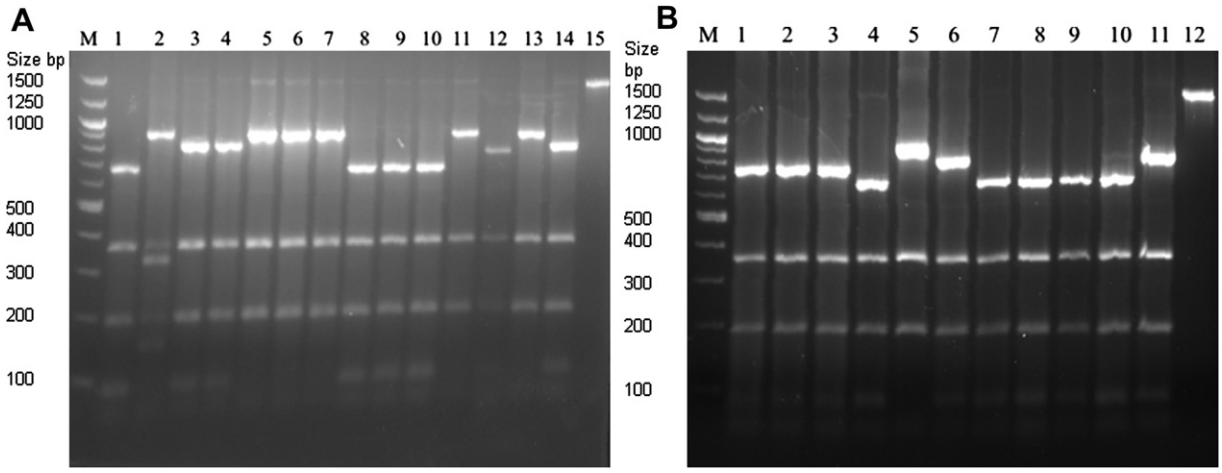

Fig. 5. Restriction digestion of 1.5 kb 16S rDNA gene fragment by *Taq*1. (A) Lane M- 100bp ladder, 1–NN1,2-SP1, 3-SP2, 4-SP3, 5-PM1, 6-PM2, 7-PM3, 8-PM4, 9-JN1, 10-JN2, 11-JN3, 12-N1, 13-N2, 14-N3, 15-Undigested product.(B) Lane M- 100bp ladder,1-SDS1, 2-SDS2, 3-SDS3, 4-NN2, 5-NN3,6-NN4, 7-K1, 8-K2, 9-K3, 10-K4, 11-N3,12-Undigested product.

constitute a closed system, where chemicals tend to accumulate with time. Also, seasonal factors like rainfall and drought drastically effect the concentration of chemicals in the pond water. This system is different from rivers, which is an open system. The chemicals entering eventually get distributed with flowing water (Fendinger et al., 1994). In another study, measurement of sewage treatment plant, indicated that concentrations of anionic detergents in waste water are typically in the 1–10 ppm range (Fendinger et al., 1994). A concentration of 4–24 ppm in raw sewage and 0–14.1 ppm in river water has been also detected. Levels of 1–25 mg anionic detergent per kg of river sediment have been recorded (Ladle et al., 1989). However, there is no data available regarding concentration of anionic detergent in pond water. Our data indicate that concentration of anionic detergent in pond water ranged from 5.5 to 9.5 mg/l (5.5–9.5 ppm), which was moderately high. A similar result was shown by Ghose et al. (2009), where high levels of anionic detergent were observed in surface and ground waters of Kolkata region. Presence of anionic detergent in pond water aroused our interest to screen and isolate SDS-degrading bacteria. Using enrichment technique in PBM, 24 SDS-degrading bacteria were isolated from 7 detergent contaminated ponds. In every pond, there was presence of SDS-degrading bacteria indicating that this detergent was easily biodegradable. Rates of SDS degradation in all the isolates were tested in PBM supplemented with SDS (1 g/l). The results showed that these isolates showed varying rates of SDS biodegradation. The time required for complete degradation of SDS ranged from 12 to 24 h. Hosseini and Malekzadeh, 2007, have isolated two isolates belonging to *Pseudomonas betelli* and *Acinetobacter johnsoni* capable of metabolizing SDS. In another study, *Pseudomonas* sp. ATCC19151 has been shown to degrade as high as 0.5% SDS (w/v) (Jovcic et al., 2009). Shukor et al. (2009) have isolated a strain

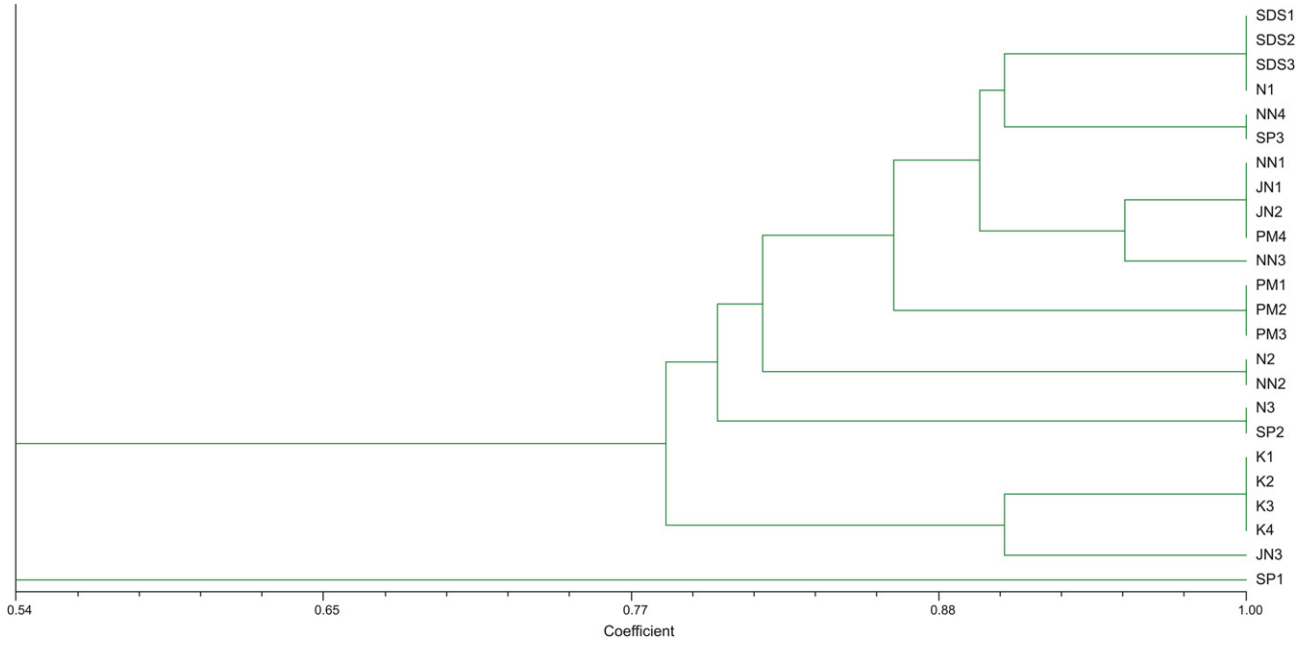

Fig. 6. Dendrogram based on ARDRA pattern.

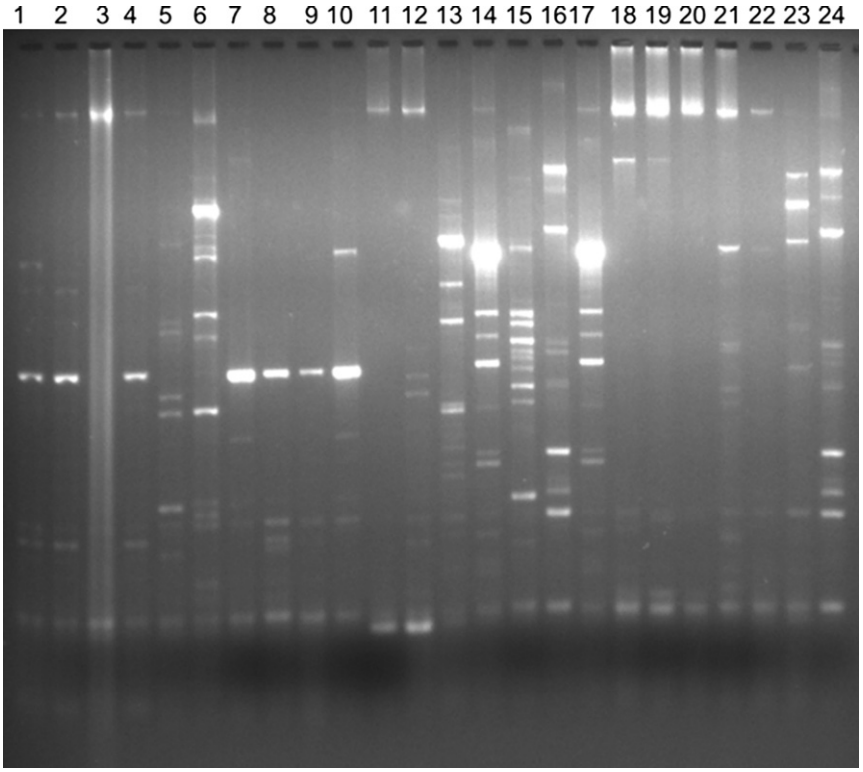

Fig. 7. ERIC PCR profile of SDS-degrading isolates. Lane1. SDS1, 2. SDS2, 3. SDS3, 4. N1, 5. N2 6. N3, 7. K1, 8. K2, 9. K3, 10. K4, 11. JN1, 12. JN2, 13. JN3, 14. NN1, 15. NN2, 16. NN3, 17. NN4, 18. PM1, 19. PM2, 20. PM3, 21. PM4, 22. SP1, 23. SP2, 24. SP3.

belonging to *Klebsiella oxytoca* from SDS polluted water samples from Malaysia. This isolate was able to degrade approximately 80% of 2.0 g/l SDS after 4 days of incubation with concomitant increase in cellular growth.

It has been previously reported that SDS degradation was initiated by the enzyme alkyl sulfatase that catalyze hydrolytic cleavage of the ester bond of SDS and liberates inorganic sulfate (Harada, 1964). The corresponding alcohol is further degraded (Dodgson et al., 1982) or enters in to lipid biosynthesis pathway (Thomas and White, 1989). The results of rates of SDS degradation indicated that all the isolates showed varying rates of SDS degradation. So, alkyl sulfatase activity in these isolates was evaluated. Alkyl sulfatase activity in these isolates ranged between 0.024 ± 0.005–0.168 ± 0.004 μmol SDS/mg protein/min. Varying

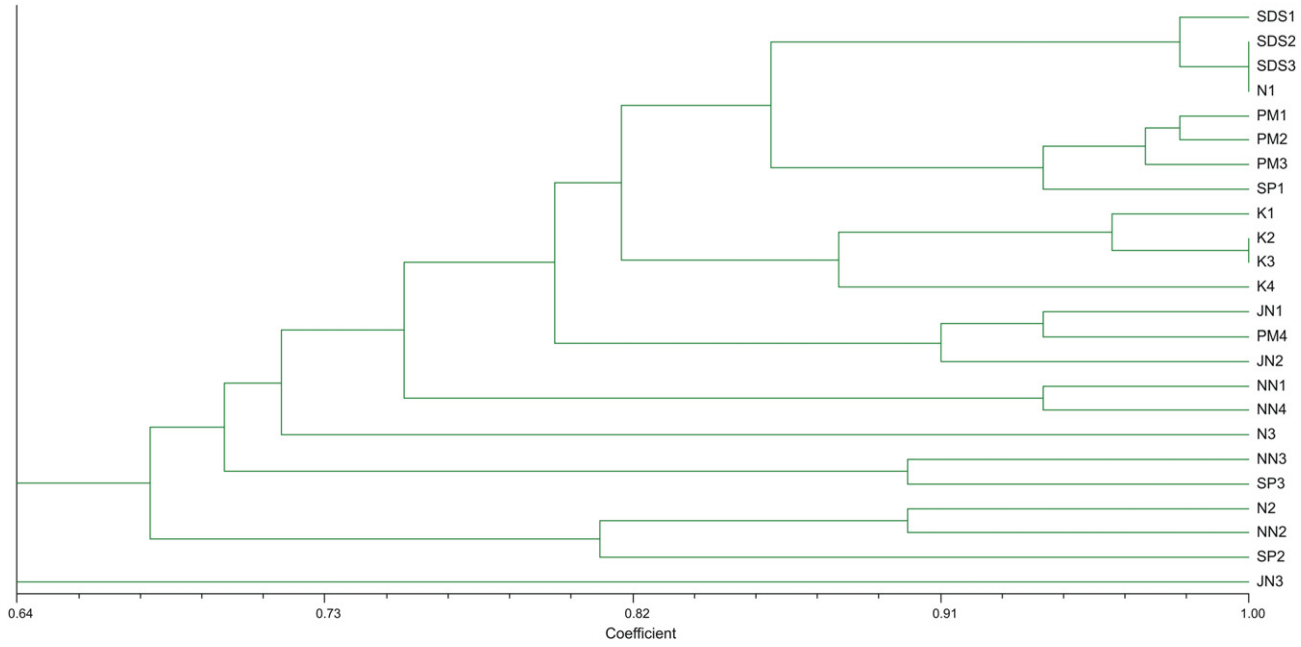

Fig. 8. Dendrogram based on ERIC PCR pattern.

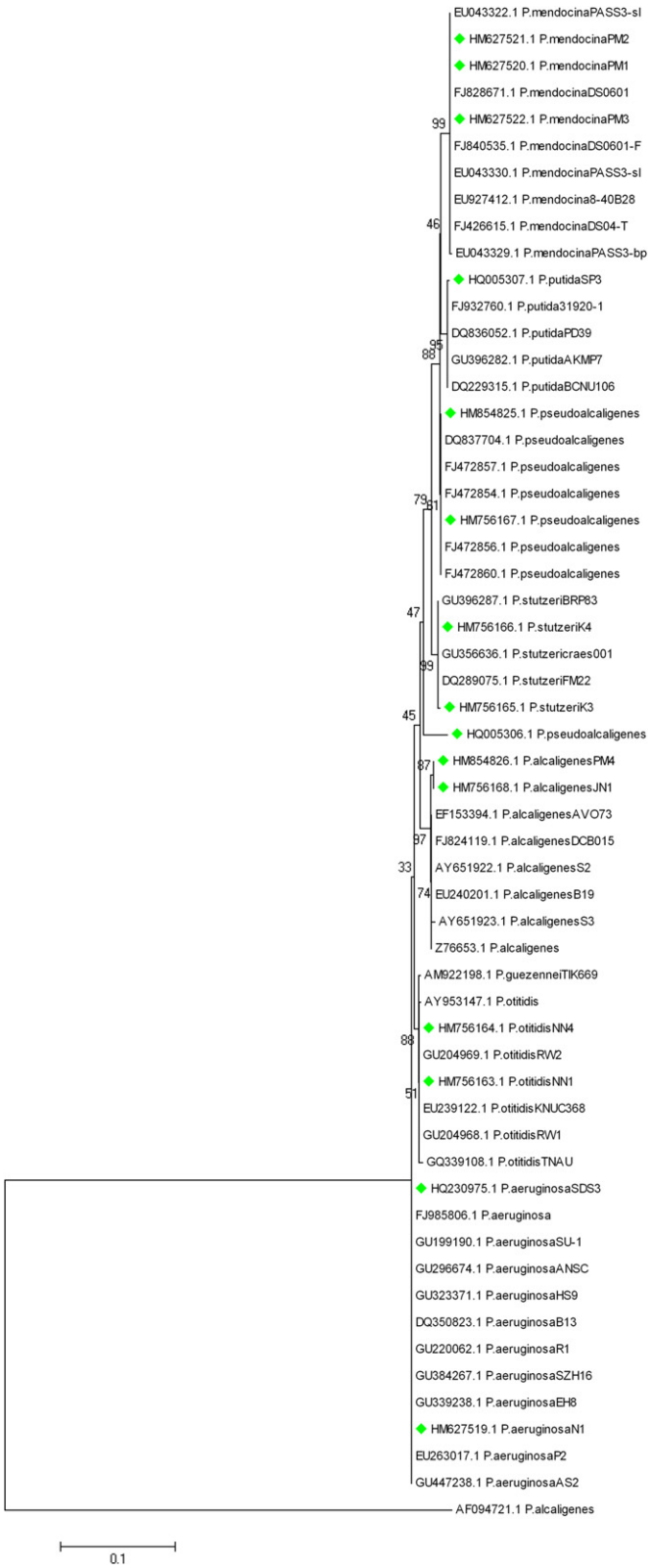

Fig. 9. Phylogenetic tree of 15 SDS-degrading strains and related organisms based on 16S rDNA sequences.

levels of alkyl sulfatase activity indicated that these isolates may harbor different alkyl sulfatases.

All the SDS-degrading isolates were initially characterized by employing various biochemical tests. The result indicated that all these isolates belonged to 9 groups with different sub groups. Further, molecular diversity among SDS-degrading bacteria was studied by ARDRA and ERIC PCR. Results of ARDRA showed that these isolates formed 10 groups. Similar to our study, Molecular characterization of plant growth promoting *Pseudomonads* has been done using various restriction enzymes (Lawongsa et al., 2008), molecular characterization of *Pseudomonas fluorescens* was also performed (Browne et al., 2009). ARDRA has also been successfully employed for characterization of *Bifidobacterium* sp. (Ventura et al., 2001)

ARDRA data did show genetic diversity among all the 24 isolates, however rDNA based fingerprinting is not very useful tool for discrimination of bacteria above strain level, as it based on a single locus i.e. 16S rDNA. Therefore multi-locus analysis like ERIC-PCR is supposed to be a preferred method for rapid study of molecular polymorphism among bacterial isolates. In general the distribution of repetitive sequences (ERIC) has been employed in elucidating the genomic diversity in a number of bacteria (Selenska-Pobell et al. 1995). In this study, the bacterial isolates were analyzed by ERIC-PCR. Presence of 2–14 bands was noted. Reproducibility of the ERIC profiles was evident by the fact that identical banding pattern was obtained when replicate samples were run together. ERIC–PCR profile of 24 SDS-degrading isolates showed that these isolates formed 8 groups. Similar to our approach, other workers have applied ERIC PCR for studying the bacterial diversity of *Rhizobium meliloti* strains (de Bruijn, 1992) and putative endophytic bacteria isolated from seedlings of rice plants (Stoltzfus et al., 1997).

Identification of selected isolates was performed by 16S rDNA sequencing. Results showed that these isolates belonged different species of *Pseudomonas* group namely *P. aeruginosa*, *P. mendocina*, *P. stutzeri*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. putida* and *Pseudomonas otitidis* respectively. These results were in accordance with majority of reports, where members of *Pseudomonas* sp. were implicated in SDS degradation (Payne and Feisal, 1963). Among these isolates, *P. otitidis* (Jovcic et al., 2009), *P. putida* S-313 (Kahnert and Kertesz, 2000), *P. aeruginosa* PA01 (Hagelueken et al., 2006) have been implicated in SDS degradation and their corresponding alkyl sulfatase has been characterized. The rest of the species namely, *P. stutzeri*, *P. mendocina*, *P. alclaigenes* and *P. pseudoalcaligenes* are reported here for the first time involved in degradation and metabolism of SDS. However, these isolates have been reported to degrade and metabolize a wide variety of xenobiotic compounds. It has been reported that *P. stutzeri* OX1 could degrade Tetrachloroethylene (PCE) (Ryoo et al., 2000); it also oxidizes TCE, 1,1-dichloroethylene (1,1-DCE), cis-DCE, trans-1,2-dichloroethylene (trans-DCE) and chloroform (Chauhan et al., 1998; Shim and Wood, 2000). *P. mendocina* NSYSU, was able to degrade and metabolize pentachlorophenol (Kao et al., 2005). Another strain of *P. mendocina* namely KR1 has been shown to degrade Trichloroethylene (Elkarmi et al., 2008). *P. otitidis* WL-13 had high capacity to decolorize triphenylmethane dyes (Jing et al., 2009).

However, this is a preliminary report; a detailed study is study on degradation of SDS is required to elucidate the kinetics of SDS degradation and to characterize the enzymes involved in SDS degradation.

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