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RESEARCH ARTICLE

BIOFOULING ACTIVITY OF *VIBRIO* SPP ISOLATED FROM POWER PLANT PIPES

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ABSTRACT

Totally ten bacterial isolates were obtained and screened for biofouling activity. Two isolates DPR1 and DPB1 showed significant biofouling activity in the tube method and microtitre plate assay. These two isolates showed similarity with *Vibrio sp.* on morphological, biochemical and molecular characteristics. The 16S rDNA sequence of the one bacterial isolate was performed and identified as *Vibrio* sp. DPR1 using Phylogenetic analysis. The 16S rDNA secondary structure and restriction sites of *Vibrio* sp. DPR1 were also studied. This investigation clearly revealed the presence of biofouling bacteria anchored in the thermal power plant pipe. *In vitro* biofouling activity was evaluated in different substrate such as stone, glass, metal sheet and pipe. Among substrate the maximum biofouling was in stone followed by glass, metal sheet and pipe. Bacterial EPS production was evaluated using different carbon, nitrogen and phosphate source. Among the various nutrition, sucrose, ammonium sulphate and K_2HPO_4 specifically the maximum EPS production under *in vitro* condition.

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INTRODUCTION

Biofilms are ubiquitous phenomena in aquatic environments. Biofilms are often a nuisance to technical applications, e.g., ship hulls, heat exchangers, pipes (Characklis and Cooksey, 1983; Lappin-Scott and Costerton, 1989). In the marine environment; biofilms cover most subtidal and intertidal solid surfaces such as rocks, ships, loops, marine animals and algae (Lee *et al*., 2003). Marine structures such as platforms, jetties and ship hulls are subject to diverse and severe biofouling (Chambers *et al*., 2006). Biofilm processed on heat exchangers, pipelines, ship surfaces and other industrial devices causes serious problems and consumes large amounts of time and money to remove the biofilm. Also, biofilm formed on implanted materials has been related to microbial diseases. Most bacteria are attached to various surfaces. Attachment leads to the formation of biofilm, which can influence the surrounding environment either beneficially or harmfully (Costerton *et al*., 1995). The role of bacterial biofilms in disease processes is becoming increasingly recognized in both clinical and environmental settings (Bourne, 2006).

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Bacterial colonization occurs via a two step process beginning with reversible attachment to the substratum followed by irreversible adhesion (Dexter, 1979; Morisaki *et al*., 1999). The reversible attachments of cells are held by physical forces and can be easily removed by gentle washing. Non reversible attachment of cells is often mediated via specific mechanisms such as hydrogen bonding, cation bridging, specific receptor ligand interactions and the production of extra cellular polysaccharides (EPS) (Biancitto *et al*., 2001). The solid surface may have several characteristics that are important in the attachment process. Characklis and Cooksey (1983) noted that the extent of microbial colonization appears to increase as the surface roughness increases. This is because shear forces are diminished, and surface area is higher on rougher surfaces. The physicochemical properties of the surface may also apply a strong influence on the rate and extent of attachment. Most investigators have found that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces such as teflon and other plastics than to hydrophilic materials such as glass or metals. (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983; Bendinger *et al*., 1993). The strength and mechanisms of attachment of bacteria (Fletcher and Loeb, 1979; Duddridge *et al*., 1982; Rittle *et al*., 1990), diatoms (Pyne *et al*., 1984; Woods and Fletcher, 1991), marine and freshwater fungi (Read *et al*., 1991) to surfaces has been studied by several authors.

Marine organisms like *Vibrio proteolyticus* (Paul and Jeffrey, 1985), *Shewanella oneidensis* (Lee and Newman, 2003), *Bacillus subtilis* (Omoike and Chorover, 2004), *Bacillus* sp (Dhanasekaran *et al*., 2009) have been found to be involved in the biofouling process. *Vibrio alginolyticus* marine biofouling bacterium was isolated from a tin panel associated biofilm in the surface coastal waters of Bay of Bengal at Tiruchendhur by Muralidharan and Jayachandran, (2003). *Bacillus alvei* and *Aerobacter aerogens* biofilms were isolated from various sites with in a paper mill in Chandigarh (Chaudhary *et al.,* 1997).

There is a need to develop new environmentally compatible alternatives that would be equally efficient against several biofouling organisms. Fouling of the surfaces of aquatic environment may be of life threatening, because it can impair vital processes. Keeping these points in view the present study has been undertaken to isolate and screen the biofouling activity in marine bacteria from power plant pipes, Tamilnadu, India and also an attempt has been made to characterize the biofouling bacterial isolates by analyzing morphological, biochemical and molecular characteristics. In order to achieve this goal the present investigation has been planned with the following objectives, to isolate, screen and characterize the biofouling bacteria from thermal power plant pipes, to optimize the nutritional requirement and substrate for biofilm production.

MATERIALS AND METHODS

Collection of biofilm

The biofilm samples were collected from the thermal power plant pipe lines, Neyveli, Tamil Nadu, India (located at 11.30° N - 79.29° E) during the period of March, 2009. The samples were brought to the laboratory in ice box for further processing. Isolation of bacteria was carried out immediately upon retrieval of the samples to the laboratory.

Isolation of bacteria

Zobell agar medium was prepared and sterilized at 121°C at 15 lbs for 15 minutes. The medium was poured into sterile Petri plates. The collected biofilm samples were diluted up to 10^{-6} and 0.1 ml of the diluted samples were spread over the agar plates. The inoculated plates were incubated at 28 ± 2°C for 24-48 hours. The replicates were maintained for each dilution. After incubation, the selected bacterial colonies were purified and maintained in Zobell agar medium for further investigation.

Screening of biofouling activity of bacterial isolates by tube method and microtiter plate assay

Tube method

The bacterial colonies were inoculated into 5ml of nutrient broth in glass tubes. Cultures were incubated at 37°C for 18-20 hours and the culture contents were aspirated. Tubes were stained with safranin. The presence of a visible stained film on the wall of the tube was considered to be positive for biofouling.

Microtiter plate assay

The bacterial isolates were grown over night in nutrient broth at 37°C. Aliquotes of 3µl were inoculated in six parallel wells of a 96 well microtitre plate. Then the plate was incubated at 37°C for 72 hours. After the incubation period the wells were rinsed with physiological saline to remove the detached cells and fixed with 2µl of 99.99% ethanol for 10 minutes. The attached bacterial material was then stained by adding 2µl of crystal violet (2%) for 20 minutes. The plate was rinsed with tap water and the amounts of attached cells were measured using an ELISA reader at 570 nm (Abdi-Ali *et al.,* 2006).

Characterization of biofouling bacteria

Isolated bacteria were identified based on morphological, physiological, biochemical and molecular characterization by Bergey's manual of systematic bacteriology.

Gram staining

The isolated bacterial strains were made into a thin smear in a clean glass slide, air dried then heat fixed. The primary stain crystal violet was flooded on the smear washed with running tap water. The mordant stain gram's iodine was flooded on the smear and left for one minute and then washed with water. Decolorizing agent alcohol was added drop until the dye gets removed from the smear and then washed with tap water. At last the smear was flooded with counter stain, Safranin and left for 30 seconds and washed with water. The slide was air dried observed under microscope.

Determination of Motility by hanging drop technique

A small drop of liquid bacterial culture was placed in the center of a cover slip and then Vaseline was applied at each corner of the cover slip. The cavity slide was inverted with a central depression over the cover slip. The cover slip was stick to the slide and when the slide was inverted the drop of bacterial culture was suspended in the well. The motility of the bacteria was examined using microscope (X 400).

Biochemical characterization

Indole test

The bacterial culture was inoculated into peptone broth and incubated at 37°C for 24-48 hrs. After incubation few drops of Kovac's reagent was added and gently agitated. Examine for the presence of red color in the upper layer of liquid. Positive result was indicated by the formation of cherry red colour and negative result was indicated by the formation of yellow colour.

Methyl red test

The bacterial culture was inoculated into MR-VP broth and incubated for 24-48 hrs at 37°C. After incubation few drops of methyl red reagent was added into the culture medium. Positive result was indicated by the formation of red colour and negative result was indicated by the formation of yellow colour.

Voges Proskauer Test

The isolated bacterial culture was inoculated into the MR-VP broth and incubated at 37°C for 48 hrs. After incubation few drops of alpha napthol reagent and 40% KOH solution were added. Positive result indicated by the formation of strong red colour and negative result was indicated by a no colour change.

Nitrate reduction test

Nitrate broth was inoculated with the bacterial isolate and incubated at 37°C for 48 hours. 10-15 drops each of sulphanilic acid and N, N-dimethyl- 1-naptthyalamine was added. The positive result was indicated by the formation of deep red colour within 5 minutes and negative result was indicated by the yellow colour.

Citrate utilization test

Simmon citrate agar slant was inoculated with the bacterial isolate and the tubes were incubated at 37°C for 24 hours. After incubation, the change of green to blue colour indicates positive result and negative result was indicated by no colour change.

Triple sugar iron agar test

The TSI agar slants were prepared and inoculated with the bacterial isolate by stab and streak method. The slants were incubated at 37°C for 24-48 hours. After incubation the results were recorded as alkaline production (red), acid production (yellow) and gas production.

Urease test

Christensen urea agar was inoculated with the bacterial isolates and incubated at 37°C for 24-48 hrs. After incubation, the positive result was indicated by the formation of Purple/ pink colour and negative result was indicated by colour of medium remains unchanged.

Catalase test

One drop of hydrogen peroxide solution was placed on a slide. A small portion of the suspect colony was spotted onto the center of the slide. The vigorous gas bubble was observed within 10 seconds indicates positive result and negative result was indicated by no gas bubble formation.

Oxidase test

The bacterial culture was rubbed over the filter paper containing a reagent N-N- tetramethylpapaphenylene diamine dihydrochloride. Purple color indicates positive result.

Carbohydrate fermentation test

The sugar fermentation base broth was prepared, sterilized and supplemented with 1% filter sterilized sugars such as lactose, glucose, maltose and sucrose.

The bacterial isolates were inoculated and incubated at 37°C for 24-48 hours. After incubation the positive result were recorded based on indicator changes and gas production in Durhams tubes. The change of color from red to yellow and gas production indicates the positive test.

Esculin Hydrolysis

The Esculin medium was inoculated with the bacterial isolates and incubate at 35-37^{o}C for 48 hours. After incubation, the positive result was indicated by the formation of black colour and negative result was indicated by no colour change.

Gelatin Hydrolysis

Nutrient gelatin deep tubes were prepared, sterilized and inoculated with the bacterial isolate. It was then incubated at 37°C for 24-48 hours and observed for gelatin liquefaction by placing at 4°C for 30 minutes.

Casein Hydrolysis

Casein medium was prepared and inoculated with the bacterial isolates and incubate at 35-37°C for 48 hours. After incubation, the positive result was indicated by the formation of clear zone and negative result was indicated by no zone formation.

Physiological characterization

Effect of pH on bacterial growth

Nutrient broth was prepared and distributed into test tubes. Different pH such as 5.5, 6.5, 7.0, 7.5, and 8.5 were maintained by using 0.1 N HCl and NaCl. The tubes were sterilized and inoculated with bacterial cultures. Then tubes were incubated at 37°C for 24–48 hours. After incubation, the OD value was determined by colorimetric method at 540 nm.

Effect of temperature on bacterial growth

Nutrient broth was prepared and distributed into test tubes. The tubes were sterilized and inoculated with bacterial cultures. Then tubes were incubated at different temperature as 15°C, 25°C, 30°C, 35°C and 45°C for 24–48 hours. After incubation, the OD value was determined by colorimetric method at 540 nm.

Molecular characterization of biofouling bacteria

Isolation of Genomic DNA

Nutrient broth and starch casein broth was prepared and sterilized. The bacterial isolates and actinobacteria were inoculated and incubated in a shaking incubator for overnight. A small amount of culture broth was centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and 200 µl of Tris 0.1mM, 200 µl of lysis solution 1% sodium dodecyl sulfate (SDS) and sodium hydroxide (NaOH) was added to the pellet. The suspension was mixed in vortex and deproteinazed with 700 ml phenol, chloroform, isoamyl alcohol

(25:24:1v/v/v), homogenized and centrifuged 10 minutes at 13,000 rpm for 10minutes. The DNA was precipitated by addition of 700µl of ice cold 95% ethanol was added and spinned, washed in 70% ethanol and centrifuged 10 minutes at 10,000 rpm and the supernatant was discarded. The pellet was dried in room temperature and suspended in TE buffer and stored in refrigerator.

Separation of Genomic DNA by agarose gel electrophoresis

TBE buffer was prepared, 1% agarose was dissolved in TBE buffer and melted. 1 µl of ethidium bromide stain was incorporated into the gel. The gel casting tray was sealed on both sides with tape and agarose was poured into the tray. The comb was placed in the gel and allowed for solidification. After solidification, the comb and the tape were removed. The gel tray was placed in the electrophoresis tank and TBE buffer was poured over to cover the gel. 3 µl of Bromophenol blue (tracking dye) and 7µl of DNA sample were mixed well. Then the samples were loaded into the wells using micropipette. The power was switched on and the gel was run at 50V. The power was switched off when the tracking dye reached three fourth of the gel. The DNA bands were observed on gel doc imaging system (UVP).

PCR amplification of 16S rRNA

The bacterial 16S rRNA was amplified by PCR using universal primer pair of 1.0µl forward primer (5' AGAGTTTGATCCTGGCTCAG3') and 1.0µl reverse primer (5'ACGGCTACCTTGTTACGACT3'). The 50µl reaction mixture contained the following components: 5.0µl template DNA, 5.0µl of 10X buffer, 2.0µl of Taq DNA polymerase, 2.0µl of dNTP mixture and sterile water 34.0µl. The 50µl reaction mixture was taken in 0.5ml of microcentrifuge tube. The total 50µl mixture in the tube was gently spun for 10 seconds and allowed to settle the contents. The samples were kept in PCR thermal cycler (Applied biosystem). The PCR program was carried out in the following manner of 30 cycles with an initial denaturation step at 94^{o}C for 2 minutes, followed by denaturation step at 94^{o}C for 45 seconds, annealing at 52^{o}C for 30 seconds, extention at 72^{o}C for 1 minute and final extention at 72^{o}C for 2 minutes. 10µl of PCR product with 2µl of loading dye was mixed and loaded on a 1% agarose gel and analyzed electrophoretically at 60V for 45 minutes. The gel was observed on gel doc imaging system (UVP).

DNA sequencing

The amplified 16S rDNA sample was sent to MWG Biotech Pvt. Ltd, Bangalore for nucleotide sequencing. Specific sequencing primer was provided along with the samples for sequencing.

Phylogenetic analysis

The 16S rDNA nucleotide sequence was obtained from sequencing the PCR product. A BLAST of obtained sequence was performed with that of available EMBL database using the site http://www.ncbi.nlm.nih.gov/genebank and the 16S rDNA nucleotide sequence was subjected to Phylogenetic analysis using Bioinformatics tool available in online using software Mega clustral W and neighbor joining method.

Prediction of 16S rRNA secondary structure

The secondary structure of the bacterial 16S rRNA was predicted using the bioinformatics tool available in online www.genebee.msu.su/services/rna 2-reduced.html

Restriction sites analysis

The Restriction sites present on the bacterial 16SrRNA were analyzed using the NEB Cutter program version 2.0 tools in online http://tolls.neb.com/NEB Cutter2/index.php

Testing the influence of different nutritional source on EPS production

Effect of carbon source on EPS production

The effect of carbon source on the production of EPS was studied using Basal salt solution (BSS) medium supplemented with 1% of fructose, glucose, maltose and sucrose as the carbon and energy source. The growth medium was inoculated with the 18 hours old culture grown in the same medium. The culture was grown at room temperature for 8 days. Exopolysaccharide was estimated by the phenol sulphuric acid method (Dubois *et al*., 1956).

Effect of nitrogen source on EPS production

The BSS growth medium containing 1% sucrose and 0.013% of nitrogen either as ammonium chloride, ammonium sulphate, sodium nitrate or urea was used to assess the effect of nitrogen source on EPS production. The growth medium was inoculated with the 18 hours old culture grown in the same medium. The culture was grown at room temperature for 8 days. Exopolysaccharide was estimated by the phenol sulphuric acid method (Dubois *et al*., 1956).

Effect of phosphate source on EPS production

The BSS medium containing 1% sucrose and 0.013% ammonium sulphate was supplemented with dipotassium hydrogen phosphate and potassium dihydrogen phosphate (1µg/ml) was used to assess the effect of phosphate source on the EPS production. The growth medium was inoculated with the 18 hours old culture grown in the same medium. The culture was grown at room temperature for 8 days. Exopolysaccharide was estimated by the phenol sulphuric acid method (Dubois *et al*., 1956).

***In Vitrio* analysis bacterial biofouling on different substratum**

Bacterial cultures providing a maximum OD value from above Microtiter plate assay was selected and *in vitrio* biofouling assay was performed. Sufficient amount of nutrient broth was prepared containing carbohydrate (0.25% glucose). They were poured into Schott Duran bottles (100 ml) upto ¾th of its volume.

Different substratum were taken

Glass slide: The surface of glass slide was made rough by grinding with salt paper. The glass slide was rinsed in detergent solution and washed thoroughly in tap water and air dried and wiped the surface with 75% ethyl alcohol. Wrapped in aluminium foil.

Metal sheet: Metal Steel sheets measuring 3x1inch was taken, surface was washed repeatedly in detergent solution and washed thoroughly in tap water and air dried and wiped the surface with 75% ethyl alcohol. Wrapped in aluminium foil.

Stones: A stone of medium size was collected, each weighing around 50g, having irregular shape. It was washed repeatedly to remove soil particles in tap water, air dried and wiped the surface with 75% ethyl alcohol and kept in a glass beaker.

Plastic pipe: Plastic pipes measuring 3x1inch was taken, surface was scrubbed repeatedly in detergent solution and washed thoroughly in tap water and air dried and wiped the surface with 75% ethyl alcohol. Wrapped in aluminium foil.

The media and substratum (glass slide, metal, stones and plastic pipe) were independently sterilized in autoclave at about 121°C for 15 minutes. The bacterial isolate were inoculated in the conical flask with the help of a loop and was properly mixed. The substratum was inserted gently (without splashing) with the help of sterile forceps into individual bottle containing respective bacterial isolates. The substratum should be immersed to its ¾th of its volume. The bottles were incubated in an incubator at 37°C for 5 days. All the bacterial isolates were tested for its growth in all the four substrates. Control was maintained so as to avoid false positive results. The bottles ones kept for incubation should not be shaken or disturbed, since it might affect the growth of biofilms on the substratum. The number of colonies present in $1cm^2$ of area of different substratum was estimated. Then about $1cm^2$ area of biofilm was scrapped and diluted in distilled water and replated in the TCBS agar by pour plate method.

Colony forming units/cm^2 was calculated using the formula,

$$\text{c.f.u/cm}^2\text{ of substratum} = \frac{\text{Colonies counted x Dilution x Volume of suspension}}{\text{Volume plated x Area of slide in cm}^2}$$

RESULTS

Isolation of biofouling bacteria

In the present study, the biofilm samples were collected from power plant pipes. The samples were plated on Zobell agar medium for bacterial isolation. Totally ten isolates were obtained from power plant pipes. The diversity of bacterial isolate was increased due to the nutritive status of water.

Biofouling assay

Totally ten bacterial isolates were obtained from power plant pipes and named as DP1, DP2, DP3, DP4, DP5, DPR1, DPB1, DPS1, DPS2 and DPS3. Among the 11 isolates only bacterial isolate DPR1 and DPB1 showed visible biofilm on the wall of the test tube. The other isolates did not show significant visible biofouling activity. Among the ten isolates only bacterial isolate DPR1 and DPB1 showed maximum biofouling activity in the microtitre plate assay with a significant OD of 0.170 and 0.162 respectively (Table 1). The other isolates did not show significant biofouling activity. This result reveals that the bacterial isolates DPR1 and DPB1 possess significant biofouling activity.

Table 1. Screening of biofouling activity of bacterial isolates

S. No	Bacterial isolates	Biofouling activity (Optical density)
1	Control	0.000
2	DP1	0.069^{c}
3	DP2	0.021^{g}
4	DP3	0.013^{i}
5	DP4	0.045^{d}
6	DP5	0.032^{e}
7	DPR1	0.170^{a}
8	DPB1	0.162^{b}
9	DPS1	0.030^{f}
10	DPS2	0.018^{h}
11	DPS3	0.045^{d}

A value in the each column represents the mean of three replications. Statistically the means of the three experiments were not significantly different ($P< 0.05$). Values in the same column with different letters are significantly different at $P< 0.05$ in accordance with Fisher's least significant difference test.

Characterization and identification of biofouling bacteria

Since only two bacterial isolates were found to possess significant biofouling activity, they were justifiably chosen for the taxonomic characterization. The different parameters namely morphological, biochemical and molecular characters were used for characterization and identification of bacterial isolates.

Morphological characterization

The biofouling bacteria showed a notable array of distinguishing microscopic morphology**.** The bacterial isolate DPR1 was observed as yellow color circular flat colonies on TCBS agar medium. The staining report showed Gram negative, rod shaped (Table 2). The bacterial isolate DPB1 was observed as green colored circular colonies on TCBS agar medium. The staining report showed Gram negative, rod shaped.

Biochemical characterization

Various biochemical characteristics of biofouling bacteria were used for their identification. In the present investigation it was found that the bacterial isolate DPR1 was negative for voges-proskauer, H_2S production, urease, starch, gelatin, casein and lactose fermentation. In TSI agar, alkaline slant and acid butt was observed and found to be positive for indole, methyl red, citrate utilization, nitrate reduction, oxidase, catalase, sucrose, maltose and glucose fermentations (Table 2).

Physiological characterization

Various factors influencing by the culture of *Vibrio* sp. DPR1 and *Vibrio* sp. DPR1 were assessed. Among the factors, the optimum pH and temperature for the growth of *Vibrio* sp. DPR1 and *Vibrio* sp. was 7.5 and 35 ºC respectively. The values showed in Table 3.

approximately 1200 bp in length. The 16S rDNA gene amplification of the bacterial isolate DPR1 and actinobacteria isolates were performed by PCR technique using universal primers. PCR product was analyzed in 1% agarose gel. A sharp band was observed, which confirmed the PCR product.

Table 2. Morphological and biochemical characteristics of biofouling bacteria

S. No	Name of the test	Bacterial isolates (DPR1)	Bacterial isolates (DRB1)
1	Gram Staining	Negative Rods	Negative Rods
2	Motility	Positive	Positive
3	TCBS agar	Yellow, Small, circular	Green, Small, circular
4	Catalase	Positive	Positive
6	Oxidase	Positive	Positive
7	Indole Test	Positive	Positive
8	Methyl Red Test	Negative	Negative
9	Vogues-Prosauker Test	Negative	Negative
10	Citrate Utilization	Positive	Positive
11	Nitrate Reduction	Positive	Positive
12	Urease Test	Negative	Negative
13	Glucose	Positive	Positive
14	Sucrose	Positive	Positive
15	Maltose	Positive	Positive
16	Lactose	Negative	Negative
17	Starch Hydrolysis	Negative	Negative
18	Gelatin Hydrolysis	Negative	Negative
19	Casein Hydrolysis	Negative	Negative

Table 3. Effect of pH and temperature on *Vibrio* sp. growth

S.No	Factors	*Vibrio* sp. DRR1	*Vibrio* sp. DPB1
	pH	OD Value	OD Value
	5.5	0.672	0.563
1	6.5	0.543	0.578
	7.0	1.564	0.672
	7.5	1.674	1.248
	8.5	1.73	1.679
	Temperature (^{0}C)	OD Value	OD Value
	15	0.673	0.788
2	25	0.985	1.080
	30	1.427	1.362
	35	1.466	1.562
	45	1.249	1.014

Molecular characterization

The classification of bacteria using morphological and physiological characteristics does not necessarily lead to the identification of phylogenetically coherent taxa. However some of these characters were shown to be variable with changing media and environmental conditions. Several chemotaxonomic isozyme patterns were used for species and strain level differentiation with little success. To evaluate the bacteria, different PCR based molecular methods namely 16S RNA sequencing, RAPD, STRR, etc., may be used at all taxonomic levels. The sequence analysis of genes encoding 16S rRNA is currently the most promising approach for phylogenetic classification of bacteria.

The comparison of rRNA sequence is a powerful tool for detecting phylogenetic and evolutionary relationship among bacterial species. In the present study the DNA was isolated from the bacterial isolate DPR1, actinobacteria DPR2, DPR3 were analyzed by agarose gel electrophoresis. A sharp band was obtained in the gel. The DNA was found to be

The amplified 16S rDNA sample was sent to MWG Biotech Pvt. Ltd, Bangalore for partial 16S rDNA nucleotide sequencing. The nucleotide sequence was obtained from sequencing the PCR product. A BLAST of the obtained nucleotide 16S rDNA sequence for the bacterial isolate DPR1 was performed and the sequence showed similarity with the *Vibrio* spp. Phylogenetic analysis is all about understanding the evolutionary relationship between the microorganisms. The easiest way to depict any evolutionary relationship between groups of organisms is building up a phylogenetic tree. A phylogenetic tree of the bacterial isolate DPR1 was constructed using its 16S rDNA sequence with that of the other *Vibrio sp.* from the NCBI data base (Table 4). The resultant phylogenetic tree showed similarity of the bacterial isolate DPR1 only at the genus level, where as it did not show any similarity at the species level (Figure 1). Secondary structure of the 16S rDNA sequence of the *Vibrio* sp. DPR1 was predicted using the gene bee tool. *Vibrio* sp. DPR1 showed 21 loops and 34 stems with an over all free energy of -160.6 kkal/mol. (Figure 2). The restriction sites present on the 16S rDNA of *Vibrio* sp.

Table 4. Nucleotide sequence of the 16S r RNA region from *Vibrio* sp. obtained from Genbank

S. No	Name of the organism	Accession No	Habitat	Country
1	*V. harveyi* D7047	FJ161348	Unknown	China
2	*V. parahaemolyticus* D6071	FJ161313	Coastal water	China
3	*Vibrio* sp. D6069	FJ161312	Coastal water	China
4	*V. fortis* D6013	FJ161299	Coastal water	China
5	*V. aestuarianus* D5057	FJ161294	Coastal water	China
6	*V. cyclitrophicus* D4053	FJ161267	Coastal water	China
7	*V. cholerae* PIM9	GQ359963	Intestine mucus	China
8	*Uncultured Vibrio* sp.	GQ290399	Surface sediment of a shrimp pond	China
9	*V. tapetis*	FN432995	Diseased cultured Wedge sole	Spain
10	*V. anguillarum* CECT522	EU851049	Fish	USA
11	*V. mimicus* CECT4218	EU851050	Homo sapiens	USA
12	*V. cincinnatiensis* CECT4216	EU851051	Homo sapiens	USA
13	*V. alginolyticus*	FN432778	Sediment sample	India
14	*Vibrio* sp. DPR1	GQ495091	Thermal power pipe line	India

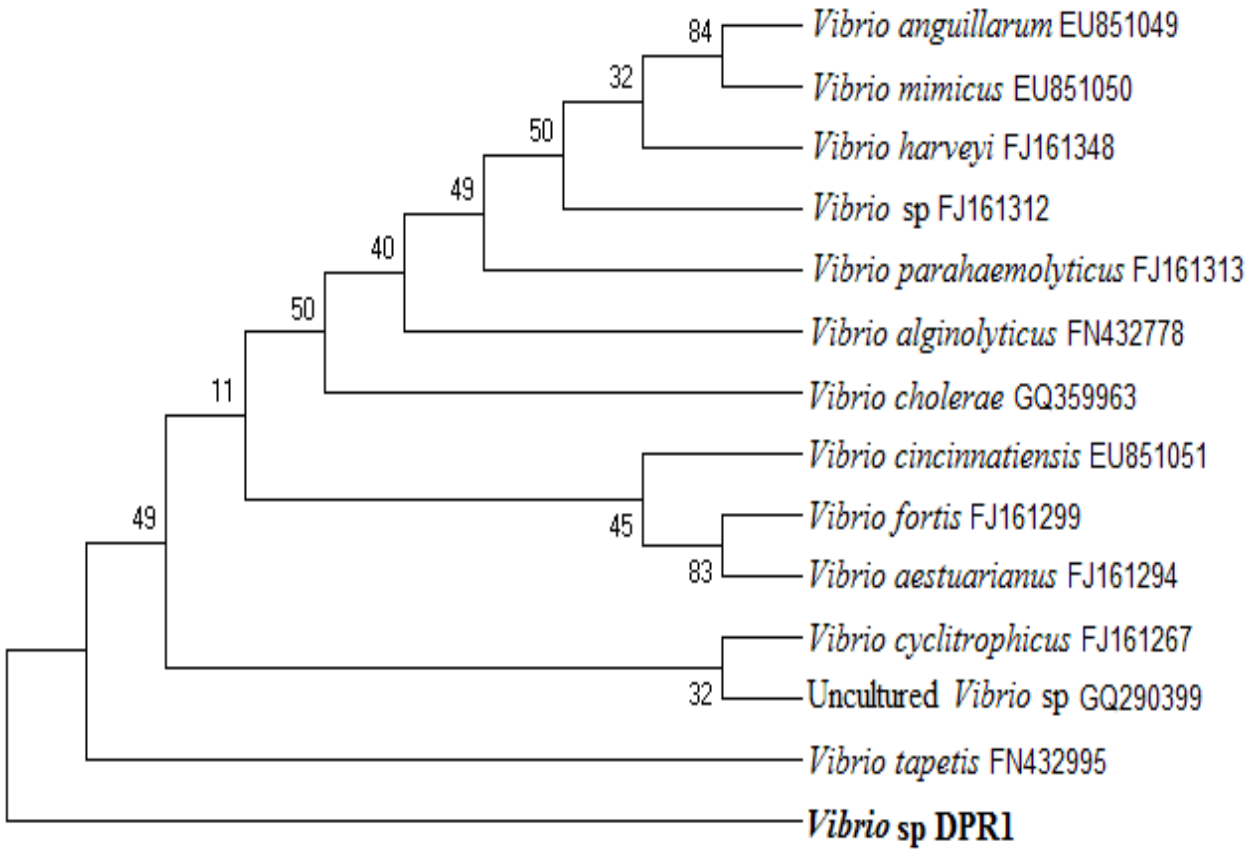

Figure 1. Phylogenetic tree derived from partial 16S rRNA gene sequence of *Vibrio* sp. DPR1 using Neighbor joining method

Table 5. Effect of Carbon, Nitrogen and Phosphate source on EPS production

S.No	Name of the Nutrient source	*Vibrio* sp. DPR1		*Vibrio* sp. DRB1	
A	Carbon source (1%)	OD(540nm)	EPS(mg/l)	OD(540nm)	EPS(mg/l)
1	Lactose	0.66	6.95	0.791	13.5
2	Sucrose	1.801	64.0	1.848	66.35
3	Maltose	1.21	34.45	1.052	26.55
4	Glucose	2.199	83.9	2.128	80.35
B	Nitrogen source (0.013%)				
1	$NaNO_3$	1.263	37.1	1.573	52.6
2	NH_2CONH_2	0.961	22.00	0.661	7.00
3	$(NH_4)_2SO_4$	0.818	14.85	0.901	19.0
4	NH_4Cl	1.332	40.55	1.673	57.6
C	Phosphate source(1µg/ml)				
1	K_2HPO_4	1.167	32.3	1.401	44.0
2	KH_2PO_4	1.222	35.05	1.324	40.15

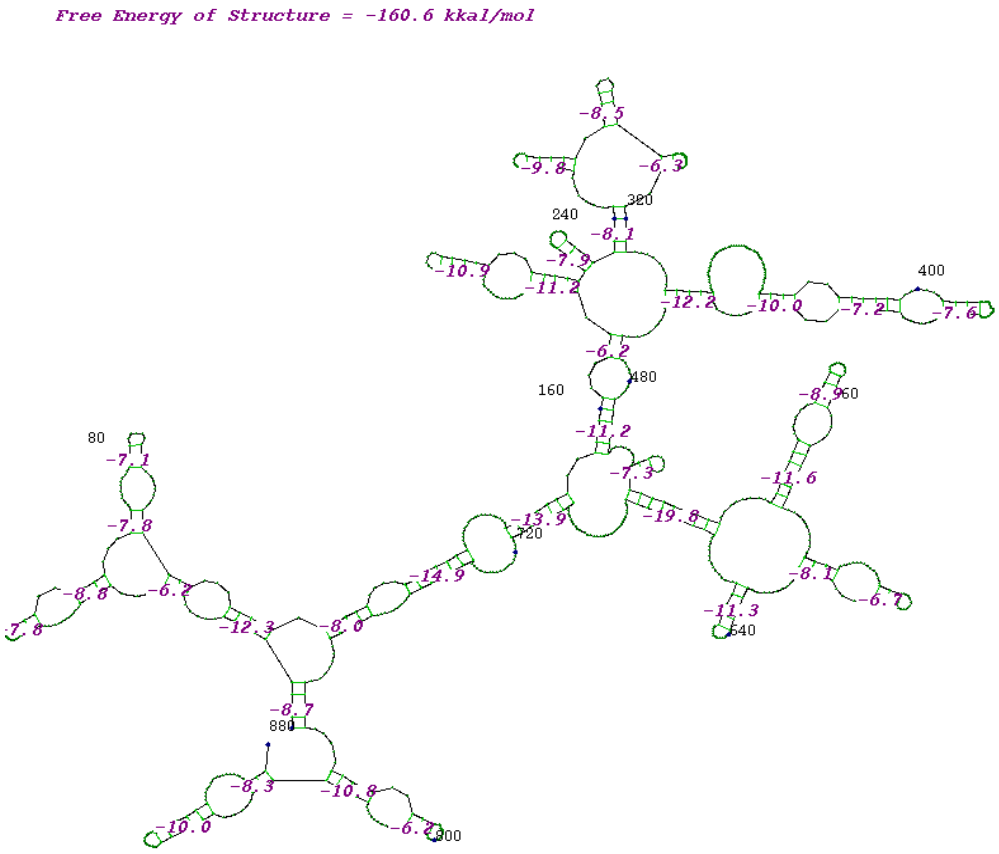

Figure 2. Secondary Structure of 16S rRNA of *Vibrio* sp. DPR1 using Genbee software

DPR1 were analyzed using the NEB cutter program and it showed the site for various commercial and NEB (New England Biolabs) restriction enzymes. *Vibrio* sp. DPR1 had 48 restriction sites and GC and AC contents are 53% and 57% respectively (Figure 3).

Effect of different nutrient source for growth and EPS production

Various factors influencing EPS production by the culture of *Vibrio* sp. DPR1 and *Vibrio* sp. DPR1 were evaluated. Among the various carbon sources used, sucrose influenced the highest amount of EPS. Among the various nitrogen sources used, ammonium sulphate when used at 0.006%, produced the highest quantities of EPS production. When the growth medium was supplemented with 1 µg/ml of phosphate, the EPS production was the highest maximum (Table 5).

In vitro analysis bacteria biofouling on different substratum

The idea behind this work was to check the growth of biofilms in *in vitro* conditions. The biofilm growth of various marine bacteria on different substratum was observed. Initially about ten different strains were obtained from the biofilm sample collected from power plant biofilm sample. The selection was done on the basis of colony showing mucoid outgrowth, since they have a greater capacity to produce slime layer.

From those ten isolates, only 2 bacterial isolates were able to secrete EPS in the presence of 3% glucose in the nutrient broth. The selected bacterial isolates were further checked for their ability to grow on different substrates.

Table 6. *In vitro* analysis of biofouling on Substrate

S. No	Name of Strains	Stone	Glass slide	Metal sheet	Pipe
1	*Vibrio* sp. DPR1	Strong	Medium	Weak	Weak
2	*Vibrio* sp. DPB1	Strong	Medium	Weak	Weak

Table 7. Total bacterial population in 1cm^2 area of substrate

S. No	Name of the substrate	*Vibrio* sp. DPR1 (cfu/cm^2)	*Vibrio* sp. DPR2 (cfu/cm^2)
1	Glass	235	228
2	Pipe	143	170
3	Stone	318	316
4	Metal sheet	213	220

It was found that both isolates showed high growth on stones; glass slides, plastic pipes and steel sheet gradually (Table 6). The colony forming units in 1 cm^2 area of different substrate was calculated for two bacterial isolates (Table 7). Therefore affinity of bacterial isolates towards substratum can be arranged in ascending order stone followed by glass, metal sheet and pipe.

Figure 3. Restriction site analysis and GC content of 16S rRNA of *Vibrio* sp. DPR1 using NEB Cutter program

DISCUSSION

Biofouling bacteria corroding solid surfaces - iron, aluminium, steel, plastics and nylon strings at a depth of 2 m in sea water at Bhavnagar Port, were isolated and identified. Simoes *et al.,* (2004) has isolated *Pseudomonas fluorescence* and *Bacillus cereus* from bioreactor rotating system. Karunasagar *et al.,* (1995) have isolated *Vibrio harveyi* from cement slab, high density polyethylene (HDPE) plastic and steel coupons. Chaudhary *et al.* (1997) have isolated *Aerobacter aerogens* and *Bacillus alvei* from various sites within a paper mill. Nidal *et al,* (2003) have isolated *Escherichia coli* from membrane systems. Bagge *et al*. 2001 have isolated *Shewanella putrefaciens* adhesion and biofilm formation under batch and flow conditions. Muralidharan and Jayachandran (2003) have isolated *Vibrio alginolyticus* from a tin panel associated biofilm in the surface coastal waters. Dhanasekaran *et al*., (2009) isolated the biofilm forming marine *Bacillus* from ship hull. Thus it is obvious that *Vibrio* sp. is adapted to diverse habitats which vary widely in space and time and also diverse environmental conditions. The present investigation is different from the previous findings as the *Vibrio* sp. has been isolated from biofilm samples of thermal power plant pipe. Screening of Biofouling organism was done by several methods like microtitre plate method was done by Abdi-Ali *et al.* (2006).

A similar biofilm screening has been done by Saravanan *et al.* (2008) using glass cover slips as substratum to check the adherence of *Pseudoalteromonas ruthenica* biofilms. Prasad *et al*. (2009) using glass tubes as substratum to check the adherence of *pseudomonas aeruginosa* biofilms and then staining the cover slips with crystal violet. Characterizations of biofouling organisms are done by morphological, biochemical and molecular. The present finding is similar to the findings of Jorquera *et al*. (1999). According to Jorquera *et al*. (1999) *Vibrio* sp. is Gram negative, motile rods, with the ability to ferment glucose, maltose and sucrose and reduced nitrates to nitrites. The bacterial isolate DPB1 was negative for voges-proskauer, H_2S production, urease, starch, gelatin, casein and lactose fermentation. In TSI agar, alkaline slant and acid butt was observed and was found to be positive for indole, methyl red, citrate utilization, nitrate reduction, oxidase, catalase, sucrose, maltose and glucose fermentations. Buchanan and Gibrons, 1975 reported the presence of *Vibrio* sp. was characterized depending upon morphology, Gram staining and biochemical tests by Bergey's manual of determinative bacteriology. 16S rRNA analysis is given a significant value for the molecular identification of organisms. The present findings based on 16S rRNA analysis are similar to the findings of Leal *et al.* (2004). According to Leal *et al.* (2004) *Vibrio* sp. isolated from the aquatic basins of the state pernambuco, Brazil was determined by their 16S rRNA

sequence analysis. Extracellular Polymeric Substances (EPS) are playing a major role in Biofouling organisms. The EPS comprise mainly polysaccharides and proteins, which form hydrogel matrices. EPS matrix offers important advantages for biofilm organisms and can maintain stable arrangements of synergistic microconsortia of different species. EPS production depends on the substrates used by the organism as nutrient and biofouling organisms can be characterized by their EPS nature and effect of different macro and micro molecules on their growth. Majumdar *et al*. (1999) reported the presence of the *Vibrio* sp. in biofilm and were characterized by different nutrient source. Jahn *et al*. (1999) reported the presence of the *Vibrio* sp. was characterized nutrient status of surface associated bacteria influence the quality and chemical composition of EPS produced. Biofilm formation depends on surfaces of different substrates. Karunasagar, (1996) reported the presence of the *Vibrio harveyi* formed biofilms on three substrates such as cement stone, plastic and steel coupons.

This study, it was observed that stone was shown maximum groth, might be due to the rough surface, which offers higher surface area for bacterial attachment. Glass slide showed positive as a substratum for bacterial isolates. The other substrate showed a very loose slime layer forming on the substrate, which easily washed out, under running water. The broth containing steel sheets and plastic pipe showed less turbidity as compared to other substrata such as glass and stone containing broth culture. Thus the present study gives an idea about the biofouling bacteria *Vibrio* sp. *in* the biofilm sample collected from thermal plant pipe lines. They were characterized and identified by different parameters such as morphological, biochemical and molecular characters. Hence the present study gives an account on biofilm forming *Vibrio* sp present in pipe lines and their characteristic features.

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