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Isolation and Identification of hydrocarbon degrading bacteria from Ennore creek

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Abstract:
The widespread problem caused due to petroleum products, is their discharge and accidental spillage in marine environment proving to be hazardous to the surroundings as well as life forms. Thus remediation of these hydrocarbons by natural decontamination process is of utmost importance. Bioremediation is a non-invasive and cost effective technique for the clean-up of these petroleum hydrocarbons. In this study we have investigated the ability of microorganisms present in the sediment sample to degrade these hydrocarbons, crude oil in particular, so that contaminated soils and water can be treated using microbes. Sediments samples were collected once in a month for a period of twelve months from area surrounding Ennore creek and screened for hydrocarbon degrading bacteria. Of the 113 crude oil degrading isolates 15 isolates were selected and cultivated in BH media with 1% crude oil as a sole carbon and energy source. 3 efficient crude oil bacterial isolates *Bacillus subtilis* I1, *Pseudomonas aeruginosa* I5 and *Pseudomonas putida* I8 were identified both biochemically and phylogenetically. The quantitative analysis of biodegradation is carried out gravimetrically and highest degradation rate, 55% was recorded by *Pseudomonas aeruginosa* I5 isolate.

Background:
India has a coastline of about 5500 km in the main land and about 2000 km in the offshore islands. The coastal area of the country is blessed with a vast network of backwaters, estuaries, creeks, lagoons and specialized ecosystems like mangroves and coral reefs. It has vast beach all along the coast. The biodiversities in these coastal waters are significantly high. Hence there is an urge to preserve, conserve and protect the coastal habitats and the marine environment from all manmade activities. Oil spills that occur during discharge from the refineries, accidents of ships/tankers, their grounding, rupture on seabed and on shore pipelines, offshore oil production and exploration platforms do affect these habitats causing irreversible damage to the biodiversity. Thus the pressing research needs for bioremediation of oil spill, necessitate the isolation and identification of efficient degrading microbe and devising ways to accelerate the biodegradation rate.

Ennore Creek
The present study area covers the Ennore creek (13°13'54.48" N, 80°19' 26.60" E) is located in the northeast coast of Chennai, Tamil Nadu, India. It lies between the city of Chennai and the Pulicat Lake, the second largest brackish water lake in India. The southern arm of the creek is well developed with industries, utilities, residential areas and fishing hamlets. The northern section of the creek or Kortalaiyar back water is connected to the Pulicat Lake and has the North Chennai Thermal Power plant, Ennore port and Petrochemical industries **[1].**

The total area of the creek is 2.25 sq km and is nearly 400 m wide. Meteorological data for Ennore shows average minimum air temperatures varying between 20°C and 28° C and maximum temperatures ranging from 28°C to 37°C. The seasons of Ennore influences its oceanographic characteristics, i.e., strong winds during the SW and NE monsoons and cyclonic winds producing larger waves. The region mostly receives rainfall from the Northwest monsoon during the months of October and November. Ennore creek was once encompassed with rich biodiversity and in due course of time it has been totally wiped out by the petrochemical industries pumping their effluents into the creek. The creek receives waste

water from industries in the Manali Industrial area. Some of the petrochemical industries discharge their effluents into the sea through submerged pipelines, south of Ennore creek mouth. The present study describes the isolation and identification of Total Heterotrophic Bacterial (THB) population and Hydrocarbon Degrading Bacterial (HDB) population, identification of the efficient degrader, its molecular characterization and phylogenetic analysis based upon 16s RNA gene sequencing from the samples collected in Ennore creek.

Methodology:

Sample collection

Surface sediment samples were collected from oil contaminated site in Ennore creek. Discharges from the petrochemical industries can be seen at the southern side of the creek at a latitude, North of 13°13.413' and longitude of East of 80°19.139'. The water depth in this region was found to be 0.16m. Surface sediment samples were collected over a period of 12 months from the same point, between July 2009 and June 2010, at four different points in the oil contaminated site using a sterile stainless steel spatula. The collected samples were pooled and transferred to pre-sterilized, labeled, self sealed plastic bags and transported at 4°C to the laboratory and maintained at 4°C until analysis.

THB population

THB population was enumerated by pour plate method **[2].** 1g of the sample was aseptically transferred into 100ml of physiological saline and transferred in a series of eight 10 fold serial dilution using physiological saline. 1ml of the aliquot from each of the dilution was inoculated by pour plate method onto Nutrient agar (NA) in duplicates. The plates were incubated at 37°C for 24 - 48 hrs.

HDB population

HDB population was enumerated by spread plate technique **[3]** by inoculating 0.1 ml of aliquot onto sterile Bushnell Hass agar (BHA) plates spreaded with 100µl of sterile crude oil. The crude oil used was sterilized by filtering through Millipore filter, 0.45µ diameter and stored in sterile bottles. Crude oil was obtained from Chennai harbor with a specific gravity of 0.892 at 25° C and used as the sole carbon source to isolate HDB. The plates were incubated at 25° C for 7 days.

Identification of THB and HDB population

The enumerated bacteria were isolated and stored in NA slants at 4°C for further identification. Primary identification was done on the basis of colony and cell morphology and Gram staining. Secondary identification is carried out by performing a series of Biochemical tests **[4].**

Preliminary screening of crude oil degraders

The hydrocarbon degraders which are stored on glycerol stock were subjected to its efficiency of crude oil degradation. The isolates were single streaked on BHA plates overlaid with 100 µl of crude oil and on plain NA plates and were incubated at 25°C for 14 days and 37°C for 24 - 48 hrs respectively. Any isolate which grow on NA plates but failed to grow on BHA plate were confirmed as non degraders. The isolates which grow on both the agar plates were confirmed as hydrocarbon degraders **[5].** The zone of clearance around the degraders, grown on BHA plate with varying diameters was observed.

Selection of efficient degraders

Isolates showing greater zone of clearance were subjected further for the estimation of oil degrading efficiency. The growth was analyzed in terms of biomass and degradation rates by gravimetric method.

Biomass determination

Cells of density 10^8 mL^{-1} of the above each isolates was inoculated, into 250 ml flask containing 100ml of BHM with 1.0% crude oil. Optical density was initially recorded at day 1 at 620 nm to find out the initial population. The flasks were then incubated at 25˚C at 150 rpm for 60 days. 3ml of the samples were withdrawn periodically at day 7, 15, 30, 45 and 60. 3 ml obtained from the uninoculated flask was used as a blank. The actual biomass in dry weight (g/L) were obtained from the constructed calibration curve (y=31.2x) **[6].**

Estimation of crude oil degradation

The estimation of crude oil degradation was accomplished by gravimetric analysis **[7].** The residual crude oil was extracted in a preweighed flask with petroleum ether in a separating funnel. Extraction was repeated twice to ensure complete extraction. After extraction, petroleum ether was evaporated in a hot air oven at 60-70˚C, the flask was cooled down in a dessicator and weighed. The percentage of degradation was calculated as shown in **(Please see supplementary material for formulas).**

Molecular characterisitation

Strains with efficient degrading ability were identified upto its species level by 16s RNA sequencing. The sequencing reaction was performed using BigDye terminator V3.1 cycle sequencing Kit containing AmpliTac DNA polymerase (Applied Biosystems, P/N: 4337457). The sequencing reaction - mix was prepared by adding 1 µl of BigDye v3.1, 2ul of 5x sequencing buffer and 1µl of 50% DMSO. To 4µl of sequencing reaction – mix was added 4 pico moles of primer (2µl) and sufficient amount of plasmid. The constituted reaction was denatured at 95°C for 5 minutes.

Cycling began with denaturing at 95°C for 30 seconds, annealing at 52°C for 30 seconds and extension for 4 minutes at 60°C and cycle repeated for a total 30 cycles in a MWG thermocycler. The reaction was then purified on sepheadex plate (Edge Biosystems) by centrifugation to remove unbound labelled and unlabelled nucelotides and salts. The purified reaction was loaded on to the 96 capillary ABI 3700 DNA analyzer and electrophoresis was carried out for 4 hours.The 16s RNA gene sequences obtained, were compared with the sequences from Basic Local Alignment Search Tool (BLAST) search of National Centre for Biotechnology Information (NCBI) data bases. The strains showing more than 97% 16s RNA gene sequence similarity, was considered to be of the same species.

Phylogenetic analysis

For construction of a phylogenetic tree, the sequences were aligned with known bacterial 16s RNAs obtained from the GenBank database by using MEGA5.1 **[8]** software, Neighbour Joining and Maximum Likelihood method.

Gene bank, NCBI accession number

The 16s RNA sequence of the efficient degrader was submitted to the Gene bank, NCBI, USA to obtain the accession number.

Results and Discussion:

THB population

The average number of heterotrophic bacteria during the study period was in the order of 22.32x 10^5 $CFUg^{-1}$, with the highest count 32.4x10^5 $CFUg^{-1}$ being recorded in the post monsoon, August, 2009 and the lowest 12.21x10^4 $CFUg^{-1}$ in the beginning of summer, March and April, 2010 **Table1 (see supplementary material)** & **(Figure 1A)**. Similar studies recorded that the THB counts ranged from 9.0x10^3 to 2.6x10^6 $CFUg^{-1}$ in the hydrocarbon contaminated surface sediment samples of Gokana, River state **[9].** THB population ranged from 10^5 to 9.0x10^8 $CFUg^{-1}$ on sediment samples collected from Cuddalore fishing harbor over a period of 8 months from October 2011 onwards was reported **[10].**

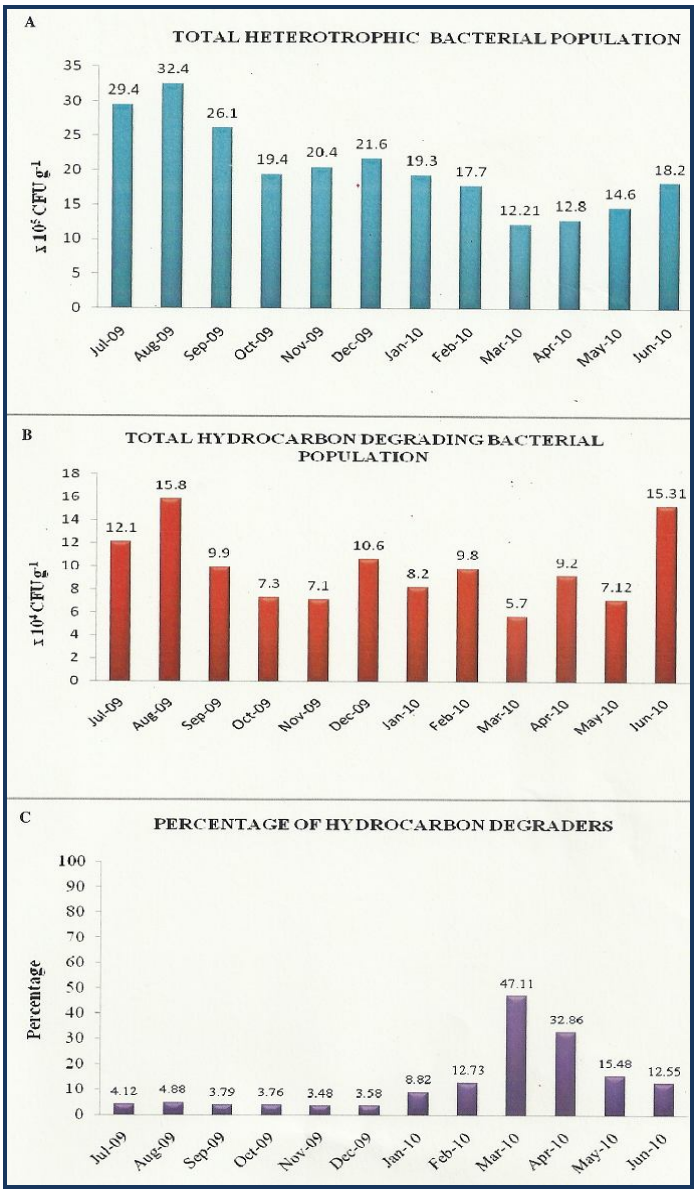

Figure 1: (A) Total heterotrophic bacterial population; **(B)** Total hydrocarbon degrading bacterial population; **(C)** Percentage of hydrocarbon degraders.

HDB population

The average number of hydrocarbon degraders during the study period was in the order of 10.75x10^4 $CFUg^{-1}$, with the highest count 15.8x10^4 $CFUg^{-1}$ being recorded in the post monsoon, August, 2009 and June, 2010 and the lowest 5.7x10^4 $CFUg^{-1}$ in March, 2010 **Table 1 (see supplementary material)** & **(Figure 1B).** Similarly HDB count between 2.0x10^7 – 1.8x10^8 CFU mL^{-1} was enumerated from petroleum effluent discharge **[11].** Highest level of HDB was recorded in Dec, 2003 and Jan 2004 during the dry season by in tropical mangrove estuarine intertidal and sub tidal sediments **[12].**

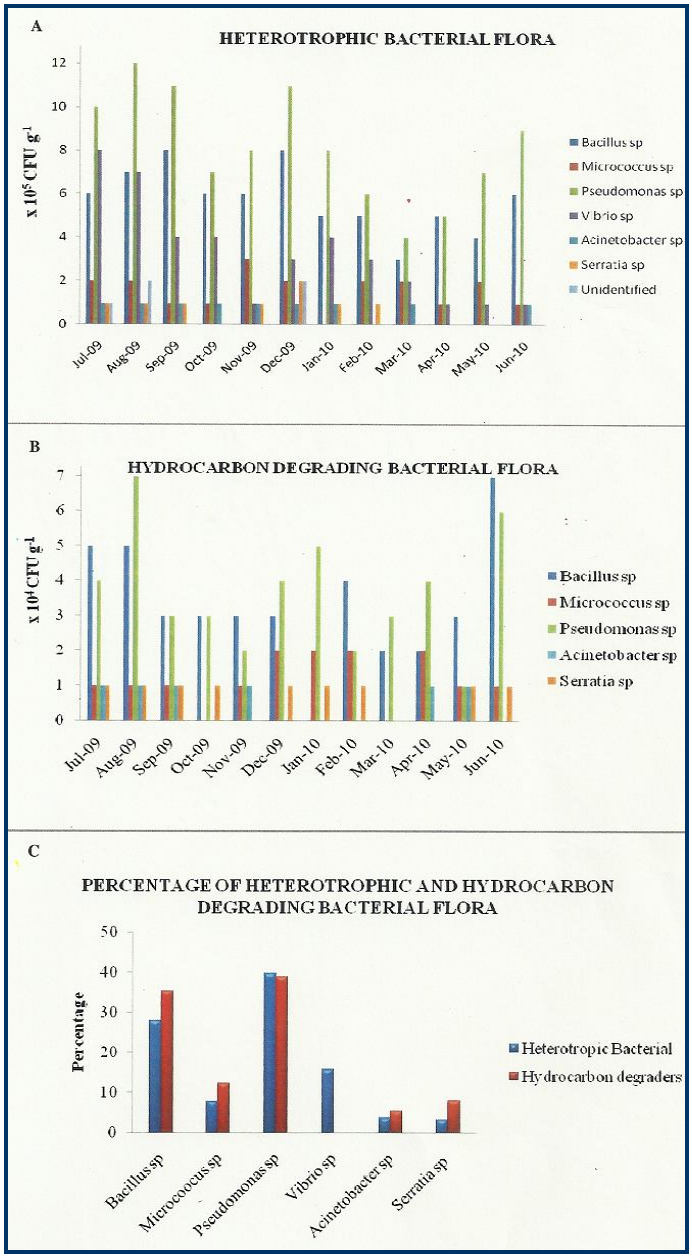

Figure 2: (A) Heterotrophic bacterial flora; **(B)** Hydrocarbon degrading bacterial flora; **(C)** Percentage of heterotrophic and hydrocarbon degrading bacterial flora.

HDB/THB ratio percentage

High HDB/THB ratio were recorded is 47.11% in March, 2010, post monsoon, which may be associated with the slow oil degrading activities of the oil degraders despite of the presence

of hydrocarbon. Lowest HDB/THB ratio were recorded is 3.48% in November, 2009, monsoon, which may be due to the proliferation of heterotrophic bacteria **Table1 (see supplementary material)** & **(Figure 1 C).**

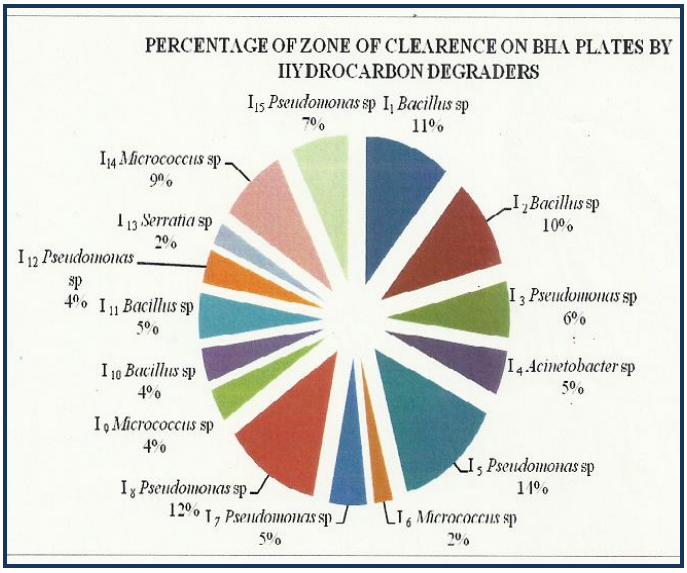

Figure 3: Percentage of zone of clearance on BHA plates by hydrocarbon degraders.

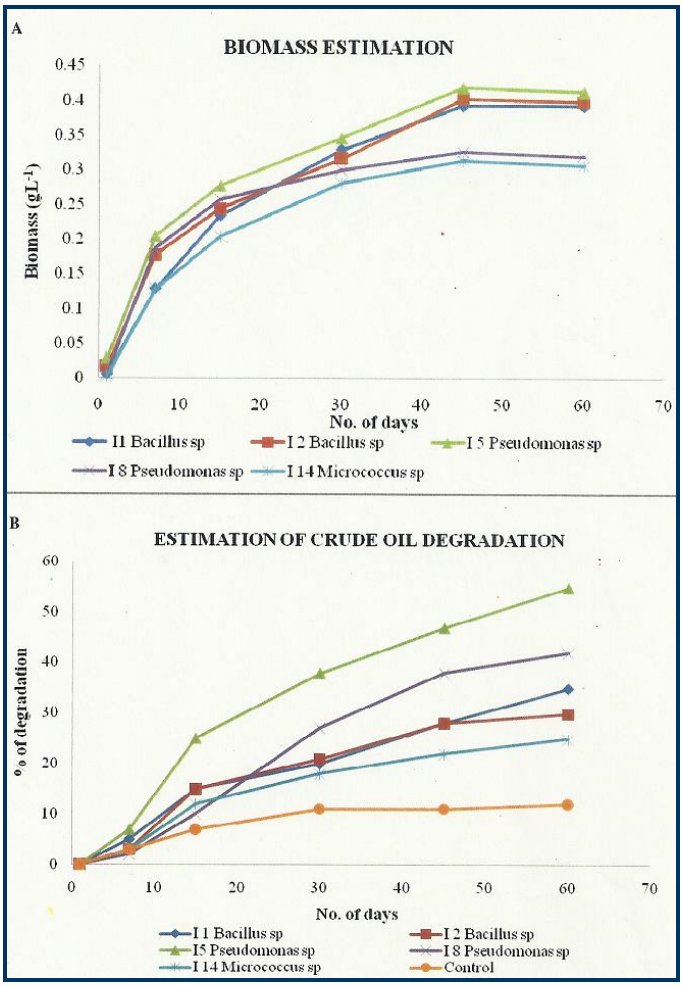

Figure 4: (A) Biomass estimation; **(B)** Estimation of crude oil degradation.

Identification of THB population

A total of 247 strains of heterotrophic bacteria were isolated. The bacterial flora in all the tested samples was predominated by gram-negative. In the samples collected 62.34% were gram-negative and 35.63% were found to be gram-positive. The gram-negative isolates mainly belonged to 4 genera repeatedly viz. *Pseudomonas sp*, *Vibrio sp*, *Achromobacter sp* and *Serratia sp*, accounting to 39.68, 15.78, 3.64 and 3.24% respectively. The gram-positive isolates belonged to 2 genera viz, Bacillus sp. and *Micrococcus sp.* accounting for 27.94 and 7.69% respectively **Table 2 & 3 (see supplementary material)** & **(Figure 2A)** Studies had reported on the isolation of 32 strains of bacteria from hydrocarbon contaminated soil and approximately 67% of them were gram-negative **[13].**

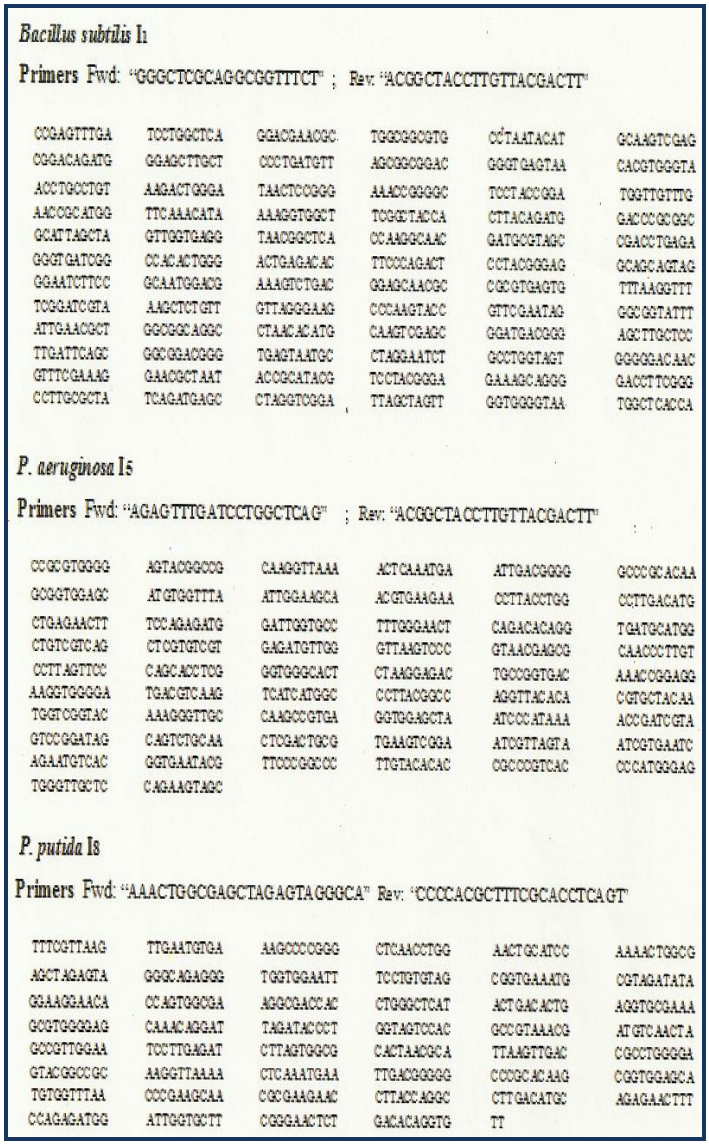

Figure 5: 16s RNA partial sequences.

Identification of HDB population

A total of 113 strains of hydrocarbon degrading bacteria were isolated from the surface sediment samples, during the period between July 2009 – June 2010, slightly predominated by gram-negative bacteria. In the samples collected 52.21% were gram-negative and 47.78% were found to be gram-positive. The gram-

negative isolates mainly belonged to 3 genera viz. *Pseudomonas sp, Achromobacter sp* and *Serratia sp,* accounting to 38.94, 5.31 and 7.96% respectively. The gram-positive isolates belonged to 2 genera viz, *Bacillus sp.* and *Micrococcus sp.* accounting for 35.39 and 12.39% respectively **Table 2 & 3 (see supplementary material)** & **(Figure 2B)**

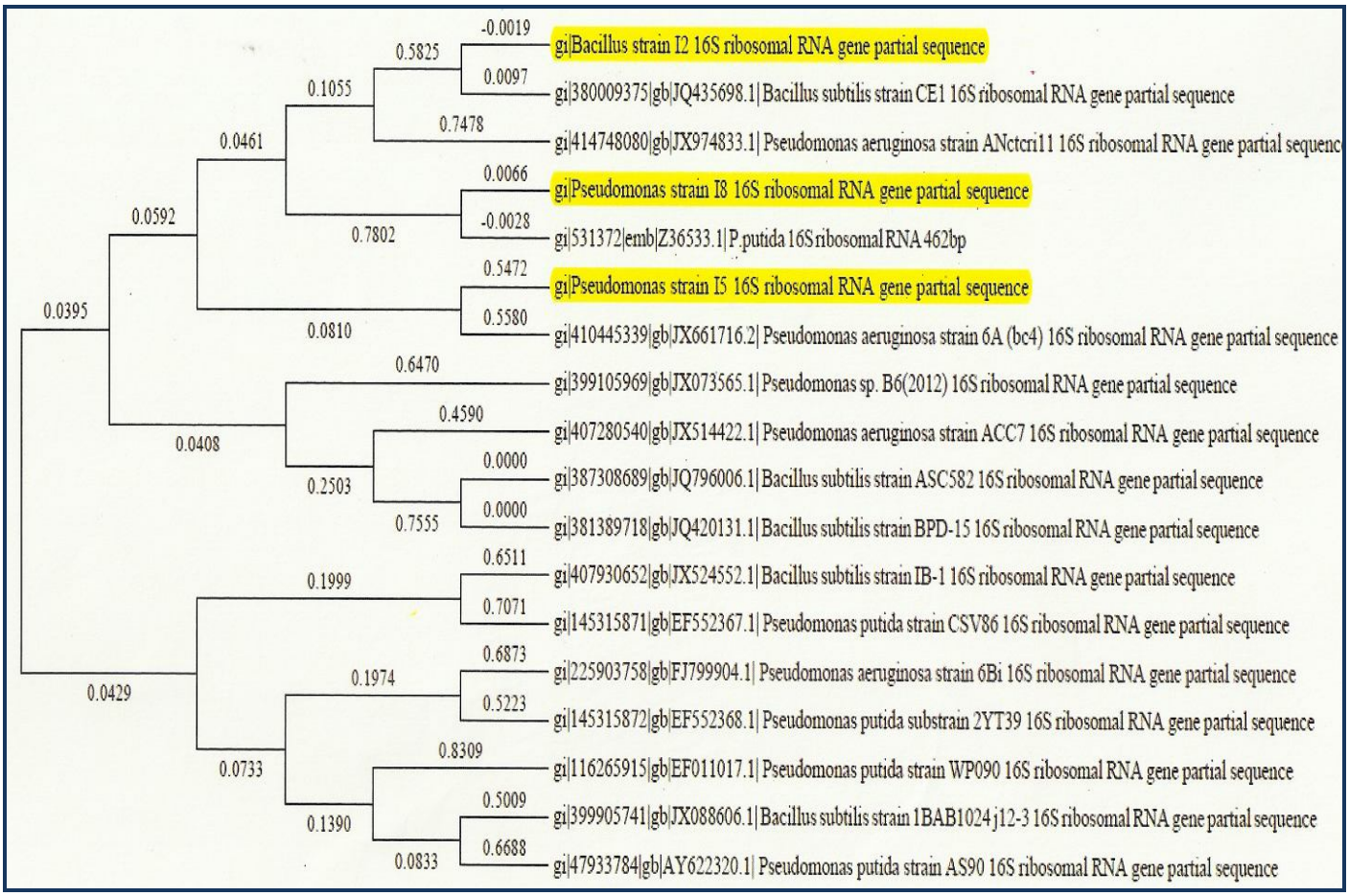

Figure 6: Molecular phylogenetic ananlysis by maximum likelihood method

To date, genera belonging to *Pseudomonas, Micrococcus, Sphingomonas, Bacillus* and *Mycobacterium* have been characterized and reported in the literature as hydrocarbon degrading strains. Studies on biodegradation of the toxic polycyclic aromatic hydrocarbon, by an indigenously isolated *Alcaligenes faecalis* MVMB1strain, from a petroleum contaminated site of Ennore creek **[14].** Similarly *P. putida* was isolated from the oil-contaminated sites of BHEL Tiruchirapalli, Tamilnadu, India **[15].** Pseudomonas sp. as the predominant (64%) crude oil degrading microbe was isolated from oil polluted sites from Cuddalore fishing harbor **[10].**

Screening of crude oil degraders

15 isolates of 113 hydrocarbon degraders, were found to maintain its crude oil degrading ability, which was established by the isolates growing both on BHA with 100µl of crude oil and on NA plates. The efficiency to degrade was recorded by the zone of clearance exhibited by the degraders on BHA paltes. The zone of clearance, shown a minimum of 2 mm, by *Serratia sp* and a maximum of 11mm, by *Pseudomonas sp. Pseudomonas sp* found to predominate the group by 40%, followed by *Bacillus sp.* 26.67%, *Micrococcus sp.* by 20% and lastly by *Serratia sp.* and *Achromobacter sp.* by 6.7%. Five isolates, I1, I2, I5, I8 and I14 were found to exhibit maximum clearing zone, **Table 4 (see supplementary material)** & **(Figure 3)** compared to the remaining isolates.

Selection of efficient degraders

Biomass concentration

The growth of the 5 isolates shows **Table 5 (see supplementary material)** & **(Figure 4A)** that all the isolates found to have their exponential phase between 7 and 45 days and a stationary phase between 45 and 60 days.

Crude oil degradation

Estimation of crude oil degradation by gravimetric analysis for the five isolates has shown **Table 6 (see supplementary material)** & **(Figure 4B)** that, higher degradation rate, 55% was observed by I5, *Pseudomonas sp*. and the least by I14 *Micrococcus sp*., 25%.

Molecular characterization

The 16s RNA partial sequencing **(Figure 5)** of the 3 different bacterial generae viz., 1 *Bacillus sp.* and 2 *Pseudomonas sp.* had shown that the organisms were identified as *B. subtilis* (I1), *P. aeruginosa* (I5) and *P. putida* (I8). The sequences aligned with the BLAST search of NCBI data bases were found to show 98% similarity for all the three strains. Bacterial strain PS-I isolated

from soil samples collected from oil production site of Oil and Natural Gas Commission (ONGC) was identified as *P. putida* with 97% similarity by 16s rDNA analysis with blast search of NCBI, RDP and microseqTM **[16].** Studies on 16s RNA gene sequence of the isolate VITDDK3 from Ennore creek were aligned with ClustW data base and the strain was designated as *Streptomyces sp* **[17].**

Phylogenetic analysis

The systematic phylogenetic tree **(Figure 6)** of *Bacillus sp* (I1), *Pseudomonas sp* (I5) and *Pseudomonas sp* (I8) and other 15 strains with high homology was constructed. Evolutionary analyses were conducted in MEGA5.1. The evolutionary history was inferred using the Neighbor-Joining method .The optimal tree with the sum of branch length = 11.01557767 are shown (next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of transversional substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 462 positions in the final dataset.

Gene bank, NCBI accession number

JQ062961 was the accession number obtained from Gene bank, NCBI, USA for the partial sequence of the 16s RNA, *Pseudomonas aeruginosa* I5 isolate.

Conclusion:

The understanding of the influence of different perturbations on the fate of the released oil in marine environment is useful in the assessment of the environmental impact of oil released and its remedial investigation. The present study monitored the isolation and identification of the heterotrophic and hydrocarbon degrading microbes of the crude oil contaminated Ennore creek area. *P.aeruginosa* JQ062961 was identified as the efficient degrader among the various strains isolated which can be applied towards oil discharge and spill treatment. However, further scale-up studies as applicable need to be carried out in increasing the degrading ability and stability of the crude oil degrading isolate and its usage as a possible commercial strain.

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Supplementry material:

Methodology:

Estimation of crude oil degradation

The percentage of degradation was calculated as shown in

Wt. of residual oil = Wt. of the container with extracted crude oil - Wt. of empty container.

Amount of crude oil degraded = Wt. of crude oil added in media - Wt of residual crude oil.

$$\text{Percentage of degradation} = \frac{\text{Amount of crude oil degraded} \times 100}{\text{Amount of crude oil added in media.}}$$

Table 1: Total Heterotrophic and hydrocarbon degrading bacterial count

S.NO	MONTH	THB (x 10^5 CFU g^{-1})	HDB (x 10^4 CFU g^{-1})	(HDB/THB RATIO x 100)
1	Jul-09	29.4	12.1	4.12
2	Aug-09	32.4	15.8	4.88
3	Sep-09	26.1	9 .9	3.79
4	Oct-09	19.4	7.3	3.76
5	Nov-09	20.4	7.1	3.48
6	Dec-09	21.6	10.6	3.58
7	Jan-10	19.3	8.2	8.82
8	Feb-10	17.7	9.8	12.73
9	Mar-10	12.21	5.7	47.11
10	Apr-10	12.8	9.2	32.86
11	May-10	14.6	7.12	15.48
12	Jun-10	18.2	15.31	12.55

Table 2: Identification of bacterial isolate

S.no.	Biochemical reactions	*Bacillus* sp	*Micrococcus* sp	*Pseudomonas* sp	*Vibrio* sp	*Acinetobacter* sp	*Serratia* sp
1	Gram reaction	+ve rods	+ve cocci	-ve rods	–ve rods	-ve short rods	–ve short rods
2	Motility	Motile	Non-motile	Motile	Motile	Non-motile	Motile
3	Indole	-ve	-ve	- ve	+ ve	- ve	-ve
4	Methyl Red	-ve	-ve	- ve	+ ve	- ve	+ve
5	Voges-Proskauer	+ve	-ve	- ve	+ ve	- ve	+ve
6	Citrate	+ve	+ve	+ ve	+ ve	+ ve	+ve
7	Urease	-ve	+ve	+ ve	- ve	- ve	-ve
8	Nitrate	+ve	-ve	+ve	+ ve	- ve	+ ve
9	Triple sugar iron	*	*	K / K	A / K, No gas, No H_2S	K / K, No gas, No H_2S	A / A, No gas
10	Catalase	+ve	+ve	+ ve	- ve	+ ve	+ve
11	Oxidase	+ve	+ve	+ ve	+ ve	- ve	-ve
12	OF test	Oxidative	Oxidative	Oxidative	Fermentative	Oxidative	*
13	Casein Hydrolysis	+ve	-ve	+ve	*	*	+ve
14	Glucose	NF	NF	- ve	Acid, No gas	+ ve	F with gas
15	Mannitol	F	NF	NF	+ ve	- ve	+ve
16	Maltose	+ve	+ve	NF	+ ve	-ve	+ve
17	Lactose	NF	NF	NF	NF	+ ve	NF
18	Starch hydrolysis	+ve	-ve	-ve	*	*	-ve
19	Sucrose	-ve	+ve	-ve	+ ve	- ve	+ve
20	Mannose	+ve	-ve	-ve	+ ve	+ ve	+ve
21	L-Arabinose	+ve	+ve	-ve	- ve	+ ve	-ve
22	Lysine	-ve	*	+ve	- ve	-ve	+ve
23	Arginine	-ve	-ve	+ve	- ve	-ve	+ve
24	Ornithine	-ve	*	+ve	- ve	-ve	-ve

+ve – positive; -ve - negative; A-acid; K- alkali; F- fermentative; NF- non-fermentative; H2S- Hydrogen Sulphide; * - Not applicable

Table 3: Total Heterotrophic and Hydrocarbon degrading Bacterial flora

HETROTROPHIC BACTERIAL FLORA (x 10^5 CFU g^{-1})										HYDROCARBON DEGRADING BACTERIAL FLORA (x 10^4 CFU g^{-1})									
S.NO	MONTH	*BACILLUS* SP	*MICROCOCCUS* SP	*PSEUDOMONAS* SP	*VIBRIO* SP	*ACINETOBACTER* SP	*SERRATIA* SP	Total no. of isolates identified	Unidentified isolates	S.NO	MONTH	*BACILLUS* SP	*MICROCOCCUS* SP	*PSEUDOMONAS* SP	*ACINETOBACTER* SP	*SERRATIA* SP	Total no. of isolates identified
1	**Jul-09**	6	2	10	8	1	1	28	1	1	**Jul-09**	5	1	4	1	1	12
2	**Aug-09**	7	2	12	7	1	1	30	2	2	**Aug-09**	5	1	7	1	1	15
3	**Sep-09**	8	1	11	4	1	1	26	0	3	**Sep-09**	3	1	3	1	1	9
4	**Oct-09**	6	1	7	4	1	0	19	0	4	**Oct-09**	3	0	3	0	1	7
5	**Nov-09**	6	3	8	1	1	1	20	0	5	**Nov-09**	3	1	2	1	0	7
6	**Dec-09**	8	2	11	3	1	2	27	2	6	**Dec-09**	3	2	4	0	1	10
7	**Jan-10**	5	0	8	4	1	1	19	0	7	**Jan-10**	0	2	5	0	1	8
8	**Feb-10**	5	2	6	3	0	1	17	0	8	**Feb-10**	4	2	2	0	1	9
9	**Mar-10**	3	2	4	2	1	0	12	0	9	**Mar-10**	2	0	3	0	0	5
10	**Apr-10**	5	1	5	1	0	0	12	0	10	**Apr-10**	2	2	4	1	0	9
11	**May-10**	4	2	7	1	0	0	14	0	11	**May-10**	3	1	1	1	1	7
12	**Jun-10**	6	1	9	1	1	0	18	0	12	**Jun-10**	7	1	6	0	1	15
Total		69	19	98	39	9	8	242	5	**Total**		40	14	44	6	9	
Percentage %		27.94	7.69	39.68	15.78	3.64	3.24	**247**		**Percentage %**		35.39	12.39	38.94	5.31	7.96	**113**

Table 4: Growth (620 nm) and Biomass concentration of isolates

S.no	Isolate no.	Organism	Zone of clearance (mm)
1	I_1	*Bacillus* sp	9
2	I_2	*Bacillus* sp	8
3	I_3	*Pseudomonas* sp	5
4	I_4	*Acinetobacter* sp	4
5	I_5	*Pseudomonas* sp	11
6	I_6	*Micrococcus* sp	2
7	I_7	*Pseudomonas* sp	4
8	I_8	*Pseudomonas* sp	10
9	I_9	*Micrococcus* sp	3
10	I_{10}	*Bacillus* sp	3
11	I_{11}	*Bacillus* sp	4
12	I_{12}	*Pseudomonas* sp	3
13	I_{13}	*Serratia* sp	2
14	I_{14}	*Micrococcus* sp	7

Table 5: Growth (620 nm) and Biomass concentration of isolates

S.NO.	DAY(s)	ISOLATES									
		I_1		I_2		I_5		I_8		I_{14}	
		OD	gL^{-1}	OD	gL^{-1}	OD	gL^{-1}	OD	gL^{-1}	OD	gL^{-1}
1	1	0.02	0.007	0.05	0.017	0.09	0.03	0.02	0.007	0.01	0.003
2	7	0.39	0.129	0.54	0.178	0.62	0.205	0.57	0.188	0.39	0.129
3	15	0.71	0.234	0.74	0..244	0.84	0.277	0.78	0.257	0.62	0.204
4	30	1.00	0.33	0.96	0.317	1.05	0.347	0.91	0.3	0.85	0.281
5	45	1.19	0.393	1.22	0.403	1.27	0.419	0.99	0.327	0.95	0.314
6	60	1.19	0.393	1.21	0.399	1.25	0.413	0.97	0.32	0.93	0.307

Table 6: Crude oil degradation of isolates

S.NO.	ISOLATE	DAYS - % OF DEGRADATION					
		1	7	15	30	45	60
1	I1	0	5	15	20	28	35
2	I2	0	3	15	21	25	30
3	I5	0	7	25	38	47	55
4	I8	0	2	10	27	38	42
5	I14	0	3	12	18	22	25
6	Control (Abiotic loss)	0	3	7	11	11	12