TITLE: Identification & Isolation of Hydro Carbon Bacteria From the Soils Contaminated Industrial Effluents

REVIEW OF THE LITERATURE

Review by Claude E. Zobell 1946 and Koren et.al 2003 recognized that many microorganisms have the ability to utilize hydrocarbons as a sole source of carbon and energy. Biodegradation of petroleum in natural environments depends on the nature of the soil, nature of microbial communities and the environmental factors that influence microbial activities. Hydrocarbon degrading bacteria and fungi are widely distributed in marine, fresh water and soil habitats. (Atlas R. M.1981).

Petroleum is a complex mixture composed primarily of aliphatic, alicyclic and aromatic hydrocarbons which influences the biodegradability of individual hydrocarbons. (Moshrafuddin Ahmed and S. K Basumatary 2006). Walker and Colwell in their study observed that the greater rates of mineralization were observed for bacteria and samples collected from oil polluted harbor than for samples collected from relatively unpolluted area.(walker & Colwell 1976).

Dua M, Singh (2002) and Atlas (1978) found that bioremediation process rely on the ability of microorganisms which are present naturally and highly efficient due to their simplicity and cost-effectiveness when compared to other technologies. Bioremediation is a mineralization of organic chemicals, leading ultimately to the formation of CO_2, H_2O and biomass .But the rate of degradation of hydrocarbons in nature is limited due to their hydrophobic property which leads to their limited solubility in ground water and tendency to partition to the soil matrix.

It had been proposed by Kerry 1990 that bacteria are major colonizers of oil contaminated soils and contain non aqueous phase liquid in the form of droplets on soil surfaces. Studies have shown that most of the microorganisms produce biosurfactants of diverse chemical nature and molecular size with effective surface active and biologic properties. (Desai 1999; Ron & Rosenberg 1999, 2001).

Biosurfactants help to disperse hydrocarbons and increase their solubilisation and subsequently stimulate the growth of bacteria and responsible for bioremediation. (Ron & Rosenberg 2002; Hommel, R.K., 1990; Desai, J.D., Banat, I.M., 1997). Physical and chemical methods are expensive and time consuming to reduce hydrocarbon pollution than biological methods. (Bonnier et.al1980, EI – Nawawy et.al 1987; Rhykerd et.al 1998).

The microorganisms degrade hydrocarbons by producing enzymes in their metabolism that can help in cleaning up of contaminated sites and that could be measured by monitoring of hydrocarbon disappearance rate. (Atlas 1981, Atlas & Bartha 1992 & Steffan et.al 1997, Alexander 1999).

Lyle G et.al. (1997) found that microorganisms are able to biodegrade the various components of petroleum hydrocarbons such as poly nuclear aromatic hydrocarbons (PAHs), naphthalene, monoaromatic hydrocarbons such as toluene & aliphatic hydrocarbons such as the *n*-alkanes. The microorganisms can be readily isolated from the environment, particularly from the petroleum contaminated sites.

It was reported that the hydrocarbons were originated biologically due to their short and long chain hydrocarbons (alkanes: C10 –C40) and from the sedimentary material of marine environment which consists of dead microbial cells. Furthermore a variety of microorganisms undergoes biochemical changes in the sedimentary rock that associate with the formation of petroleum (Surridge, 2007 & M. Ahmed 2006). The international traffic of oil in supertankers with the occasional accidents result for oil spills. Hydrocarbon contamination is harmful to the life of plants, animals and are carcinogenic, mutagenic & potent immune toxicants to human health also. (Atlas,1981 ; LIebeg & Cutright ,1999; Vasudevan and Rajaram ,2001).

Gold (1985) reported that petroleum and coal contain molecules known as hopanoids which are the molecules commonly present in the bacterial cell walls indicate that the origin of these fuels in part from microorganisms .The hydrocarbons would be converted by the microorganisms from hazardous form into less or non toxic form by which petroleum and diesel products are removed from the environment inexpensively.(Flood gate 1984, Leahy and Colwell 1990 ;Lidderdale ,1993).

Ismail saadoun (2002) reported that Pseudomonas putida , P. mallei and Enterobacter cloacae degrade diesel fuel and were isolated from the soil samples exposed to crude petroleum oil spills. A test was conducted to detect the biodegradation of diesel by monooxygease pathway using an approach of detection of alcohol production as a result of alkane oxidation. (Jacobs et.al 1983).

Pseudomonas strains capable of degrading oil , alkanes and aromatics were studied by Mittal & Singh (2009) .They found that Pseudomonas strains are potent aromatic hydrocarbon degraders than Bacillus and used gas chromatographic analysis of crude oil and its fractions to determine biodegradability.(Dubey RC 2009 & Boboye et.al 2010).

Teli Nikhil et.al (2013) identified and isolated Micrococcus sp and Pseudomonas sp that degrade diesel engine oil from the garage soil contaminated with petroleum .It was found that the degradation rate was high for pseudomonas and showed higher rates of degradation for the mixture of both bacterial isolates of Pseudomonas and Micrococcus a single organism.

Jyothi K et.al (2012) examined the biodegradability of hydrocarbons (Petrol and diesel) by different bacteria such as Bacillus megaterium, B.cereus ,Micrococcus luteus, Staphylococcus aureus, Lactobacillus acidophilus, Neisseria flavescence and Corynebacterium xerosis . These bacteria were screened for the presence of hydrocarbon degrading enzyme catechol 2, 3 dioxygenase by DNA isolation and amplification of gene by PCR.

Zhao Dongfeng et .al (2011) reported that many environmental factors like P^H 6-8, quantity of the inoculum(3%), substrate concentration, Nitrogen and phosphorus , low concentration of metal ions(Fe^{+2}, Mg^{+2}, and Ca^{+2}) influence& improve the degradation of petroleum hydrocarbons by Bacillus fusiformis isolated from the oil contaminated soil in the Karamay oilfield.

Oliveira et.al 2012 reported that biodegradability of nonadecanoic acid and Squalene by 10 different genera of Marinobacter , Halomonas ,Citricella ,Achromobacter , Stenotrophomonas , Bacillus , Staphylococcus , Micrococcus, Kocuria and Streptomyces belonged to three phyla Proteobacteria, Firmicutes and Actinobacteria isolated from oil and water samples of petroleum reservoirs of campos basin (Brazil) and screened them by Amplified Ribosomal DNA Restriction analysis (ARDRA).Bacillus Pumilus had shown the degradation of nonadecanoic acid and squalene in the percentage of 63% and 79% respectively. They found the occurrence of Bacillus safensis in petroleum associated environments. Saisa-ard et al. (2013) found that Psychrobacter adeliensis DSM 15333T as capable organism to degrade turbine oil that consists of recalcitrant cycloalkanes and isoalkanes.

Imneh Amini et.al (2015) reported the degradation of Polycyclic aromatic hydrocarbons of Phenanthrene, Anthracene and pyrene by Sphingobium xenophagum, Bacillus pumilus and Pseudomonas plecoglossicida and found the production of dioxygenase enzyme and biosurfactants.

PRESENT WORK

Introduction:

Hydrocarbons are the world's most widely used primary energy and fuel resources, due to the energy they produce. Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon as energy for metabolic activities and these micro organisms are omni present and widely distributed in the nature. The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinants[1]. Hydrocarbons enter into the environment through waste disposal, accidental spills, as pesticides and via losses during transport, storage, and use[2]. Diesel engine oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. With the combined dependence on diesel engine oil by some vehicles and generators, greater quantities are being transported over long distances. Therefore diesel engine oil can enter into the environment through wrecks of oil tankers carrying diesel oil, cleaning of diesel tanks by merchants, war ships carrying diesel oil and motor mechanics[3].

Diesel oil spills on agricultural land generally reduce plant growth. Suggested reasons for the reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants[4] and reduced germination to unsatisfactory soil condition due to insufficient aeration of the soil because of the displacement of air from the space between the soil particles by diesel engine oil[5].

In the soil, petroleum hydrocarbons pose a problem since the evaporation is limited and photo oxidation does not occur. Biodegradation by naturally occurring bacteria is very attractive but has limitations due to limited

[1] Adeline,S.Y.Ting et.al (2009) Hydrocarbon degradation by P.lundensis
[2] Atlas R.M.(1978)
[3] HillG.B., Moxey J.G(1980)Petroleum product Handbook,Mc-Grew hill
[4] Baker J.M.,(1982) Mangrove swamps & oil industry
[5] ZahirA.Z.,et.al (2001)Journel of Environmetal Quality

oxygen availability and nutrients such as nitrate and phosphate .Free oxygen is crucial for hydrocarbon degradation by oxygenases. The breakdown of spilled hydrocarbons on land can be greatly enhanced by occasionally tilling the soil to keep aerated and by fertilizing with nitrogen and phosphorus while keeping the conditions moist but not wet .The two methods can be used to increase oxygen supply which had some success are injection of oxygen saturated water and water enriched with hydrogen peroxide[6].

Biodegradability of petroleum hydrocarbons depends primarily on chemical structure and also influenced by its toxicity & the physical state of the compound. N-alkanes, n-alkyl aromatic and aromatic compounds of the C10-C22 range are the least toxic to microorganisms and are the most degradable, whereas the same types of compounds in the C5-C9 range have high solvent-type membrane toxicity and are biodegradable only in very low concentrations[7]. Biodegradation is carried out largely by diverse bacterial populations, mostly by *Pseudomonas* species[8].

The bacteria capable of degrading components of diesel fuel can be isolated and screened to determine their ability to grow in the presence of diesel fuel as a sole source of carbon .The purpose of this research was to identify the feasibility of bacteria growing in soils of industrial area and also from soils of petrol pumps that degrade hydrocarbons like petrol and diesel and to detect the presence of catechol 2,3 dioxygenase enzyme in the identified bacteria.

[6] Bartha.R(1986)Microbial Ecology 12:155-172
[7] Haines &Bartha.(1974) Applied Microbiology 28(6)
[8] Dubay RC (2009) Textbook of Biotechnolgy

MATERIALS AND METHODOLOGY

Sample collection

The study includes the collection of three types of soil samples from the industrial area and from Indian oil petroleum booths of Patancheru to isolate the hydrocarbon degrading bacteria. Soil sample extending from the ground surface to a depth of 10–20 cm were collected in sterilized plastic containers from the Industrial areas of GVK , Biological E limited(BE) (effluent after treatment) near parker and petroleum booths of Patancheru . Patancheru is one of largest industrial zone, located in Medak district of Telangana. The soil samples were duly labeled and transported to the laboratory aseptically and stored at 4^0c for further analysis**.**

Isolation of bacterial cultures

One gram soil sample from each source was suspended and vortexed with 10ml sterile distilled water .The suspension was allowed to settle down and 5 ml of supernatant was used as inoculum in 100ml Mineral salts broth containing 1% Petrol and diesel added separately in each flask. The flasks were incubated for 48 hrs at 37 ^{0}c on a rotary shaker at 100rpm .The broth was centrifuged at 5,000 rpm for 15 min and the cell pellets were obtained. The cell pellets were washed with 0.1 m phosphate buffer solution (P^H 6.8)[9]. The cell pellets were used to inoculate Bushnell Hass (BH) medium supplemented with 1 % hydrocarbons (petrol and diesel) as a single carbon source. Composition of BH medium is $MgSO_4$.7H20 -0.2 g ;K_2HPO4 -1g ;KH_2PO4 -1g; $FeCl_3$ -0.05 g;NH_4NO_3- 1g ;$CaCl_2$ -0.02g, P^H -7.2 : Agar -20g ;Distilled water -1000ml.

[9] Geetha S.J. et.al (2013) Isolation of hydro Carbon degrading bacteria from oil contaminated sites.

The medium without hydrocarbon was sterilized by autoclaving at 121^0c for 15 min.The medium was supplemented with 1% filter sterilized hydrocarbons (petrol and diesel) to serve as only source of carbon and energy[10]. The medium was incubated at 37^0 c for 10-15days. The bacterial colonies were appeared after 5-7days of incubation and were identified by cultural characteristics, staining and biochemical tests. The pure and representative colonies were sub cultured onto nutrient agar slants and preserved at 4 ^{0}c in refrigerator.

Determination of isolates ability to grow on hydrocarbons of both petrol and diesel :

Diesel and petrol obtained from Patancheru were sterilized by filtration, size of 0.2mm and used as a sole source of carbon to determine their ability of isolates for the growth. The cultures in logarithmic phase were used as inoculum for the experiment. The isolates were cultivated in 250ml Erlenmeyer flasks containing 50ml liquid mineral salt medium supplemented with 1% of petrol and diesel as a sole source of carbon and energy for their growth. The log phase of isolates was determined by growing the cultures in peptone broth incubated at 37^0c. The optical density was measured at 600nm for about an interval of 60 min (1 hr) and obtained the growth curve by plotting the optical density against time in hours. The log phase occurred approximately after 12 hrs of inoculation. The inoculum was obtained centrifuging the broth at 3000rpm for 15 min and the pellet was suspended in 5ml sterile saline. The loopful of culture was used to inoculate MS medium supplemented with 1% hydrocarbon (Petrol and diesel).

[10] B. Olukunle, et. Al (2010) degradative activity of bacteria from Hydro carbon Polluted site

IDENTIFICATION OF THE ISOLATES

Morphological characterization of isolates:

The isolated bacteria were examined after growth on nutrient medium for grams reaction and cell morphology .Some biochemical characteristic's of the cultures as growth on different carbon sources such as Glucose , Sucrose & lactose ,starch hydrolysis ,gelatin liquefaction, IMViC tests, Nitrate reduction, Catalase ,Production of H_2S,Growth on MacConkey agar, Mannitol salt agar and production of urease were studied according to Bergey's Manual of Determinative Microbiology[11].

The biochemical tests used to determine the metabolism of microorganisms by identifying certain enzymes. These are

- Starch hydrolysis test was used to determine the production of amylase enzyme which has the ability to degrade starch.
- Gelatinase test was used for the identification of proteolytic exoenzyme known as gelatinase which hydrolyses proteins into amino acids .The enzyme can be detected by observing the liquefaction of gelatin at low temperature 4^0 c.
- Urease test was used to identify the hydrolytic enzyme urease which splits the carbon and nitrogen bond amide compounds (urea) and liberate ammonia.
- Hydrogen sulphide production test was used to study the ability of microorganisms to reduce sulphur containing amino acids which can be detected by incorporating a heavy metal salt containing Fe^{+2} or lead ion as H_2S indicator to a nutrient medium containing cysteine and sodium thiosulfate as sulphur substrate.
- Catalase test was used for the enzyme Catalase which splits hydrogen peroxide to water and oxygen. Hydrogen peroxide is produced by the microorganisms in the presence of oxygen during aerobic respiration.

[11] Willam & Wilkins, Baltimore Bergey's Manual of determinative bacteriology .

Isolation of Genomic DNA for identification of the isolate:

1. 400µl of bacterial cells was taken (overnight culture) in eppendorf tube and centrifuged at 12,000 rpm for 30sec.The cell pellet was treated with 200µl of 1X PBS. The pellet was resuspended in 100µl of Buffer S1, Vortexed for 1 min to resuspend it completely. 5µl of Buffer SII was added and mixed by inverting the tube for 3-4 times. 37µl of Buffer SIII was added and mixed by inverting the tube for 3-4 times.
2. An equal amount of Buffer SIV (~150µl) was added and mixed by inverting the eppendorf for 3-4 times.
3. 30µl of DBM was added to clear the lysate and mixed by inverting the tube 3-4 times and kept for incubation at room temperature for 2-3 min followed by centrifugation at maximum speed for 30 sec.
4. The supernatant was discarded and the pellet was washed with 400µl of 70% ethanol.
5. Centrifugation was done at maximum speed for 30sec and 60 sec to remove the traces of 70% ethanol completely.
6. 15µl of Elution Buffer was added to the pellet, mixed and incubated at room temperature for 2-3 min.
7. After Centrifugation at maximum speed for 60 sec at room temperature the elute was stored at -20° C.

Amplification of 16S region of bacterial genomic DNA:

1. The DNA isolated was amplified using 16s rDNA universal primers - Bac8F & 1492 R and sequenced for the identification of bacterial strain at molecular level. Upon obtaining amplicons of 1.5kb (S1-S7) and 320bp (P2 & P4) the products were desalted (purified) and were sequenced.
2. The 16S r-RNA gene sequence of each sample was aligned with the database (**NCBI**) using an alignment tool- **BLAST** (Basic Local Alignment Search Tool).

Determination of bacterial biodegradative activity:

Turbidometry was used to determine bacterial growth by utilsing the hydrocarbons (1%petrol and diesel) given as carbon source in Mineral salts broth. The degrading activities of each isolate was obtained by using MS broth adding 1% of hydrocarbon (petrol & diesel) separately and kept for incubation in a shaker for about 25 days at 37^0.The growth of bacterium was monitored and measured by taking O.D readings at 595 nm from 0 hrs to 25 days at regular intervals of 3 days against mineral salt broth as blank[12]. The graph obtained indicates the growth pattern of organisms relates to the utilization of hydrocarbons in the medium.

[12] Nikhil et.al (2013) Isolation of degrading bacteria form garage soil.

RESULTS AND DISCUSSION

The hydrocarbon degrading bacteria were isolated from soils contaminated with industrial effluents and petrol pumps on the Bushnell Hass (BH) agar medium and Nutrient agar (NA) medium.10 colonies from samples of industrial area &5colonies showing different morphological characteristics on BH medium were selected for further characterization. Out of 10 cultures 5from industrial area & out of 5 cultures 2 from petroleum booth had shown the growth on mineral salt medium with 1% of hydrocarbons as sole carbon source namely petrol and diesel individually. The most potent bacterial petrol and diesel degraders were identified by observing morphological characters and biochemical tests. Different types of biochemical tests were done such as Gram's staining, Indole test, Methyl red test, VP test, Citrate utilization test, Urease test, Nitrate reduction test, Gelatinase test, starch hydrolysis test, Catalase test and H_2S production test etc. Morphological features include cell morphology, colony morphology and structural appearance.

Hydrocarbons can be toxic to microorganisms due to the solvent effects of diesel and petrol as they destroy bacterial cell membrane. The studies reported on diesel and petrol using the concentrations 0.5 to 1.5 %

Characteristics of organisms isolated from Industrial area:

S1, S2, S3, S4, S7 were isolated from the industrial area of Patancheru from the GVK, BE and Parker areas.

Table 1: Colony characteristics

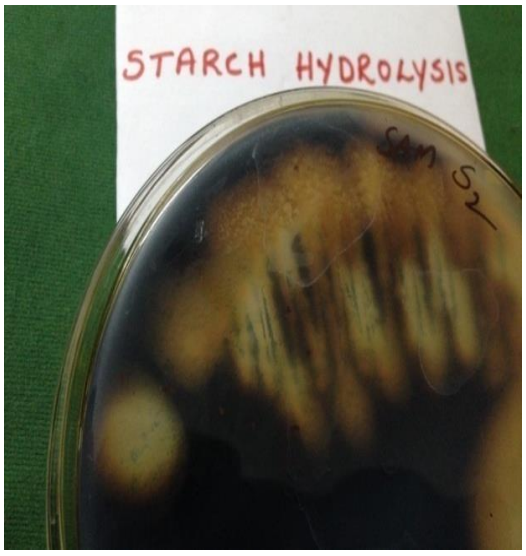
S.No	Character	S1	S2	S3	S4	S7
1	**Size**	Small	Small	Large	small	Medium
2	**Shape**	Circular	Irregular	Circular	Irregular	Circular
3	**Opacity**	opaque	opaque	opaque	opaque	opaque
4	**Margin**	Entire	wavy	Regular	undulate	undulate
5	**Elevation**	Raised	Raised	Convex	Rough	umbonate
6	**Texture**	Dry	Smooth	Moist	Dry	Dry
7	**Pigmentation**	Cream	Cream	White	White	Cream

Table 2: Morphological and biochemical characteristics of isolates

Feature	S1	S2	S3	S4	S7
Gram Stain	+ve	+ve	+ve	+ve	+ve
Shape	ROD	ROD	ROD	ROD	ROD
Spore	+ve	+ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve	+ve
Starch Hydrolysis	+ve	+ve	-ve	-ve	-ve
Indole	-ve	-ve	-ve	-ve	-ve
MR	-ve	-ve	-ve	-ve	+ve
VP	-ve	+ve	-ve	-ve	-ve
Glucose	A	NA	A	AG	AG
Lactose	NA	NANG	NA	NA	NA
Citrate	+ve	+ve	-ve	+ve	+ve
Nitrate reduction test	+ve	-ve	-ve	+ve	+ve
Gelatinase	+ve	+ve		-ve	+ve
H2Sgas production	-ve	-ve	-ve	-ve	-ve
Urease	-ve	-ve	-ve	-ve	+ve
Growth on MacConkey Agar	No growth	No growth	No growth	No growth	No growth

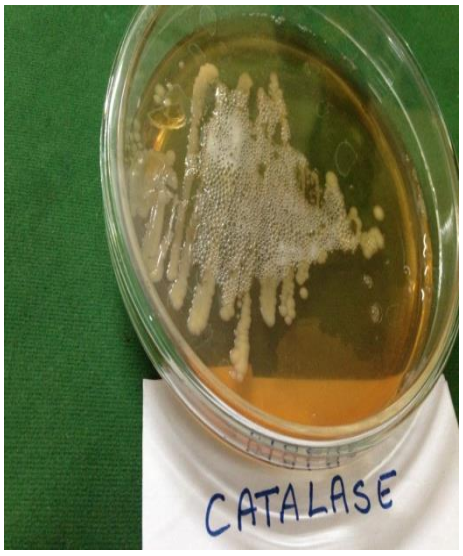
*NA-No Acid , NG –No Gas

FIGURES :
Results of positive reaction of the biochemical tests by the isolates



Zone of starch hydrolysis by S2

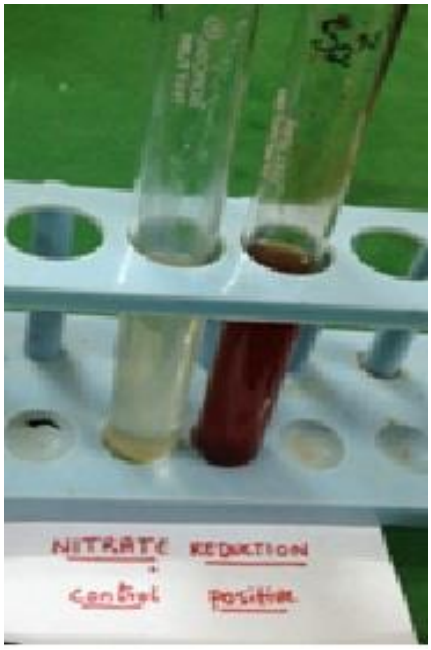
Fig 1a



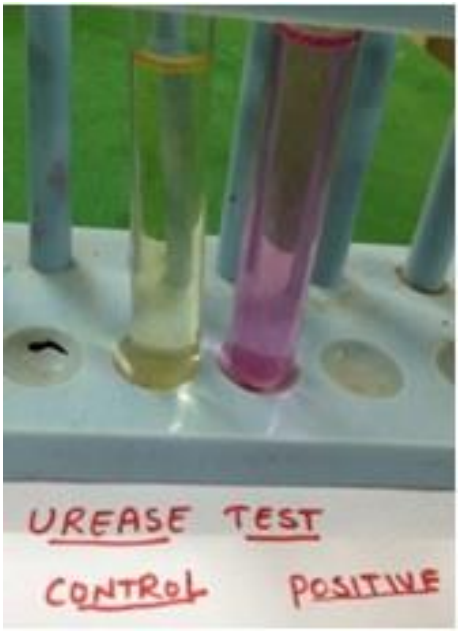
Positive reaction for catalase by S2

Fig1b

Results of positive reaction of the biochemical tests by the isolates



Positve reaction for Nitrate reduction by S2
Fig1c



Urease positive reaction by S7
Fig 2

Growth of S1 on NAM
Fig 3a

Citrate positive by S1
Fig 3b

16s rDNA sequence:

1. The genomic DNA from each sample was amplified for 16S r-RNA gene using universal primers- Bac8F and 1492R and obtained amplicons of 1.5kb.
2. The bacterial 16S r-RNA gene sequences of each sample was aligned with the database (**NCBI**) using an alignment tool- **BLAST** (Basic Local Alignment Search Tool).
3. The sequence alignment gave above 98% similarity with Bacillus subtilis, B.pumilis, B.altitudinis, B.licheniformis and B.safensis respectively.These results highlight the different species of Bacillus are able to degrade hydrocarbons.

Boboye,B etal (2010)reported the degradation of hydrocarbons by Bacillus species and other group of bacteria. Likewise Ojo (2006) reported hydrocarbon degradation by B.megaterium, B.brevis and B.pumilis. The ability of members of genus Bacillus of B.megaterium and B.cereus to degrade hydrocarbons of petrol and diesel has been reported by Jyothi etal (2012) and B.pumilis degrade diesel by Mandri &Lin (2007) and Singh et al (2008) , pyrene by khanna etal(2011) ,pyrene & phenanthrene by Yaliani etal(2012) and poly aromatic hydrocarbons. The species of B. fusiformis also degrade petroleum hydrocarbons provided ions and optimum conditions for their growth.

Fig4: DNA isolation from the isolated bacteria

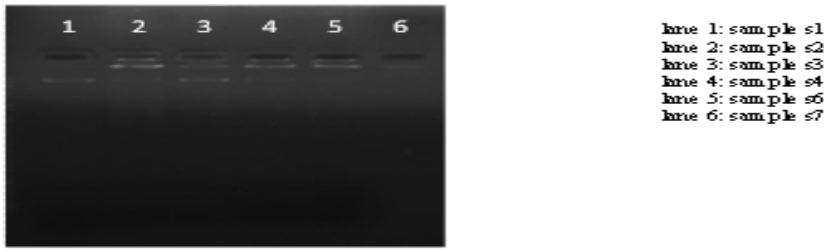


Fig 4a -Gel Image showing isolated genomic DNA

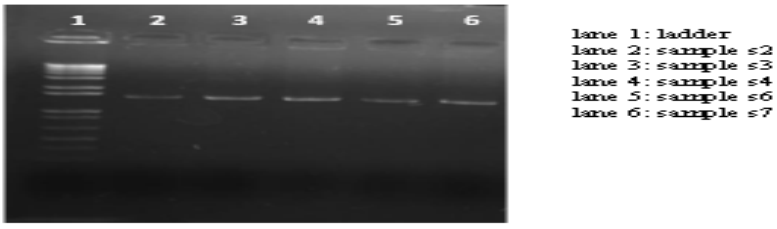


Fig 4b -Gel Image of 16S PCR amplicons 1.5 Kb

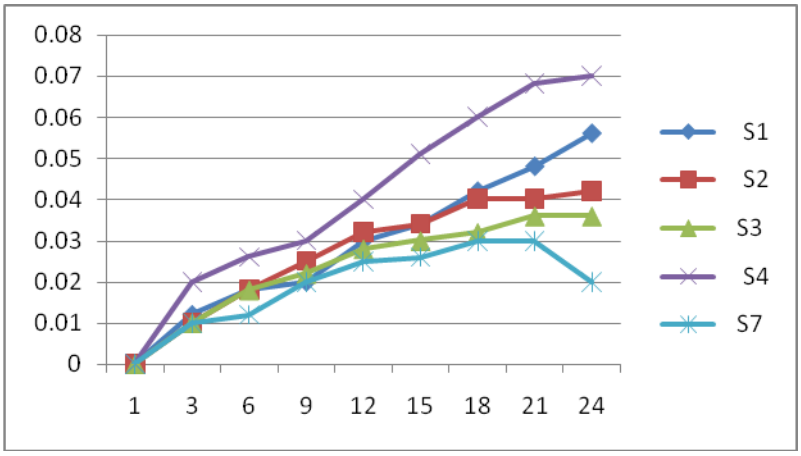
BIODEGRADATIVE ACTIVITY OF ISOLATES BY TURBIDOMETRY

Table 3: ***Growth curve readings at595nm for 25 days incubation***

Name of the organism	Hydro carbon degrading source	OD at zero hours	OD at 3th day	OD at 6th day	OD at 9th day	OD at 12th day	OD at 15th day	OD at 18th day	OD at 21th day	OD at 24th day
S1	Petrol	0.0	0.01	0.01	0.015	0.02	0.025	0.032	0.037	0.04
	Diesel	0.0	0.012	0.018	0.02	0.03	0.034	0.042	0.048	0.056
S2	Petrol	0.0	0.01	0.015	0.02	0.03	0.04	0.045	0.05	0.05
	Diesel	0.0	0.01	0.018	0.025	0.032	0.034	0.04	0.04	0.042
S3	Petrol	0.0	0.02	0.03	0.035	0.04	0.048	0.05	0.06	0.06
	Diesel	0.0	0.01	0.018	0.022	0.028	0.03	0.032	0.036	0.036
S4	Petrol	0.0	0.01	0.02	0.025	0.028	0.03	0.034	0.037	0.032
	Diesel	0.0	0.02	0.026	0.03	0.04	0.051	0.06	0.068	0.07
S7	Petrol	0.0	0.01	0.01	0.02	0.025	0.025	0.03	0.035	0.04
	Diesel	0.0	0.01	0.012	0.02	0.025	0.26	0.03	0.03	0.02

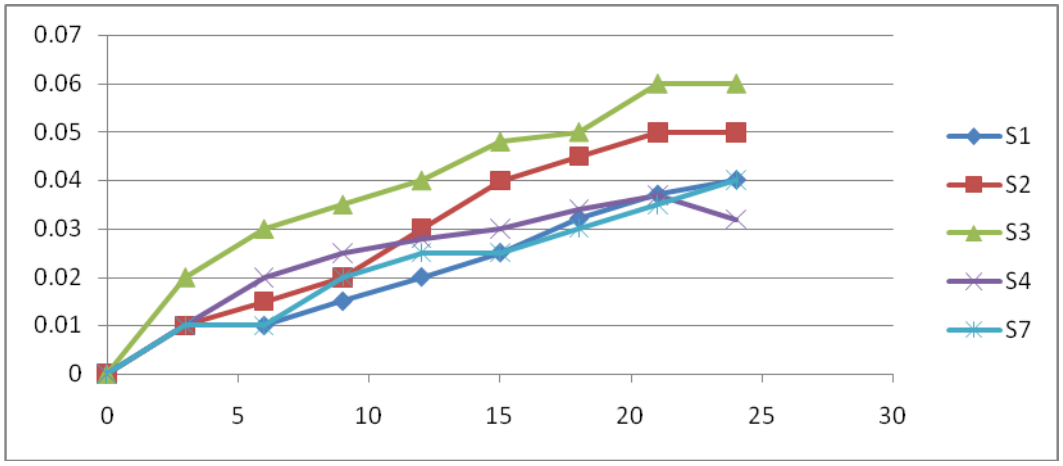
The above table shows the optical density of bacteria grown on media containing petrol and diesel separately.

Fig-5: Graph showing the growth curve (O.D) values of bacteria on petrol degrading broth for a period of 24 days of incubation.



S1-Bacillus subtilis, S2- Bacillus pumilis ,S3- Bacillus altitudinis, S4- Bacillus licheniformis, S7 Bacillus safensis.

Fig 6: Graph showing the growth curve (O.D) values of bacteria on diesel degrading broth for a period of 24 days of incubation.



S1-B.subtilis, S2-B.pumilis, S3-B.altitudinis, S4-B. licheniformis , S7-B.safensis

Table 4:

Identification of bacteria by 16s rDNA sequencing :

Name of the isolate	Organism Identified (PCR and BLAST)	Presence of Catechol 2,3 dioxygenase	KEGG identifier
S1	*Bacillus subtilis*	✓	K07104
S2	*Bacillus pumilus*	✓	K07104
S3	*Bacillus altitudinis*	✓	K07104 QR42_04240
S4	*Bacillus licheniformis*	✓	K07104
S7	*Bacillus safensis*	✓	QR42_04240 K07104

The above analyses show that all the given isolates Possess the gene coding for Catechol 2,3 dioxygenase. The identifiers or entry numbers can be used to retrieve the sequences for the gene in the respective organism.

Sequence alignment of S1 -:

Blast Hit list: The organism was found to share 98% similarities with **Bacillus subtilis.**

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus sp. HN91 16S ribosomal RNA gene, partial sequence	1666	1666	96%	0.0	97%	KF933650.1
Bacillus sp. 195(2010) 16S ribosomal RNA gene, partial sequence	1642	1642	91%	0.0	98%	HM011274.1
Uncultured prokaryote clone seq_K-R50_16SF 16S ribosomal RNA gene, partial sequence	1640	1640	91%	0.0	98%	KP409515.1
Bacillus altitudinis gene for 16S rRNA, partial sequence, strain: MKCM4002	1640	1640	96%	0.0	96%	AB691777.1
Bacillus subtilis strain BaAP3 16S ribosomal RNA gene, partial sequence	1640	1640	91%	0.0	98%	JQ734770.1
Bacillus altitudinis strain xfchu11 16S ribosomal RNA gene, partial sequence	1639	1639	91%	0.0	98%	GQ480481.1
Bacillus pumilus strain EI-14 16S ribosomal RNA gene, partial sequence	1639	1639	96%	0.0	96%	FJ613542.1

Sequence alignment of S2 -:

Blast Hit list: The organism was found to share 95% similarities with **Bacillus pumilis** for both forward & reverse primer.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus pumilus strain DDEN06 16S ribosomal RNA gene, partial sequence	1293	1293	90%	0.0	93%	KR234048.1
Bacillus pumilus strain DDEN05 16S ribosomal RNA gene, partial sequence	1293	1293	90%	0.0	93%	KR234047.1
Bacillus pumilus strain MTCC B6033, complete genome	1293	10305	81%	0.0	95%	CP007436.1
Bacillus pumilus strain RHS_06 16S ribosomal RNA gene, partial sequence	1293	1293	90%	0.0	93%	KF957734.1
Bacillus sp. S48 16S ribosomal RNA gene, partial sequence	1293	1293	81%	0.0	95%	KC466253.1
Bacillus pumilus strain CS_151 16S ribosomal RNA gene, partial sequence	1293	1293	81%	0.0	95%	JF899249.1
Bacillus sp. WJ03 16S ribosomal RNA gene, partial sequence	1293	1293	90%	0.0	93%	HM045827.1

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus sp. R30(2014) 16S ribosomal RNA gene, partial sequence	1458	1458	86%	0.0	95%	KJ373681.1
Bacillus pumilus gene for 16S ribosomal RNA, partial sequence, isolate: B0645	1458	1458	86%	0.0	95%	AB695335.1
Bacillus sp. KITA4-17 16S ribosomal RNA gene, partial sequence	1454	1454	86%	0.0	95%	KF032704.1
Bacillus pumilus strain B5 16S ribosomal RNA gene, partial sequence	1454	1454	86%	0.0	95%	KC596003.1
Bacillus pumilus strain MB7 NIOT 16S ribosomal RNA gene, partial sequence	1454	1454	86%	0.0	95%	HQ858063.1
Bacillus pumilus strain S811 16S ribosomal RNA gene, partial sequence	1454	1454	86%	0.0	95%	FJ485830.1

Upon identifying the organism with the help of BLAST- an alignment search tool, further searched for the presence of **Catechol 2,3, dioxygenase** gene in that organism, with a database called **KEGG** (Kyoto Encyclopedia for Genes and Genomes.

Sequence alignment of S3:

Blast Hit list: The organism was found to share 97% similarities with **Bacillus altitudinis.**

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus altitudinis strain SCSAAB0001 16S ribosomal RNA gene, partial sequence	1616	1616	95%	0.0	97%	JQ647873.1
Bacillus sp. strain TZQ22 16S ribosomal RNA gene, partial sequence	1620	1620	96%	0.0	96%	HQ143630.1
Bacillus sp. B2066 16S ribosomal RNA gene, partial sequence	1618	1618	96%	0.0	96%	JX266376.1
Bacillus sp. IHB B 2282 16S ribosomal RNA gene, partial sequence	1618	1618	96%	0.0	96%	HM233973.1

Sequence alignment of S4 :

Blast Hit list : The organism was found to share 96% similarities with **Bacillus licheniformis.**

Sequences producing significant alignments:

Select: All None Selected:0

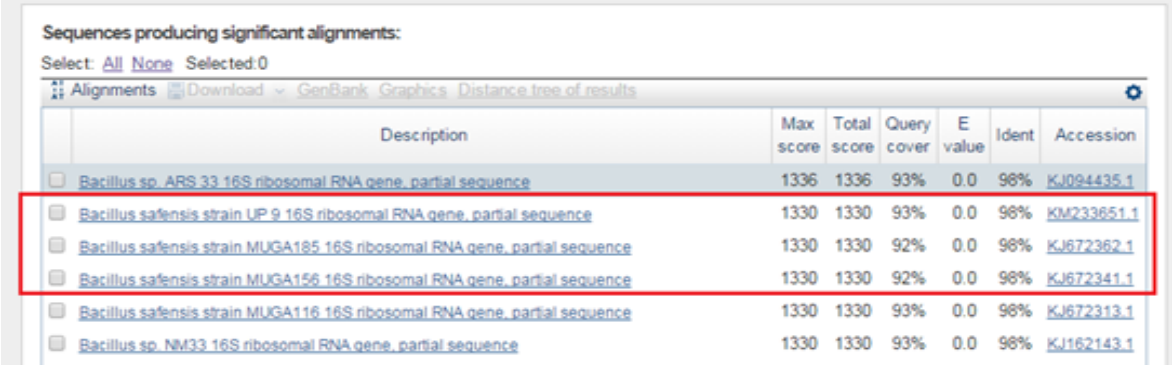
Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus licheniformis strain B-5 16S ribosomal RNA gene, partial sequence	1568	1568	90%	0.0	96%	KC834069.1
Bacillus licheniformis strain W36 16S ribosomal RNA gene, partial sequence	1568	1568	90%	0.0	96%	KC441846.1
Bacillus licheniformis strain B68 16S ribosomal RNA gene, partial sequence	1568	1568	90%	0.0	96%	KC441743.1
Bacillus aerius strain RS12 16S ribosomal RNA gene, partial sequence	1568	1568	90%	0.0	96%	JX472927.1
Bacillus licheniformis strain SANN8 16S ribosomal RNA gene, partial sequence	1563	1563	90%	0.0	96%	KP975925.1
Bacillus licheniformis strain AS4 16S ribosomal RNA gene, partial sequence	1563	1563	90%	0.0	96%	KJ729817.1

Upon identifying the organism with the help of BLAST- an alignment search tool, we further searched for the presence of **Catechol 2,3, dioxygenase** gene in that organism, with a database called **KEGG** (Kyoto Encyclopedia for Genes and Genomes)

Sequence alignment of S7:

Blast Hit list: The organism was found to share 98% similarities with **Bacillus safensis.**



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus sp. ARS 33 16S ribosomal RNA gene, partial sequence	1336	1336	93%	0.0	98%	KJ094435.1
Bacillus safensis strain UP 9 16S ribosomal RNA gene, partial sequence	1330	1330	93%	0.0	98%	KM233651.1
Bacillus safensis strain MUGA185 16S ribosomal RNA gene, partial sequence	1330	1330	92%	0.0	98%	KJ672362.1
Bacillus safensis strain MUGA156 16S ribosomal RNA gene, partial sequence	1330	1330	92%	0.0	98%	KJ672341.1
Bacillus safensis strain MUGA116 16S ribosomal RNA gene, partial sequence	1330	1330	93%	0.0	98%	KJ672313.1
Bacillus sp. NM33 16S ribosomal RNA gene, partial sequence	1330	1330	93%	0.0	98%	KJ162143.1

Characteristics of Organisms Isolated From Petroleum Booths:

P2&P4 was isolated from soil samples collected at Indian oil petroleum booths of Patancheru and Isnapur.

Table 5: Colony Characteristics of isolates

S. No	Character	P4	P2
1	**Size**	Small	Small
2	**Shape**	Circular	Round
3	**Opacity**	Transparent	Opaque
4	**Margin**	entire	rough
5	**Elevation**	Convex	umbonate
6	**Texture**	Dry	Dry
7	**Pigmentation**	yellow	Black/ white

Table 6: Morphological and biochemical characteristics of isolates.

S.No	Feature	P4	P2
1	Gram Stain	+ve	-ve
2	Shape	rods	Cocci
3	Spore	No	No
4	Catalase	+ve	+ve
5	Starch Hydrolysis	+ve	-ve
6	Indole	-ve	-ve
7	MR	+ve	-ve
8	VP	-ve	-ve
9	Glucose	A,NG	A,NG
10	Lactose	NA,NG	A,NG
11	Citrate	-ve	-ve
12	Nitrate reduction test	+ve	+ve
13	Gelatinase	+ve	-ve
14	H2S gas production	-ve	-ve
15	Urease	-ve	+ve
16	Growth on MacConkey Agar	No growth	No growth

Figure 7:

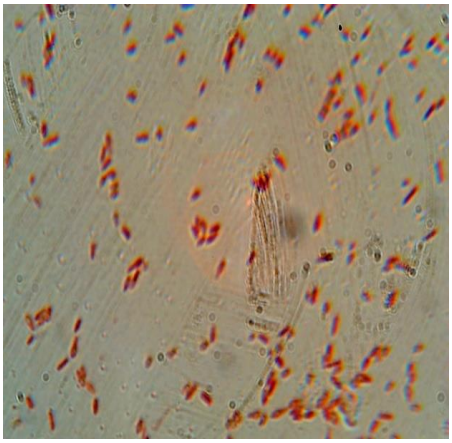


Fig 7a : Microscopic view of Aeromicrobium

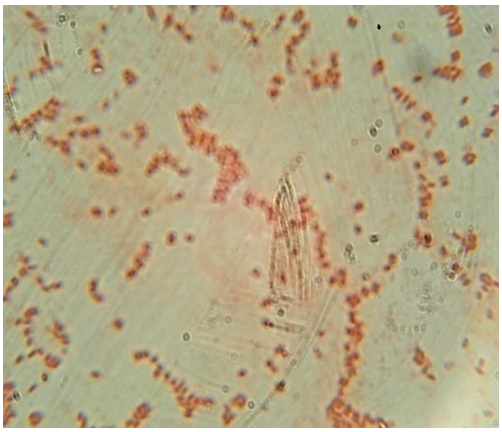


Fig 7b: Microscopic view of Psychrobacter

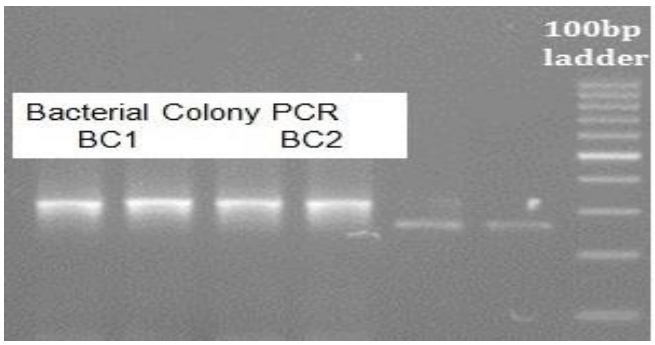
Positive reaction for urease by P2

Fig 7c

Positive reaction for Nitrate reduction byP2

Fig 7d

Figure 8:



Lane1 - BC1 PCR at 58 degrees C
Lane 2 - BC1 PCR at 62 degrees C
Lane 3 - BC2 PCR at 58 degrees C
Lane 4 - BC2 PCR at 62 degrees C
Lane 7 - 100bp ladder

Where BC1 is the P2 organism & BC2 is P4 at 58 º & 62 º C

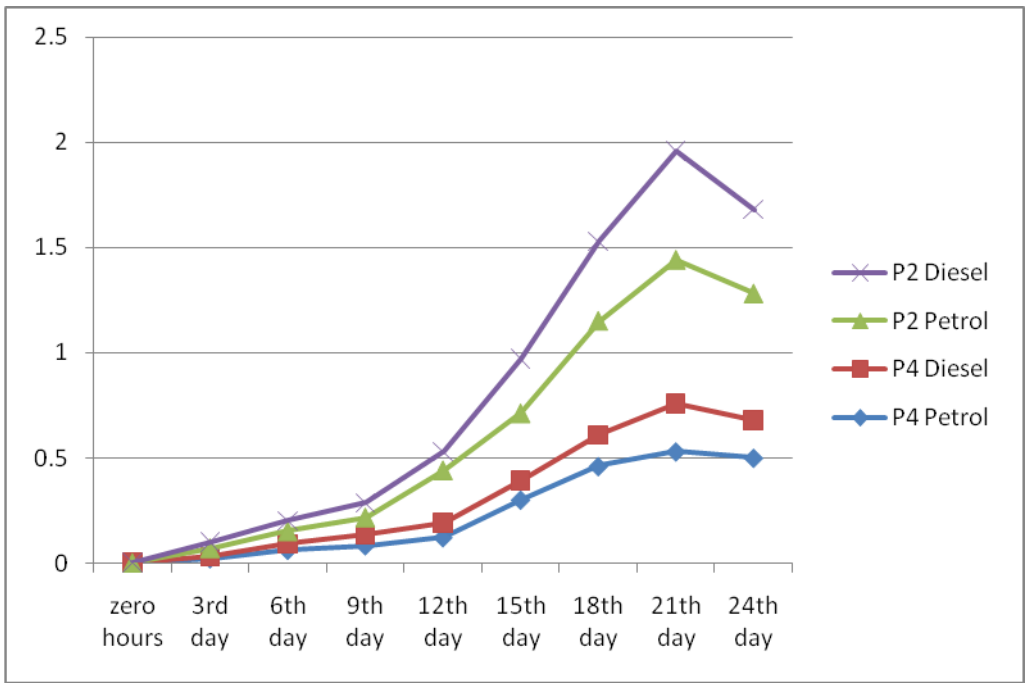
Gel image of 16 S PCR Amplicons of 320bp.

BIODEGRADATIVE ACTIVITY OF ISOLATES BY TURBIDOMETRY

Table 7: Growth curve readings at595nm for 25 days incubation

Name of the organism	Hydro carbon degrading source	OD at zero hours	OD at 3th day	OD at 6th day	OD at 9th day	OD at 12th day	OD at 15th day	OD at 18th day	OD at 21th day	OD at 24th day
P2	Petrol	0.0	0.02	0.06	0.082	0.12	0.30	0.46	0.53	0.5
	Diesel	0.0	0.01	0.03	0.052	0.07	0.091	0.15	0.23	0.18
P4	Petrol	0.0	0.01	0.04	0.082	0.2	0.30	0.5	0.68	0.6
	Diesel	0.0	0.01	0.02	0.06	0.09	0.10	0.2	0.32	0.2

Fig 9: Graph showing the growth curve (O.D) values of bacteria on diesel and Petrol degrading broth for a period of 24 days of incubation.



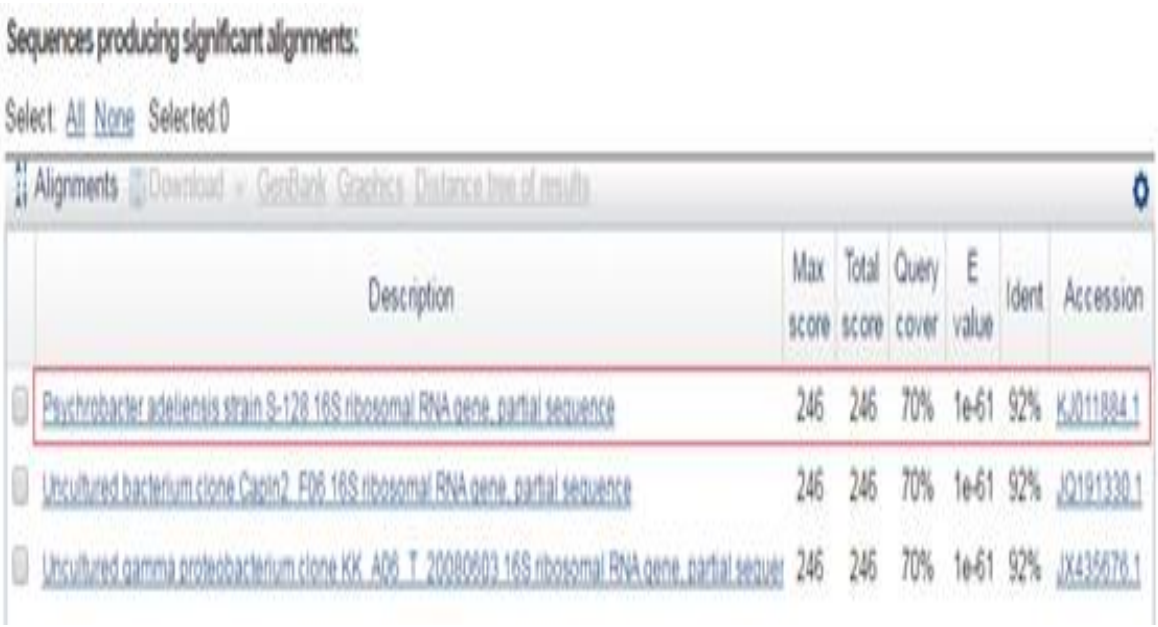
The above Graph shows the more Growth for P2 than P4 in turbidometry.

Table 8 : Identification of bacteria by 16s rDNA sequencing

S NO	Name of the Isolate	Organism based on PCR & Blast
1	P2	Psychrobacter sps
2	P4	Aeromicrobium sps

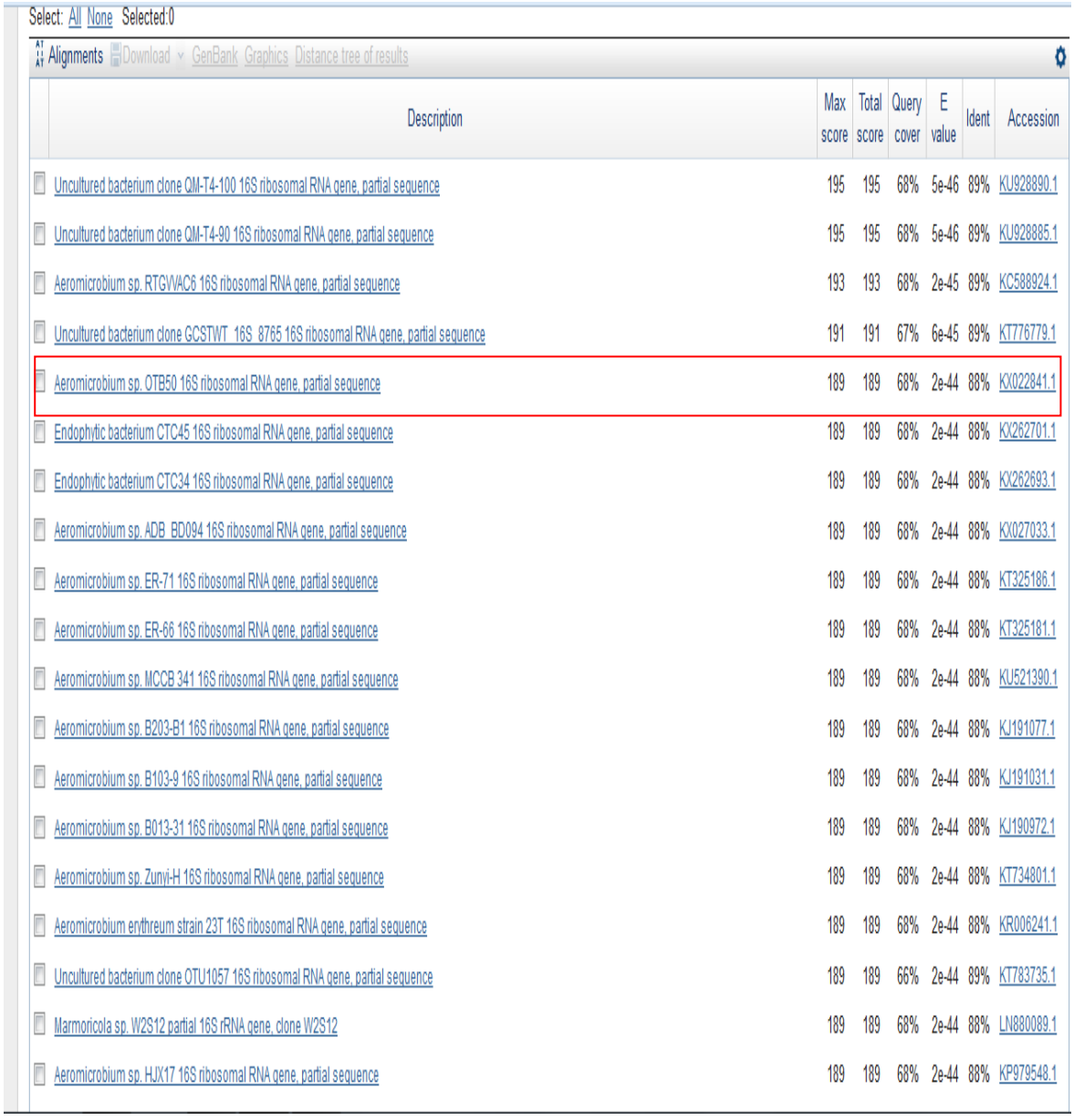
Sequence alignment of P2

Blast Hit list: The organism was found to share 92% similarities with **Psychrobacter sps.**



Sequence alignment of P4

Blast Hit list: The organism was found to share 89% similarities with **Aeromicrobium sps.**



Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured bacterium clone QM-T4-100 16S ribosomal RNA gene, partial sequence	195	195	68%	5e-46	89%	KU928890.1
Uncultured bacterium clone QM-T4-90 16S ribosomal RNA gene, partial sequence	195	195	68%	5e-46	89%	KU928885.1
Aeromicrobium sp. RTGWAC6 16S ribosomal RNA gene, partial sequence	193	193	68%	2e-45	89%	KC588924.1
Uncultured bacterium clone GCSTWT_16S_8765 16S ribosomal RNA gene, partial sequence	191	191	67%	6e-45	89%	KT776779.1
Aeromicrobium sp. OTB50 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KX022841.1
Endophytic bacterium CTC45 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KX262701.1
Endophytic bacterium CTC34 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KX262693.1
Aeromicrobium sp. ADB_BD094 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KX027033.1
Aeromicrobium sp. ER-71 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KT325186.1
Aeromicrobium sp. ER-66 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KT325181.1
Aeromicrobium sp. MCCB 341 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KU521390.1
Aeromicrobium sp. B203-B1 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KJ191077.1
Aeromicrobium sp. B103-9 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KJ191031.1
Aeromicrobium sp. B013-31 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KJ190972.1
Aeromicrobium sp. Zunyi-H 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KT734801.1
Aeromicrobium erythreum strain 23T 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KR006241.1
Uncultured bacterium clone OTU1057 16S ribosomal RNA gene, partial sequence	189	189	66%	2e-44	89%	KT783735.1
Marmoricola sp. W2S12 partial 16S rRNA gene, clone W2S12	189	189	68%	2e-44	88%	LN880089.1
Aeromicrobium sp. HJX17 16S ribosomal RNA gene, partial sequence	189	189	68%	2e-44	88%	KP979548.1

Conclusion:

The ability of native bacteria to utilize petrol and diesel as a sole source of carbon and energy were investigated in this study. The study produced promising results where in the 6 isolates of Genus Bacillus, Aeromicrobium and Psychrobacter sps were characterized. These isolates have been identified and characterized based on the cultural, staining, biochemical and genetic i.e. 16s RNA analysis as Bacillus subtilis, B.pumilis, B.altitudinis, B.licheniformis and B.safensis, Psychrobacter adeliensis and Aeromicrobium sps respectively. These have been shown as of special relevance as petrol and diesel degrading microbes. some of the strains of genus Bacillus were identified in this study through qualitative analysis as promising strains for petrol and diesel degradation .It is evident from this experiment that the efficiency degradation of hydrocarbons both petrol and diesel was more for Psychrobacter than Bacillus . The study gives a focus for the optimization of above cultures for the effective application of these strains to be used at large scale in industries.

This study showed that the environment contaminated with petroleum components contains the microbial population with effective degradability for hydrocarbons than those of microbes from unpolluted area, which could further help in disappearance of those hydrocarbons from the environment.

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March, 2017

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02 MAR 2017

Sub: Release of Grants-in-aid to **The Principal TARA GOVT. COLLEGE SANGAREDDY MEDAK502001.** Under the Scheme "**Minor Research Projects**" - Reg.

Sir/Madam,

On the basis of the accounts received for the grant released earlier under the scheme, I am directed to convey the sanction of the Commission for the payment of Rs.95917. to **The Principal, TARA GOVT. COLLEGE SANGAREDDY MEDAK 502001.** as final instalment towards the Minor Research Project entitled **IDENTIFICATION & ISOLATION OF HYDRO CARB NG BACTERIA FROM THE SOILS CONTAMINATED INDUSTRIAL EFFLUENTS** submitted by **MRS DEVAVANI Department of MICROBIOLOGY** as per the details given below:-

Item	Allocation (Rs.)	Amount already released (Rs.)	Amount sanctioned now (Rs.)	Total grant sanctioned/released so far (Rs.)
Hiring Services	0 0	0 0	0 0	0 0
Contingency	30000.	15000.	15000.	30000.
Chemicals	150000.	75000.	75000.	150000.
Travel/Field Work	15000.	7500.	5917.	13417.
Total	**195000.**	**97500.**	**95917.**	**193417.**
Equipment	0 0	0 0	0 0	0 0
Books	15000.	15000.	0 0	15000.
Total	**15000.**	**15000.**	**0 0**	**15000.**
Grand Total	**210000.**	**112500.**	**95917.**	**208417.**

1. The grant is debitable to following head of account.

Amount Sanctioned	Head of Accounts	Category
Rs.95917.	31-GIA-MRP(50)-3(B)2202.03.789.27.01	SC

2. The sanctioned amount is debitable to the **Head of Account 31-GIA-MRP(50)-3(B)-2202.03.789.27.01 (SC)** and is valid for payment during the financial year 2016-17 Only and the amount of the Grant shall be drawn by the Accounts Officer (Drawing and Disbursing Officer) UGC-SERO, Hyd. on the Grants-In Aid Bill and shall be disbursed to and credited to "**The Principal, TARA GOVT. COLLEGE, SANGAREDDY, MEDAK by Electronic Mode through PFMS Portal at the following details:**"*(a)Name & Address of Account Holder: The Principal, TARA GOVT. COLLEGE, SANGAREDDY, MEDAK (b) Account No: 62061242507(c) Name & Address of Bank Branch: STATE BANK OF HYDERABAD, SANGREDDY (d)IFSC Code:SBHY0020107*
3. The Grant is Subject to the adjust on the basis of Utilization Certificate in the prescribed Proforma submitted by the Institution.
4. The Institution shall maintain proper accounts of the expenditure out of the Grants which shall be utilized only on the approved items of expenditure.
5. The institution may follow the **General Financial Rules, 2005** and take urgent necessary action to amend their manuals of financial procedures to bring them in conformity with GFRs, 2005 and those don't have their own approved manuals on financial procedures may adopt the provision of GFRs 2005 and instructions / Guidelines there under from time to time.
6. The Utilization Certificate to the effect that the grant has been utilized for the propose for which it has been sanctioned shall be furnished to UGC as early as possible after the close of current financial year.
7. The assets acquired wholly or substantially out of UGC's Gant shall NOT be disposed or encumbered or utilized for the proposes other than those for which the grants was given without proper sanction of the UGC and should at any time the Institution ceased to function, such assets shall revert to the University Grants Commission.
8. A Register of Assets acquired wholly or substantially out of the Grant shall be maintained by the Institution in the prescribed proforma.