Isolation and Characterization of Petroleum Hydrocarbon Degrading Bacteria (PHDB) from Petroleum Contaminated Marine Environment at Mirkarwada, Ratnagiri

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Abstract: This study involves isolation and characterization of petroleum hydrocarbon degrading bacteria, by employing selective media compositions of Bushnell Hass Mineral Salt Medium along with the primary substrate followed by checking their tolerance cum degradation ability on 15 different petroleum derivatives. The isolates were screened at each step based on their abilities of degrading the substrates. Also, all the isolates were checked for presence of plasmid DNA, and were identified tentatively.

Term words: Petroleum hydrocarbon degradation, Bushnell Hass Medium, Plasmid, Tolerance, Degradation Ability, Pollution, Growth curve, etc.

I. INTRODUCTION

Several kinds of natural and manmade activities contribute to water pollution. The seas are highly susceptible for pollutants due to manmade activities like use of fishing trawlers, transportation of the fossil fuels and handling errors. Thus, by many means these petroleum compounds are liberated into the seas where they accumulate and pollute the area disturbing the local ecology and the livestock depending on that particular water body. There are several measures taken so as to deal with these issues of petroleum pollutants, but employing the biological agents for the degradation of the pollutants proves to be the most promising technique of dealing with these problems. The major reasons behind these are that the biological agents like the bacteria are fairly omnipresent and as they come in direct contact with these pollutants frequently they eventually develop such a metabolism that they can utilize the pollutants in which they persist for their growth and development (Desai and Vyas, 2006). There are number of pathways with different enzymes and the metabolic fates followed by the microorganisms for the degradation of these hydrocarbons (Das and Chandran, 2010). Unlike the other methods of dealing with the problems caused by the spillages of petroleum hydrocarbons, microbial use in degradation of such pollutants, in *in-situ* conditions too, does not affect the environment (Sekar, 2011).

Due to the increasing usage of these compounds causing increased pollution, researchers are now concentrating on isolation of such microorganisms especially bacteria for the degradation of petroleum hydrocarbons (Atlas, 1981). Taking these issue as a lead an effort was thus made for the isolation and characterization of the indigenous PHDB from Mirkarwada, Ratnagiri.

II. MATERIALS AND METHODS

The petroleum contaminated water sample was collected by means of sterile Eppendorf tubes from Mirkarwada Jetty (17.000720 ^{o}N and 73.279545 ^{o}E), Ratnagiri. The collected samples were employed for enrichment in two 100 ml Erlenmeyer's flask, each containing 50 ml of Bushnell Hass Broth Medium (Bushnell and Haas, 1941) along with 1% Petrol and 1% Diesel, respectively, on shaker at 100 rpm for about 10 days at room temperature.

The enriched sample was then spread plated on sterile Bushnell Hass Agar plated with similar substrates as that for enrichment along with one in combination of both (Petrol + Diesel). The colonies with distinct morphologies were then selected and streaked to get pure isolates. The isolates were then named after the substrate bearing plates from which they were isolated, *viz* P-Petrol, D- diesel, and PD-Petrol + Diesel. The isolates were then maintained on Nutrient agar slants and supplemented with the petroleum substrates so as to check their stability. Here, the stable isolates were then taken up for the checking of their tolerance cum degradation ability studies, for which they were spotted on the Bushnell Hass agar medium plates containing 15 different petroleum derivatives like Toluene, Benzene, Phenol, p-Nitrophenol, liq. Paraffin, Methyl Benzoate, Xylene, Benzoic Acid, α-Napthol, α -Napthylamine, o-Nitrophenol, Sodium Benzoate, 1,10-Dihydro-9-oxoanthracene, Phenylene diamine, β-Napthol.

The isolates showing best results were selected for checking presence of plasmid DNA by

Alkaline lysis method. Later, the isolates were employed for their tentative identification by referring the Bergey's Manual for Determinative Bacteriology. Further, after checking for the plasmid DNA, growth schematics of the isolates was monitored in the presence of 1% Benzoic acid for over 15 hours by checking absorbance at $A_{530\ nm}$ and then were represented graphically.

III. RESULTS AND DISCUSSION

The results and their interpretation were done simultaneously as they were observed. As for the enrichment and the screening protocols Bushnell Hass medium was used along with the petroleum product as the substrate. It confirmed that the substrate provided acted as the sole carbon source for the bacteria to grow as there are no other sources present in the media composition initially (Varjani and Upasani, 2013). At the first stage of screening in all 27 isolates were studied, which on screening further the number of isolates was reduced to 22 from which 13 had petrol degrading ability, 5 showed diesel degrading ability and the rest 4 showed petrol+ diesel degrading ability.

After checking for the colony morphology and interpreting them, it was found that most of the isolates were Gram negative short rods in their cellular characteristics. This may be due to the fact that cell wall composition helps the organism to tackle the harsh surrounding conditions. When checking for Tolerance cum Degradation ability studies, these 22 isolates were spot inoculated on 15 different petroleum derivatives as Toluene, Benzene, Phenol, p-Nitrophenol, liq. Paraffin, Methyl Benzoate, Xylene, Benzoic Acid, α-Napthol, α-Napthylamine, o-Nitrophenol, Sodium Benzoate, 1,10-Dihydro-9-oxoanthracene, Phenylene diamine, β -Napthol referring to the growth of bacterial isolates (Prakash, 2014). In selection of these substrates various properties were taken into consideration, like some of them are much complex (e.g. in tricyclic ketone form), some are organics fluorescent compounds, some are reduced derivatives, some are esterified organics, aromatic carboxylic acids, dimethyl benzene isomers, carbolic acids, potent carcinogens, organic solvents and also mineral oils. Thus, degradation was studied for every hazardous product of petroleum and diesel.

In this study, it was observed that 4 of the bacterial isolates were able to degrade all the 15 petroleum derivatives followed by 6 of the isolates with the ability for degrading 14 substrates, 6 isolates utilised 13 substrates, 3 used 12 substrates while 3 isolates had ability to degrade 11 substrates.

From this observation, 10 isolates were selected having ability to utilize and degrade maximum substrates then isolates were then employed for further studies.

TABLE I: Tolerance cum Degradation Ability (I)

Isolates	Petroleum Derivatives						
	1	2	3	4	5	6	7
D 1.1	+	+	+	+	+	+	+
D 1.2	+	+	+	+	+	+	+
D 2.1	+	+	+	+	+	+	+
D 2.2	+	+	-	+	+	+	+
D 3.1	+	+	+	+	+	+	+
D 4.1	+	+	+	+	+	+	+
D 4.3	+	+	-	+	+	+	+
D 5.2	+	+	+	+	+	-	+
D 6.1	+	+	+	+	+	+	+
D 7.1	+	+	+	+	+	-	+
D 7.2	+	+	+	+	+	-	+
D 8.1	+	+	-	+	+	+	+
D 9.1	+	+	+	+	+	+	+
P 1.1	+	+	+	+	+	+	+
P 2.1	+	+	+	+	+	+	+
P 3.1	+	+	+	+	+	+	+
P 4.1	+	+	+	+	+	+	+
P 4.2	+	+	+	+	+	+	+
PD 1.1	+	+	+	+	+	+	+
PD 2.1	+	+	+	+	+	+	+
PD 3.1	+	+	+	+	+	+	+
PD 4.1	+	+	-	+	+	-	+

Key: +, Positive growth; -, negative growth;
Petroleum Derivatives: 1-Toluene; 2-Benzene; 3-Phenol; 4- p-Nitrophenol; 5- liq. Paraffin; 6- Methyl Benzoate; 7- Xylene

TABLE II: Tolerance cum Degradation Ability (II)

Isolates	Petroleum Derivatives							
	8	9	10	11	12	13	14	15
D 1.1	+	+	+	+	+	+	+	-
D 1.2	+	+	+	+	+	+	+	-
D 2.1	+	+	+	+	+	+	+	+
D 2.2	+	+	+	+	+	+	+	+
D 3.1	+	+	+	+	+	+	+	+
D 4.1	+	+	+	+	+	+	+	-
D 4.3	+	+	+	+	+	+	+	+
D 5.2	+	+	+	+	+	+	-	-
D 6.1	+	+	+	+	+	+	-	-
D 7.1	+	+	+	+	+	+	+	-
D 7.2	+	+	+	+	+	+	+	-
D 8.1	-	+	+	+	+	+	+	+
D 9.1	+	+	+	+	+	+	+	-
P 1.1	+	+	+	+	+	+	+	+
P 2.1	+	+	+	+	+	+	-	-
P 3.1	+	+	+	+	+	-	-	-
P 4.1	+	+	+	+	+	-	-	-
P 4.2	+	+	+	+	+	-	-	-
PD 1.1	+	+	+	+	+	+	-	-
PD 2.1	+	+	+	+	+	+	-	-
PD 3.1	+	+	+	+	+	+	+	+
PD 4.1	+	+	+	+	+	+	-	-

Key: +, Positive growth**; -,** negative growth;
Petroleum Derivatives: 8- Benzoic Acid; 9- α -Napthol; 10- α -Napthylamine; 11- o-Nitrophenol; 12- Sodium Benzoate; 13- 1,10-Dihydro-9-oxoanthracene; 14- Phenylene diamine;15- β-Napthol

Further, checking the presence of plasmid DNA was carried out to check whether the genes encoding the enzymes required for the hydrocarbon degradation are present on the plasmid while in some cases the genomic DNA bears the gene for encoding the enzymes (Peixoto, 2011).

The isolates in the lanes 2, 5, 7, 8, 10 corresponding to the D 1.2, D 3.1, D 6.1, D 9.1, PD 3.1 showed presence of plasmid, while the rest in the lanes 1, 3, 4, 6, and 9 corresponding to the isolates D1.1, D2.1, D2.2, D4.1, and P1.1 were found to be devoid of the plasmid DNA in them. These indicate that the enzymes essential for the active degradation of the petroleum hydrocarbons were present on the genomic DNA of the bacteria itself.

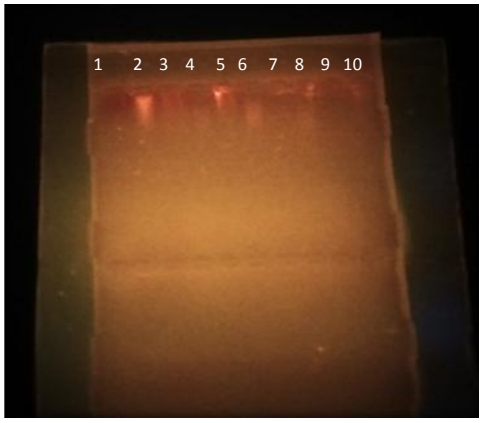
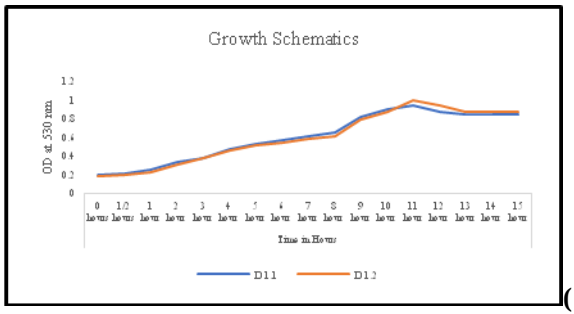
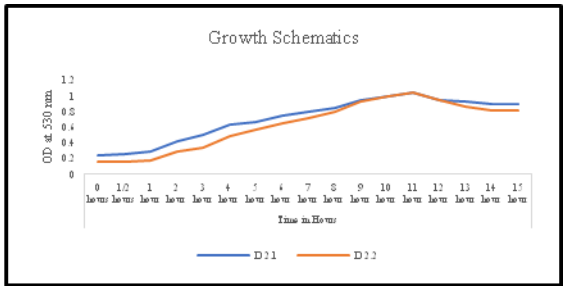


FIGURE I: Checking for Plasmid DNA

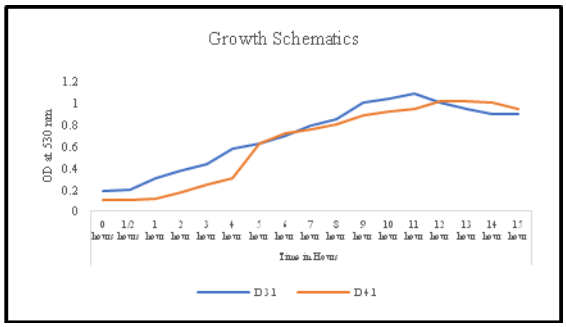
On checking for the presence of the plasmid DNA , growth schematics was studied in the presence of 1% Benzoic Acid at A_{530} nm. It was observed that the isolate PD 3.1 showed maximum growth at the given time span of 15 hour of observation. Making it more efficient in maintaining high cell density at toxic conditions too.



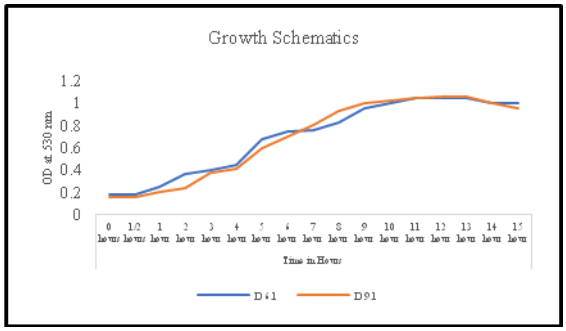
A)



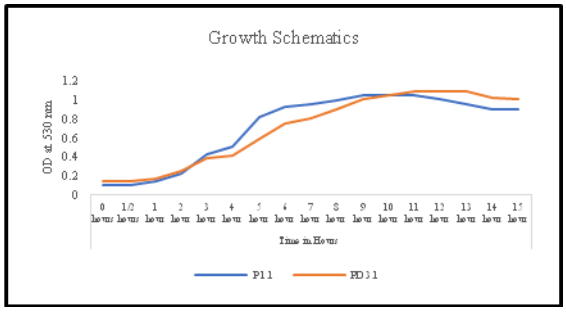
(B)



(C)



(D)



(E)

FIGURE II: A: Growth Schematics**:** for D 1.1 and D 1.2, **B**: Growth Schematics: for D 2.1 and D 2.2 , **C**: Growth Schematics: for D 3.1 and D 4.1, **D**: Growth Schematics:for D 6.1 and D 9.1, **E**: Growth Schematics: for P 1.1 and PD 3.1

These isolates were than tentatively identified using Bergey's Manual for Determinative Bacteriology. By using its flowcharts as reference, from the 10 isolates, selected 9 were found to be belonging to the genus *Pseudomonas* spp., while one is from the genus *Streptococcus* spp. Other workers have reported *Enterococcus, Vibrio, Achromobacter, Arthrobacter, Micrococcus, Corynebacter, Acinetobacter, Nocardia* (Desai and Vyas, 2006) to be involved in petroleum and diesel degradation.

IV. REFERENCES

[1]. Desai A. and Vyas P. 2006. Petroleum Microbiology. M.S. University of Baroda, Vadodara-390002.

[2]. Prakash A., Bisht S., Singh J., Teotia P., Ritukela, Vivekkuma., 2014, "Biodegradation potential of Petroleum hydrocarbons by bacteria and mixed bacterial consortium isolated from contaminated sites". Turkish journal of engineering and environmental sciences. 38: 41-50

[3]. Das N. and Chandran P. 2010. Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview, SAGE-Hindawi Access to Research Biotechnology Research International. Vol.2011, Article ID 941810: 13 pg.

[4]. Sekar. P. 2011. Efficacy of microbes in Bioremediation of tannery effluent, International Journal of Current Research, 3(4): 324 - 326.

[5]. Atlas R.M. 1981. Microbial Degradation of Petroleum Hydrocarbons: An Environmental Perspective, Microbiological Reviews. Vol.45, No. 1

[6]. Busnell D.L. and Hass H.F. (1941), The utilization of certain hydrocarbons by microorganism, Kansas Agriculture Experiment Station, 199, pp 653-673.

[7]. Varjani S.J., Rana D.P., Bateja S. and Upasani V.N. 2013. Isolation and Screening for Hydrocarbon Utilizing Bacteria (HUB) from Petroleum Samples. International Journal of Current Microbiology and Applied Sciences, 2(4): 48-60

[8]. Peixoto R. S., Vermelo A.B., Rosedo A.S.. 2011. Petroleum Degrading enzymes: bioremediation and new prospects. Enzymes and Researches, Volume, article ID 475193.