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DETERMINATION OF HYDROCARBON UTILIZATION BY PETROLEUM HYDROCARBON DEGRADING BACTERIA USING COLORIMETRIC TECHNIQUE

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ABSTRACT This study focuses on the ability of the bacteria to utilize the hydrocarbon (Benzoic Acid) a derivative of Benzene with respect to time, which is determined by using the redox potential of 2,6-DCPIP. Here, its property to change the color from blue to transparent when subjected to chemical reduction is used as a determinative feature like, the indicator, when oxidized is blue and when reduced is transparent. Thus, the rate of de-colorization is directly proportional to the utilization and oxidation of the hydrocarbons. Later, this color change is monitored by a visible spectrophotometer at 600 nm.

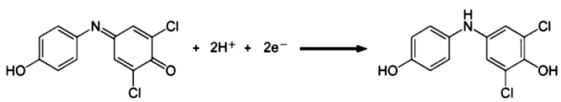
KEYWORDS : .

Introduction:

The increasing industrial development promotes serious environmental damage due to pollution of the environment. Regarding the petrochemical industry, contamination by oil and its derivatives causes the degradation of terrestrial and aquatic ecosystems. Thus, control and treatment strategies to combat the hazardous effects of oil pollution are needed (Winkelmann et al. 2009) Thus, for that, one must be well aware about various characteristic features of the microbial consortia or the culture used for the treatment of the issue caused. There are several aspects about a microbial sample to be considered (Aichberger et al., 2005) along with which the determination and knowledge of the rate of utilization of the hydrocarbons by the bacteria is essential too. To determine the rate of utilization, there are many promising techniques available and which are practiced now a day's worldwide. The DCPIP based colorimetric technique provides enough data on hydrocarbons used as metabolic substrates by microorganisms.

The concentration detection is possible due to the absorbance determination in a specified light specter. The 2,6-dichlorophenol indophenol (DCPIP) indicator is widely used in colorimetric processes. Its property is the color change from blue to transparent when subjected to chemical reduction. The indicator, when oxidized is blue and when reduced is transparent. The color change occurs due to a structural change in the molecule, in which the double bond between nitrogen and carbon passes to a simple bond.

Using this principle, the determination of the assay is designed in such a manner that, during the course of bacterial action on the petroleum derivative, which is 1% Benzoic acid here, being reduced in origin as the degradation action proceeds the oxidation of the substrate takes place leading to release of free electrons, which are accepted by 2,6-dichlorophenol indophenol which is an electron acceptor provided in our system.



2,6-DCPIP (oxidised) **Blue** 2,6-DCPIP (reduced) **Transparent**

FIGURE 1: 2,6-DCPIP Reduction Reaction

This leads to reduction of the dye, resulting in the disappearance of the blue color of the dye gradually as the oxidation of the substrate and reduction of the dye proceeds. Thus, the greater the and faster the substrate is utilized the more and rapid decolorization of the dye takes place (Bidoia et. al., 2010).

Along with rate of utilization many a times the determination of the metabolic pathway followed for the substrate utilization also plays a key role in dealing with the nuisance caused by the petrochemical wastes. For this, a chromogenic qualitative technique of enzymatic cleavage of Catechol by the enzymes Catechol-1,2- dioxygenase Catechol-2,3- dioxygenase is carried out. At initial cleavage if the cleavage of catechol takes place through *ortho* pathway by enzyme Catechol-1,2- dioxygenase, then *cis cis*- muconic acid is formed which is white in color.

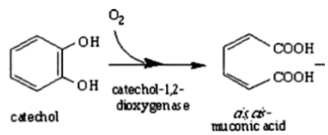


FIGURE 2: *ortho* cleavage pathway-initial cleavage

Similarly, if the initial cleavage of catechol takes place through *meta* pathway by enzyme Catechol-2,3- dioxygenase, then 2-hydroxymuconic semialdehyde is formed which is yellow in color.

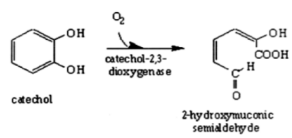


FIGURE 3: *meta* cleavage pathway-initial cleavage

This, unique property of the enzymatic cleavage of formation of colored intermediate compounds after initial cleavage is further employed here, for determination of the metabolic pathway followed for the hydrocarbon utilization on 15 different petroleum hydrocarbon derivatives.

Materials and Methods:

The Petroleum hydrocarbon degrading bacterial population was taken from petroleum contaminated marine water. Samples were collected by means of sterile Eppendorf tubes from Mirkarwada Jetty (17.000720° N and 73.279545 °E), Ratnagiri.

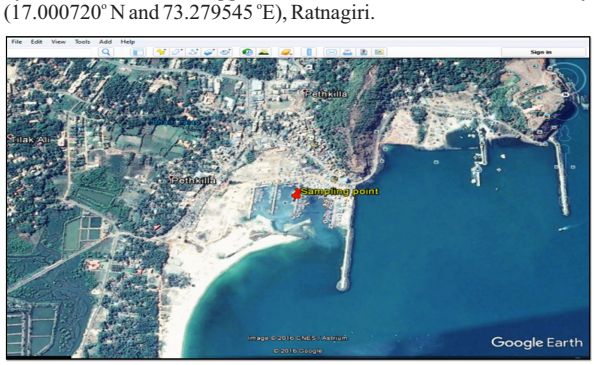


FIGURE 4: Mirkarwada Jetty (17.000720 N and 73.279545 E), Ratnagiri.

The collected samples were readily 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.

After enrichment,of isolates, purification of the selected colonies was carried out. Pure culture isolates were plated on sterile Bushnell Hass Agar plates with similar substrates as that for enrichment along with one in combination of both (Petrol + Diesel). The 27 isolates obtained were then named after the substrate bearing plates from which they were drawn, viz P-Petrol, D- diesel, and PD- Petrol + Diesel. Isolates were then maintained on Nutrient Agar Slants supplemented with the petroleum substrates so as to check their stability, on which the number of isolates was reduced to 22, of which characterization was carried out.

Tolerance cum Degradation ability studies for 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 was then carried out.

Further, on the basis of the studies of tolerance cum degradation ability, 10 isolates were selected and their characterization was carried out.

Later, the isolates were used for studying the rate of utilization of 1% Benzoic Acid. For this study 0.15mM of the 2,6-DCPIP was taken as the working concentration (Varjani et al,2013; Hanson, et al. ,1993; Mariano, et al .2008). The reaction tube composed 2 ml of DCPIP along with 7.5 ml of BH broth, 1 ml of suspension culture (in control 1 ml distilled water instead of culture) and 0.3 ml of petroleum derivative (1% Benzoic acid) as substrate. Using modified protocols, the study was carried out for over 168 hours with monitoring the optical density colorimetrically at 600 nm at the exact same time for each day.

The results recorded were the plotted using the statistics tools in the form of the graph with optical density on Y axis while the time in hours on the X axis.

After determining the rate of substrate utilization (1% Benzoic acid), the pathway by which the substrate utilization (metabolic cleavage pathway/ degradation pathway) was studied by Catechol cleavage method using 10 mM Catechol, for 10 isolates grown on 15 different petroleum derivatives. Initial enzymatic cleavage of catechol by catechol-1,2-dioxygenase or catechol-2,3-dioxygenase produced gave coloured intermediate compounds, i.e. white for *cis cis* muconic acid and yellow 2-hydroxymuconic semialdehyde, corresponding to *ortho* and *meta* pathway followed respectively.

Results and Discussion:

In the initial enrichment, the Bushnell-Hass broth medium was used. Thus, in the presence of the petroleum compounds acting as the sole carbon source, only those isolates would grow which were able to utilize that petroleum compounds. In the later steps of the screening protocols initially 27 isolates were selected from which after checking for stability 5 were eliminated and 22 were chosen for Tolerance cum degradation ability studies. Selection was carried out further and 10 isolates were chosen for the purpose of characterization and checking the rate of substrate utilization (1% Benzoic acid) which is also an petroleum derivative (as it is derived from Benzene)

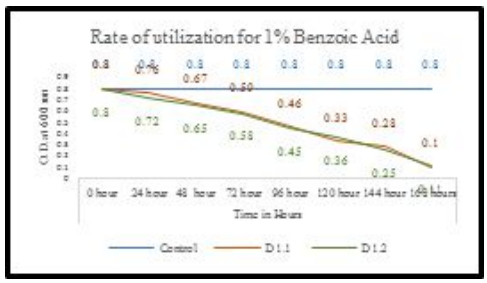
TABLE 1: Colony Characteristics

Colony characters	Isolates				
	D 1.1	D 1.2	D 2.1	D 2.2	D 3.1
Size	2 mm	1-2 mm	1-2 mm	2 mm	1 mm
Shape	Circular	Circular	Irregular	Irregular	Irregular
Colour	White	white	White	White	White
Margin	Entire	entire	Entire	Serrate	Serrate
Elevation	Raised	Flat	Raised	Raised	Flat
Opacity	opaque	Opaque	Opaque	Opaque	Opaque
Consistency	smooth	Smooth	Smooth	Smooth	Mucoid
Gram Nature	-ve Short rods	-ve Short rods	-ve Short rods	-ve Short rods	-ve Short rods

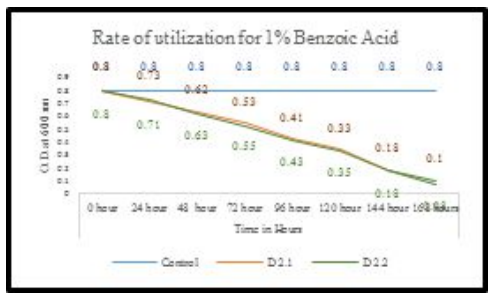
TABLE 2: Colony Characteristics

Colony characters	Isolates				
	D 4.1	D 6.1	D 9.1	P 1.1	PD 3.1
Size	3 mm	2 mm	1-2 mm	1-2 mm	1-2 mm
Shape	Irregular	Circular	Circular	Circular	Irregular
Colour	White	White	White	White	Yellowish White
Margin	Serrate	Entire	Entire	Entire	Serrate
Elevation	Flat	Raised	Raised	Flat	Raised
Opacity	transparent	Transparent	Opaque	Transparent	Transparent
Consistency	Smooth	Mucoid	Mucoid	Smooth	Smooth
Gram Nature	+ve cocci	-ve Short rods	-ve Short rods	-ve Short rods	-ve Short rods

As per the protocols, the observations were recorded at the same time intervals for about 7 days. Then, they were plotted on the graph with reference to the control (no culture inoculated). In each graph, rate of substrate utilization by two isolates was plotted against the reference control.



(A)



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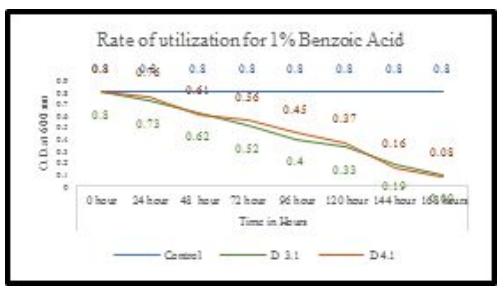

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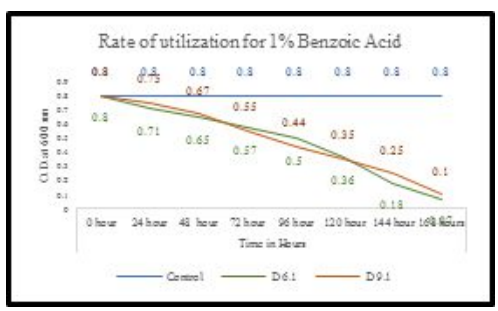
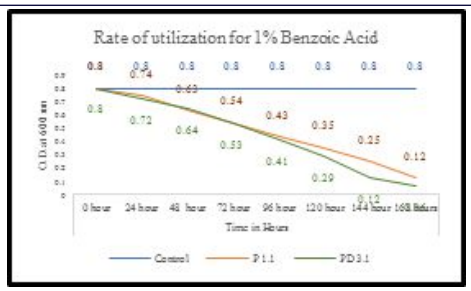


(D)



(E)

FIGURE 5: A: Rate of substrate utilization by D 1.1 and D 1.2 with reference to control, **B**: Rate of substrate utilization by D 2.1 and D 2.2 with reference to control, **C**: Rate of substrate utilization by D 3.1 and D 4.1 with reference to control, **D**: Rate of substrate utilization by D 6.1 and D 9.1 with reference to control, **E**: Rate of substrate utilization by P 1.1 and PD 3.1 with reference to control.

From the graphs, it was observed that all the isolates were potent and very efficient in their property of substrate utilization, which is a pre-requisite for any microbial culture/ consortia to be employed for the treatment of the issue caused by the petroleum pollutants. From the above results, it was also observed that from among the 10 isolates, the isolate P 1.1 showed comparatively low rate of utilization considering about the isolate PD 3.1 which showed the lowest optical density of the dye after the period of 10 days.

Hence, by determining the rate of substrate utilization at 1% Benzoic acid concentration, it was observed that the isolates were able to utilize even the toxic derivatives of Benzene at higher concentrations under normal circumstances, without much optimisation of the physicochemical conditions. So, the isolate screened are thus, reliable and with proper measures can be employed for treatment of issues created by the petroleum pollutants.

TABLE 3: Determination of Metabolic Pathway

Isolates	Petroleum Derivatives						
	1	2	3	4	5	6	7
D 1.1	W	W	W	W	W	W	W
D 1.2	W	W	W	W	W	W	W
D 2.1	W	W	W	W	W	W	W
D 2.2	W	W	-	W	W	Y	W
D 3.1	W	W	W	W	W	W	W
D 4.1	W	W	W	W	W	Y	W
D 6.1	W	W	W	W	W	W	W
D 9.1	W	W	Y	W	W	W	W
P 1.1	W	W	W	W	Y	Y	W
PD 3.1	W	W	W	W	W	W	W

Key: W, *ortho* pathway; **Y**, *meta* pathway;

Petroleum Derivatives: 1-Toluene; 2-Benzene; 3-Phenol; 4- p-Nitrophenol; 5- liq. Paraffin; 6- Methyl Benzoate; 7- Xylene

TABLE 4: Determination of Metabolic Pathway

Isolates	Petroleum Derivatives							
	8	9	10	11	12	13	14	15
D 1.1	Y	W	W	W	W	W	W	-
D 1.2	Y	W	W	W	W	W	W	-
D 2.1	W	W	W	W	W	W	Y	Y
D 2.2	W	W	W	W	W	W	W	W
D 3.1	Y	W	W	W	W	W	Y	W
D 4.1	W	W	W	W	W	W	W	-
D 6.1	W	W	W	W	Y	W	-	-
D 9.1	W	W	W	W	W	W	Y	-
P 1.1	W	W	W	W	W	W	W	W
PD 3.1	W	Y	W	W	W	W	W	W

Key: W, *ortho* pathway; **Y**, *meta* pathway

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

From these studies of determination of degradation pathway, it was observed that as all these 10 isolates, pre-grown in Bushnell- Hass Agar plates supplemented with 15 different petroleum derivatives as substrates, were metabolically active. When, such isolates were then employed for the determination of metabolic cleavage pathway, 10 mM Catechol was used which is an intermediate compound in the Petroleum hydrocarbon biodegradation. Thus, the active metabolic enzymes (Catechol-1,2- dioxygenase or catechol-2,3- dioxygenase, depending on the substrates on which the isolates were grown) readily cleaved the catechol to its product which possess chromogenic properties, as the *cis cis* muconic acid is white in colour and 2-hydroxymuconic semialdehyde is yellow in colour. Thus, the metabolic pathway followed by the bacterium for the biodegradation of the petroleum derivatives was determined righteously.

References:

1. Aichberger H, Hasinger M, Braun R, Loibner AP. (2005) Potential of preliminary test methods to predict biodegradation performance of petroleum hydrocarbons in soil. Biodegradation. 16:115–125.
2. Bidoia, E. D., Montagnolli R. N. and Lopes, P.R.M. (2010). Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique: a case study. In A. Mendez-Vilas (ed.), Current Research, Technlology and Education. Topics in Applied microbiology and Microbial Biotechnology, FORMATEX, Spain. pp. 1277-1288.
3. Busnell, D.L., and Hass, H.F. (1941) The utilization of certain hydrocarbons by microorganism, Kansas Agriculture Experiment Station, 199: 653-673.
4. Hanson, K. G., J. D. Desai and Desai,A.J. (1993). A Rapid and Simple Screening technique for potential crude oil Degrading microorganisms. Biotechnolology Techniques 7(10): 745-748.
5. Mariano, A. P., D. M. Bonotto, D. F. de Angelis, M. P. S. Pirôllo and Contiero,J. (2008). Biodegradability of commercial and weathered diesel oils. Brazilian Journal of Microbiology. 39:133-1421.
6. Varjani Sunita J., Rana Dolly P., Bateja S. and Upasani Vivek 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
7. Winkelmann M, Hunger N, Hüttl R, Wolf G. (2009). Calorimetric investigations on the degradation of water insoluble hydrocarbons by the bacterium Rhodococcus opacus 1CP. Thermochimica Acta. 482:12–16