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RESEARCH ARTICLE

A STUDY ON THE HEAVY HYDROCARBON DEGRADATION POTENTIAL OF BACTERIAL STRAINS ISOLATED FROM UTTARAKHAND

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ABSTRACT

Screening of hydrocarbon degrading microorganisms from crude oil contaminated soil (near Indian Institute of Petroleum, Dehradun) by selective enrichment technique, resulted in the collection of two distinct study species (Bacillus sp.and Micrococcus sp.). Both strains were firstly cultivated in liquid media with Glucose as the carbon and energy source. Morphological characteristics of strains were determined by preparing nutrient agar plates parallel. Further, both strains growth was examined by using four different hydrocarbons (n-Parrafin, Xylene and Naphthelenemixture (1:1), Petroleum coke and Lube aromatics) as a sole carbon and energy source in different set of experiments. Growth study and kinetics data of both strains in these set of experiments were studied and analysed. The results directly indicate the different hydrocarbon degradation potential of both strains.

INTRODUCTION

In present scenario, hydrocarbon contamination due to activities related to petrochemical industries is one of the major environmental problems. Hydrocarbon components mostly belong to the family of carcinogens and neurotoxic organic pollutants. Crude oil composed of different kind of hydrocarbons as n-Paraffin (general formula C_nH_{2n+2}), aromatics and napthenes or cycloalkanes. If amounts of oil contaminants are large in soil then currently accepted methods like incineration, landfills, mechanical and chemical degradation methods have limited effectiveness and more expensive. Bioremediation is the promising technology for the treatment of these contaminated sites since it is cost-effective and will lead to complete mineralization. Hydrocarbons utilizing microorganisms are usually found in places that are contaminated by hydrocarbons like (offshore drilling sites, sites of oil spills, places near ground storage of oil etc.). More than a hundred species of bacteria, yeasts, and fungi are able to oxidize hydrocarbons (Zobell, 1969). It had been reported by ZoBell (1950) thataliphatic hydrocarbons are more susceptible than aromatics to microbial attack and as a rule, long chain hydrocarbons are more susceptible than short chain hydrocarbons.

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Besides, unsaturated and branched hydrocarbons are more susceptible than the corresponding saturated and unbranched compounds. The literature indicates that olefins are more easily attacked than the related saturated alkanes. According to Peters and Moldowan (1993) the components of crude oil are degraded sequentially in the order of n-alkanes > monocyclic alkanes > alkyl benzenes >isoprenoidalkanes> alkyl naphthalenes> bicyclic alkanes >steranes>hopanes. The e ects of biodegradation on the isotopic composition of individual hydrocarbon have been investigated recently for potential applications in petroleum and environmental science (Boreham *et al*., 1995, Masterson *et al*., 2001; Mazeas *et al*., 2002; Mancini *et al*., 2003). The microbial metabolism of polycyclic hydrocarbons is treated in much greater detail in books by Beerstecher (1954) and Davis (Petroleum microbiology, 1967). In 1957, Gray and Thornton isolated 208 strains of bacteria including species of Micrococcus, Mycoplana, Bacterium, Pseudomonas and Mycobacterium. All of these isolates were made from soil in a mineral salts medium with substrates such as phenol, m-cresol, naphthalene, resorcinol or phloroglucinol. The microbial attack of aromatic compounds has been under considerable investigation. Research on the mechanisms of attack of the benzene ring has been the most rewarding. Wegner (1987) first demonstrated the oxidative bacterial dissimilation of cyclic substances such as phenol and toluene. Cybulski *et al*. (2003) and Carvalho and Fonseca (2004) reported that microorganisms modify their cell surface to increase its affinity for hydrophobic substrates and,

thus facilitate their absorption. Concerning the problems related to leaks and accidental spills of petroleum based products and vital role of microorganisms to naturally degrade these contaminating petroleum hydrocarbons, this paper represents growth study of isolated microorganism from crude oil contaminated soil on four different hydrocarbons (n-Parrafin, Xylene and Naphthelene mixture (1:1), Petroleum coke and Lube aromatics).

MATERIAL AND METHODS

To isolate pure cultures, serial dilution of culture suspension was plated on NB (Nutrient broth) medium solidified with purified agar. The plates were incubated for a period of 3 days at 30˚C. Morphologically different strains was further purified by streak plating onto solidified NB media and incubated. The isolated pure cultures were maintained at 30˚C on NB medium.

Isolation and identification of hydrocarbon degradable strains

The soil samples for isolation of hydrocarbon degradable strains were collected from a oil storage site near Indian Institute of Petroleum, Dehradun where the ground was contaminated with crude oil and other hydrocarbons. Individual strains were grown on Nutrient Broth (NB) medium with Glucose as the carbon and energy source, pH maintained at 7.0 and temperature at 30˚C. After growth in Nutrient broth, cultures were inoculated in Basic salt medium (BSM) with yeast extract for further studies. Morphology of isolated cultures was further studied by examining colony characteristics and Gram staining test.

Growth profile study

For determining hydrocarbon degradation potential, different hydrocarbons were used as a Carbon source in place of Glucose. After2 days of inoculation when culture growth was optimum, all cultures were inoculated in same medium, Basic salt medium, with yeast extract and trace elements, but n-Paraffin was used as a carbon source, all experimental conditions were same. For determining growth, dry cell mass and glucose consumption data were taken at regular time interval. Growth kinetics was analyzed for every strain. Those strains, which showed positive growth were further inoculated by taking culture from previous samples. In this experiment Xylene and naphthalene (1:1) mixture was used as a carbon source. Growth was determined by similar method. After this, Lube aromatics and then Petroleum coke were used as a carbon source in same medium (Basic salt medium), 170 rpm agitation was sufficient for proper growth. Temperature was maintained according to strain. One culture showed good growth at 30˚C-45˚C, however another at 60˚C.

Quantitative measurement of cell growth in various hydrocarbons

Dry cell weight method

Cell growth concentration was determined for each strain by dry cell weight method. The cell mass is dried in vacuum oven. Temperature and pressure conditions are given as 70˚C and 0.8kg/cm² respectively. Growth was monitored constantly at regular intervals. The process was repeated until a decrease in dry weight was observed. Based on the data of dry weight, we assessed the hydrocarbon degradation ability of various strains. Also we found the comparative analysis of various hydrocarbon degradable strains.

Vacuum Filtration

This is alternative method of the above, applicable in case of suspended solids. For example, microbial growth on petroleum coke is not possible to be measured by dry cell weight method, beside it vacuum filtration is more specific method to quantify cell growth in petroleum coke.

Measurement of cell number

The quantification of cell concentration in a culture medium is essential for the determination of the kinetics and stoichiometry of microbial growth. After an incubation period of a few doubling times, the slide was examined with a microscope to count cells.

Estimation of total Glucose Concentration

Total reducing sugar in the medium was estimated by DNS method and absorbance was determined at 575 nm using a spectrophotometer. Glucose concentration was calibrated from standard curve.

RESULTS AND DISCUSSION

Identification of bacterial cells

A total of 7 strains were isolated out of which 2 strains were selected on the basis of easy availability and good growth on different carbon sources. Their colony characteristics are given in below Table 1. Then they were subjected to various morphological tests following Bergy's manual. The plate culture of above colonies is shown in below Figure no. 1.The isolates were tentatively identified as Micrococcus sp. and Bacillus sp. on the basis of various morphological tests.

Growth study of Bacterial Strains

Growth study of Micrococcus sp.

A. Glucose and n-Paraffin as carbon source

The isolated strain of Micrococcus sp. was firstly grown in Basic salt medium using Glucose as carbon and energy source in all defined temperature, pH and agitation rate conditions. After growth in Glucose, same strain was examined on n-Paraffin as a carbon and energy source putting all other experimental conditions identical). Growth of particular strain was measured by determining the dry cell mass data at regular time interval during culture growth. The growth profiles of Micrococcus sp. in glucose and n- Paraffin was shown in Fig.2 (A) and (B). According to Fig. 2(A) and 2(B), growth curve for glucose is above than n-Paraffin and exponential phase is more extended for glucose which shows cell are more active in presence of Glucose as a carbon source.

Table 1. Morphology of the isolated bacterial strains

Colony characteristic	Agar plate 1	Agar plate 2
Color	White	White
Size(Relative)	Large	Small
Shape	Spreading	Circular
Surface	Smooth	Smooth
Strain characteristic	Agar plate 1	Agar plate 2
Shape	Coccus	Bacillus
Gram staining	+ve	+ve

Table 2.Dry cell mass of Micrococcus sp., in different time intervals, if Xylene and Naphthelene mixture (1:1) is used as Carbon and energy source

Temp. in hrs	Weight of Empty filter Paper	Weight of dry cell mss+filter paper	Weight of dry cell mass in gm. Per lit.
0	0.0854	0.0840	No growth
24	0.0832	0.0825	No growth

Table 3. Dry cell mass of Micrococcus sp., in different time intervals, if Petroleum coke is used as Carbon and energy source

Growth time (t) in hrs	0	72	119	141	165
Average no. of cells (N_t)	$3.20x10^2$	$6.66x10^3$	$3.08x10^5$	$4.01x10^6$	$4.5x10^6$

Table 4.Dry cell mass of Micrococcus sp., in different time intervals, Lube Aromatics is used as Carbon and energy source

Growth time(t) in hrs	Dry cell mass in g/l^{-1}
0	0.8
22	1.1
43	2.8
68	3.1
86	3.2
110	2.9

Table 5.Dry cell mass of Bacillus sp., in different time intervals, if Xylene and Naphthelene mixture (1:1) was used as Carbon and energy source

Temp. in hrs	Weight of Empty filter Paper	Weight of dry cell mss+ filter paper	Weight of dry cell mass in g/l^{-1}
0	0.0840	0.0840	No growth
24	0.0828	0.0827	No growth

Table 6. Averageno. of cells (N_t) of Bacillus sp. in different time intervals, if Petroleum coke is used as Carbon and energy source

Growth time (t) in hrs	0	72	119	141	165
Average no. of cells (N_t)	8.0×10^2	1.02×10^3	2.43×10^5	5.30×10^6	6.0×10^6

Specific growth rate coefficient (μ) is nearly equal in presence of carbon sources, Glucose (μ=0.0166hrs^{-1}) as well as n-Paraffin (μ=0.0161 hrs^{-1}). Hence, cell growth is not much different in presence of both hydrocarbons. Hence, the doubling time of cell mass is very high and almost equal in presence of both carbon sources, Glucose (τ_d =41.74hrs) and n-Paraffin (τ_d =43.04hrs).

B. Xylene and Naphthalene (1:1) mixture as Carbon source

After observing n- Paraffin degradation by Micrococcus sp., Xylene and Naphthalene mixture is used as next Carbon and energy source in same growth conditions. Growth results are shown in below Table 2, which directly predicts that no growth occurs when Xylene and Naphthalene is used as a Carbon and energy source. It means Micrococcus sp. has no potential to degrade Xylene and Naphthalene mixture.

C. Petroleum coke as Carbon source

Petroleum coke is one of the undesirable components of crude oil, the advantages of petroleum coke are that it has high calorific value, and it is cheaper than coal, but its high contents of sulfur and vanadium put them in a very disadvantageous position from the environmental perspective as compared to coal. Actually, dry cell weight determination was not possible, when petroleum coke was used as a carbon and energy source. Coke particles were suspended in the medium and it is not possible to separate the water hydrocarbon layer. Hence, the cell number density in a culture medium was determined to quantify microbial growth. Growth results are shown below in Table 3.According to Table 3, average no. of cells of Micrococcus sp. increased exponentially between time duration near about 72 hrs to 119 hrs. The specific growth rate coefficient for Micrococcus sp. is about 0.0786 and the doubling time τ_d is approximately 8.8hrs. It means Micrococcus sp. have degradation potential for petroleum coke.

D. Lube aromatics as Carbon source

To examine Micrococcus sp. aromatic degradation potential, Lube Aromatics was used as a carbon and energy source under same operating conditions. Growth results at different time

intervals are shown below in Table 4. According to Table 4, Micrococcus sp. showed good growth on Lube aromatics from 22 to 68 hours with doubling time, $\tau_d$about26.75hrs. Specific growth rate (μ) for Micrococcus sp. is about 0.0259 in such conditions.

Growth study of Bacillus sp.

A. Glucose and n-Paraffin as carbon source

Similar to Micrococcus sp., isolated Bacillus sp. was firstly grown in Basic salt medium using Glucose as carbon and energy source, all operating conditions were identical as previous experiments except temperature. About 60˚C is suiTable temperature for Bacillus growth unlike 30^0C for Micrococcus. Further growth was examined by taking n-Paraffin as alternative carbon and energy source. The growth profiles of Bacillus sp. in glucose and n- Paraffin was shown in Fig. 3 (A) and (B)

Table 7. Averageno.of cells (N_t) of Bacillus sp. in different time intervals, if Lube aromatics is used as Carbon and energy source

Growth time(t) in hrs	Bacillus sp, Dry cell mass in g/l^{-1}
0	0.3
22	0.5
43	0.9
68	1.3
86	2.3
110	2.5

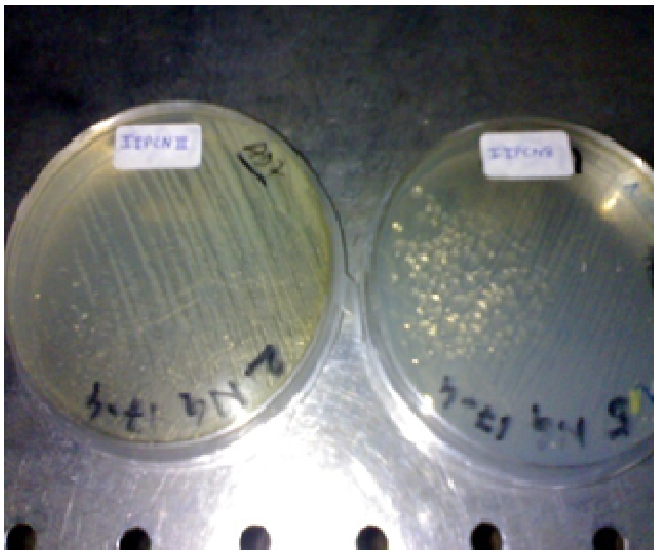

Fig.1. The plate culture of isolated colonies

According to Fig. 3(A), the lag phase is more on n-paraffin than Glucose. It means cells required additional time to adapt new growth condition (Hydrocarbon, n-Paraffin as a carbon source instead of Glucose). Fig. 3(B) shows exponential phase on n-Paraffin is lower than Glucose, which predicts cells are more active in presence of Glucose as a carbon source. Specific growth rate coefficient is nearly equal in presence of both carbon sources, Glucose (μ=0.0287hrs^{-1}) as well as n-Paraffin (μ=0.0271 hrs^{-1}). Hence, cell growth is not much different in presence of both hydrocarbons with doubling time 24.14 hrs in presence of glucose and 25.57hrs in presence of n-Paraffin.

B. Xylene and Naphthelene (1:1) mixture as carbon source

Bacillus sp. is further examined on Xylene and Naphthelene (1:1) mixture, for checking its degradability to aromatic hydrocarbons.

At different time intervals, dry cell mass was determined. The data was shown in below Table 5. Similar to Micrococcus sp., Bacillus sp.showsno cell growth when Xylene and Naphthalene is used as a Carbon and energy source. It means Bacillus sp. has no potential to degrade Xylene and Naphthalene mixture.

C. Petroleum coke as Carbon source

Cell number density was determined to estimate growth of Bacillus sp. in petroleum coke. The data was shown in below Table 6.According to Table 6, it is cleared that Petroleum coke degradation yield is very high in case of Bacillus sp. with specific growth rate coefficient (μ) about 0.0787 hrs^{-1}.

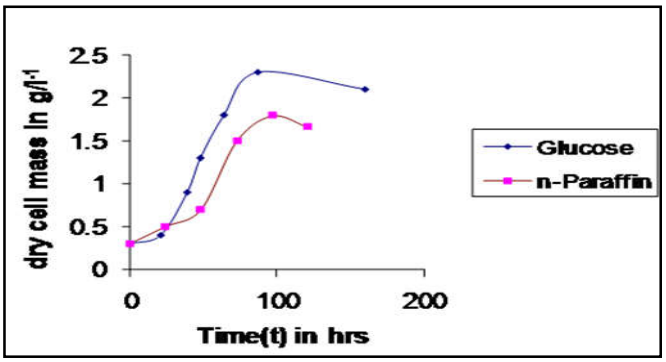

Fig. 2(A). Growth profile for Micrococcus sp., in the presence of Glucose and n-Paraffin

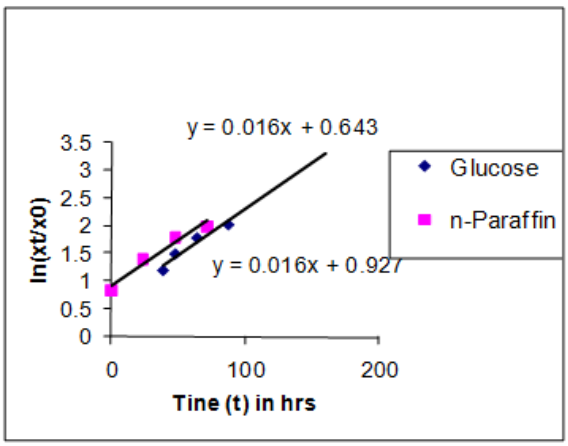

Fig. 2B. Exponential phase ofgrowth profile for Micrococcus sp., in the presence of Glucose, n- Paraffin

D. Lube aromatics as Carbon source

For Bacillus sp., Lube aromatics degradation potentialwas determined by calculating dry cell mass at regular time interval. Results were shown in below Table 7. It is quite clear from results that Bacillus sp. shows good growth on Lube aromatics with specific growth rate about 0.0291 hrs^{-1} with doubling time about 23.81hrs. It is well evident from results that growth profile of different strains truly depends upon the

carbon source utilized for cell growth. Usually the lag period increases with the age of the inoculums. To minimize the duration of the lag phase, cell should be adapted to the growth medium and conditions before inoculation. The lag phase on n-Paraffin is greater than Glucose for both strains, Bacillus sp. and Micrococcus sp.

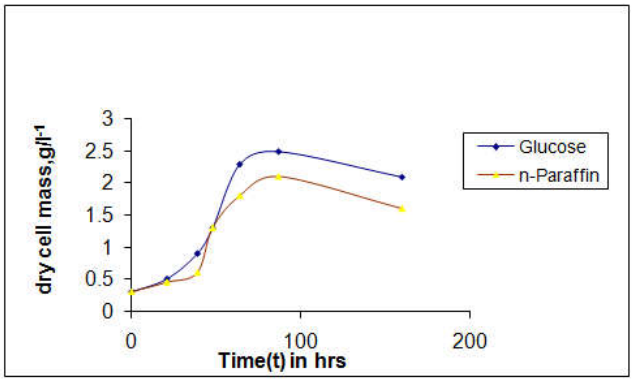

Fig. 3A. Growth profile for Bacillus subtilisin the presence of Glucose and n- Paraffin

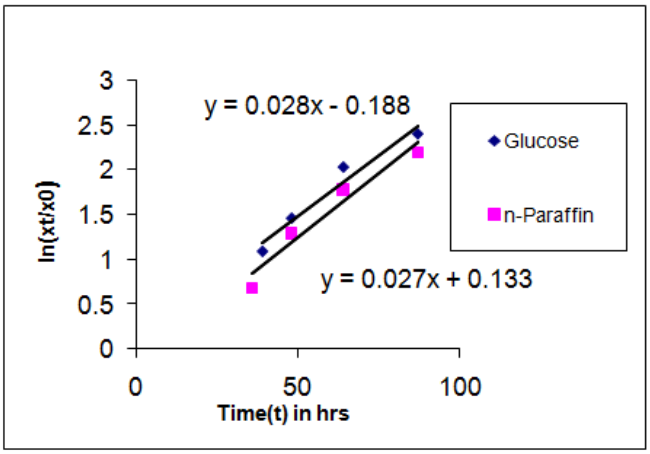

Fig. 3B. Exponential phase ofgrowth profile for Bacillus sp., in the presence of Glucose and n- Paraffin

It means cells require additional time to adapt new growth. For Bacillus sp., Specific growth rate coefficient is nearly equal in presence of carbon sources, Glucose (μ=0.0166hrs^{-1}) as well as n-Paraffin (μ=0.0161 hrs^{-1}). Hence, cell growth is not much different in presence of both hydrocarbons. It is confirmed that Bacillus sp. showed almost equal growth in presence of Glucose (τ_d= 24.14 hrs) and n-Paraffin (τ_d =25.57hrs).Further, strains, Micrococcus sp. and Bacillus sp. showed no growth, when Xylene and Naphthelene mixture (1:1) was used as a carbon and energy source. It means both strain have no ability to degrade these aromatic compounds. This study fully supports the statement given by ZoBell (1950) thataliphatic hydrocarbons are more susceptible than aromatics to microbial attack. For Lube aromatics, it is clear that lag phase of growth profile is almost equal for strains, Micrococcus sp. and Bacillus sp. It means, strains require almost equal time to adapt growth medium. Hence, the doubling time of cell mass, for Bacillus sp. (τ_d =23.81hrs) is very near to Micrococcus sp. (τ_d = 26.75hrs). Again, for petroleum coke, strains, Bacillus sp. and Micrococcus sp. showed equal doubling time 8.8hrs.

It means both strains have almost same degradation potential for petroleum coke. According to hydrocarbon degradation potential, these strains may be further examined for crude oil degradation. As, from literature, it is clear that crude oil, n-Paraffin and Xylene-Naphthalene mixture are valuable components and it is not desirable to degrade them, however Lube aromatics and petroleum coke are not useful. From above experiments and results, it is observed that both Bacillus sp. and Micrococcus sp. shows positive growth in Lube aromatics and in Petroleum coke, but negative growth in n-Paraffin and Xylene -Naphthalene mixture. It means these strains have better ability to degrade Lube aromatics and petroleum coke, which are not valuable components.

Conclusion

In present study, the Heavy hydrocarbon degradationpotential of bacterial strains, it is studied that Bacillus sp.can degrade hydrocarbon n-Paraffin (μ=0.0161 hrs^{-1}), this strain alsoshowed best growth results in Petroleum coke (μ=0.0787 hrs^{-1}) and positive growth in Lube aromatics (μ=0.0291 hrs^{-1}). Micrococcus sp. followed it by growth in hydrocarbon n-Paraffin (μ=0.0161 hrs^{-1}), petroleum coke (μ=0.0786 hrs^{-1}) and in Lube aromatics (μ=0.0259 hrs^{-1}). Both strains are quite unsusceptible to degrade aromatic compounds like Xylene and Naphthalene mixture.

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