Plant Archives Vol. 18 No. 1, 2018 pp. 457-465 *ISSN 0972-5210*

BIODEGRADATION OF OIL HYDROCARBONS BY SOIL MICROORGANISM

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Abstract

Hydrocarbons degrading micro organism was isolated from soil and Identified as *Pseudomonas* spices. This isolate was inoculated into enrichment medium containing phenantherene, or naphtha or fuel oil. The behavior of cell growth progressed with time for the five samples of cultures of different initial substrate (phenentherene) concentration. Ideal biomass behavior all with the same time interval of 48 hr. This behavior confirms that the growth is compatible with Monod's equation. When taking a sufficient amount of phenantherene as an initial concentration of (10 mg/ml) and monitoring under longer period the growth undergoes the complete cycle of its own. The decrease in naphtha specific gravity as a result of the bacterial action. Another product is selected to be more complex like fuel oil, the results showed a gentle decline in the specific gravity of the product. First order curve fitting was determined that the statistical software Statistica was used for estimating Monod's kinetics constants.

Key words : Isolation, identification, degrading, phenanthrene, naphtha, fuel oil.

Introduction

Aromatic hydrocarbons (including naphthalene and alkylbenzenes) obtained from the middle- distillate, gas-oil fraction during petroleum separation. Due to their massive production and use as fuels for transportation, they are among the most common sources of organic pollutants for the surface soil. They also impact the subsurface soil through leaking from underground storage tanks and pipelines. Due to their mobility in soil, such released fuel hydrocarbons can reach water intakes and/or groundwater reservoirs, thus generating relevant risks for humans and other living organisms (Wang and Bartha, 1990; Bequette and Bequette, 1998). Lower molecular weight types such as naphthalene (the simplest containing two benzene rings), anthracene and phenanthrene (both of which contain three benzene rings) are known to have health effects that though are comparatively mild could be potentially hazardous (Klaassen and Amdur, 1996). Furthermore, some like phenanthrene is considered as a model substrate in environmental PAHs degradation studies because its structure is found in the nucleus of carcinogenic PAHs such as benzo [a] anthracene and 3-methylcholanthrene (Nnamchi *et al.*, 2006). As a result of these hazardous effects of PAHs, there is much interest in their environmental effects.

Biostimulation is the most mature technologies available for bioremediation of hydrocarbon contaminants. However, using a systems biology approach to understand, model, predict, monitor and control bioremediation processes promises to provide faster, better, and cheaper environmental cleanup and stewardship (Hazen, 2010).

The physical and chemical characteristics of soils as well as weather conditions, affect a lot the chemical stability of the pollutants but also their availability to biodegradation (AlDisi *et al.*, 2016).The majority of petroleum-derived hydrocarbons can be biodegraded by several microbial strains each however capable of breaking down a specific group of molecules; the biodegradation potential generally decreases by moving from n-alkanes to branched alkanes, low molecular weight n-alkyl aromatics, mono aromatics, cyclic alkanes and polynuclear aromatics (Zanaroli *et al.*, 2010).

Gargouri *et al*. (2015) stated that two strains of genera Candida and Trichosporon grew on long-chain n-

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alkane, diesel oil, and crude oil but failed to grow on short-chain n-alkane and aromatic hydrocarbons. Udgire *et al*. (2015) stated that four isolated strains of *Bacillus* spp., only one was demonstrated the maximum oil degradation capacity 66% and 58%, respectively for two concentration of crude oil, after 21 days of incubation.

According to Das and Chandran (2010) bacterial strains belonging to this genus is generally able to degrade hydrocarbons.

On using hydrocarbon in aqueous solution, the micro–organisms will digest the complicated hydrocarbon molecule and gain one (or more) of its carbon atoms for its own metabolism, generating more organisms from same species and secreting. The metabolism product that consist of H_2O, CO_2 and higher hydrocarbon molecules braked down during the metabolism process (Moo-Young, 1985). These micro–organisms whom are capable of such work generally specific types of fungi and bacteria. In growth, fungi are the best micro–organism to be undertaken, but it's rather difficult to control its metabolism to achieve a specific conversion. While on other hand bacteria has a good behavior in metabolism obeying Monod's growth kinetic (Moo-Young, 1985).

Cameotra *et al*. (2009) stated that they have tried to look into the matter of hydrocarbon uptake by a hydrocarbon degrading *Pseudomonas* strain isolated from oil-contaminated soil. A common feature of all the fractions of crude oil is their low water solubility and this poses special problems for those microorganisms capable of utilizing such water immiscible substrates as source of carbon and energy. The first step in the process is the transport of the hydrocarbon from oil phase to the cell surface in some way so as to achieve effective cell surface contact and ultimately efficient transportation across cell membrane, or in other words uptake/intake.

The best method to isolate bacteria starting by using natural sources (mostly soil). Soil contaminated with organic waste material is a naturally occurring source of microorganisms with degradative properties. Bacteria capable of degrading polycyclic aromatic hydrocarbons (PAH) can be isolated from such locations (Gupta *et al*, 2017). Earth samples are transferred to liquid medium which contains, as sole energy and carbon source, the substance to be degraded. Samples of earth contaminated with oil (*e.g.* near filling stations, car parks or railway embankments) are transferred to liquid medium containing the three- ringed PAH phenanthrene (toxic) as sole carbon and energy source. Once growth of the microorganisms has been established phenanthrene–degrading bacteria are selected by repeated passages through the selective liquid medium. Suitably diluted aliquots of the bacteria are spread onto plates which are then sprayed with a solution of phenanthrene, and those colonies surrounded by a clear "halo" are picked (Zanaroli *et al.*, 2010). These degradative strains are grown in the presence of phenanthrene as the sole carbon source. The growth of the strain and the reduction in the amount of the substrate are features of successive metabolism.

The present work is concerned with polycyclic aromatic hydrocarbon degrading bacteria "PAH – bacteria" so the practice will focus on the isolation of PAH-degrading bacteria, using the biological method namely, the enriched liquid media method and then identified it. Studying the growth of isolated bacteria and estimating the Monod's kinetic parameters through the observation of bacteria growth behavior in a lab bioreactor operated batch wise. Then study the effect of limiting substrate concentration with longer time of observation. Also studying the influence of this bacterial growth on some petroleum products (phenanthrene, heavy naphtha, and fuel oil).

Experimental Work

Isolation of Phenanhrene Degrading Bacteria

The isolation was worked as in Fatal *et al*. (2004). Prepare serial dilutions (up to 10^{-5}) of the culture. Add 0.5 ml culture to 4.5 ml 0.9% NaCl and mix well (10^{-1} dilution). Plate 0.1 ml of the 10^{-3} to 10^{-5} dilutions on nutrient agar plates. The plates are incubated at 38°C until colonies are sprayed lightly with the ether solution of phenanthrene, so that as soon as the ether evaporates a visible layer of water insoluble phenanthrene remains. The plates are incubated at 38°C in a humidity chamber for several days. The plates are checked daily colonies made up of bacteria capable of degrading phenanthrene are surrounded by a clear "halo" phenanthrene is taken up by the cells and degraded, causing the agar in the immediate vicinity of the colony to appear clear. Material from colonies surrounded by "haloes" is streaked for single colonies onto nutrient agar, and the plates are incubated at 38^0C. If necessary the streaking procedure is repeated. Prepare stock cultures from colonies of different appearance (color, consistency, morphology) by streaking onto agar slants (Fattal *et al*., 2004).

The Identification of *Psuedomonas* sp.

The Identification was done according to Bergy's manual (1994) and Fattal (2004). *Pseudomonas* species are nonfermentative, gram – negative, bacillus, most frequently recovered in clinical specimens, it's important to know its characteristics so that it will be possible to

Fig. 1 : *Pseudomonas* sp. appeared throughout microscopically observation, Flagella persons.

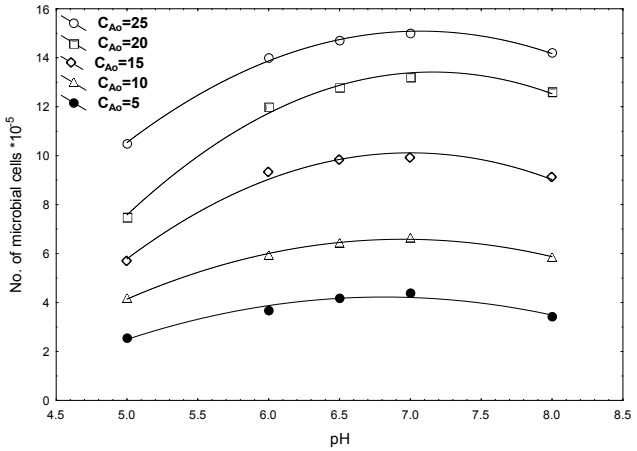

Fig. 2 : Effect of pH value on the yield of number of cells produced for different initial phenanthrene concentrations.

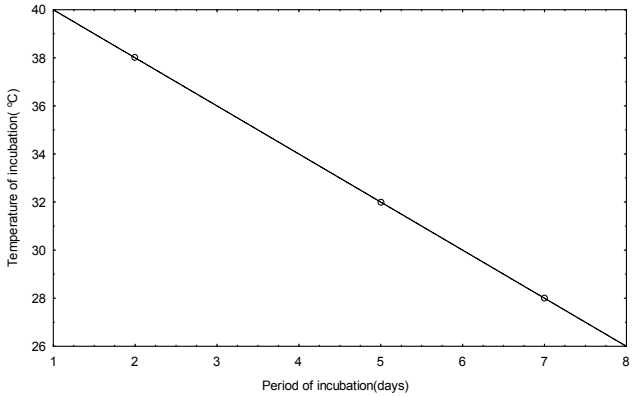

Fig. 3 : Effect of temperature on the initial growth activation for phenanthrene cultivation.

identify this organism. A number of useful tests to know the genus of bacteria are illustrated in the followings:

Desoxycholate – citrate agar : With slightly spreading, dull gray, non-lactose utilizing colonies of

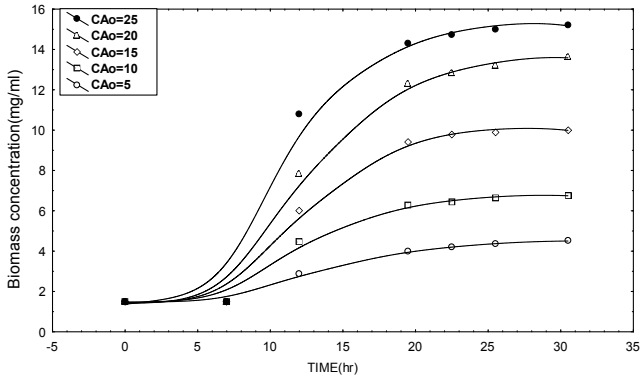

Fig. 4 : Growth concentration for different phenanthrene concentrations versus time, shows the intensity of fermentation kind to act as substrate-limiting.

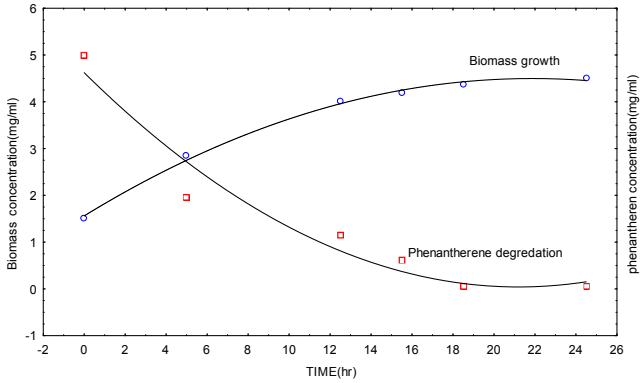

Fig. 5 : Growth concentration with phenanthrene concentration for initial phenantherene concentration of 5 mg/ml.

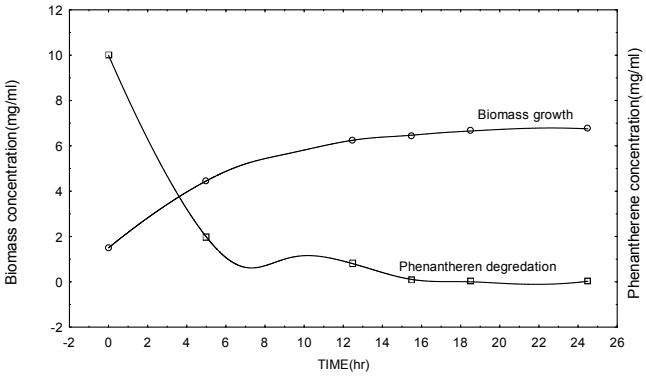

Fig. 6 : Growth concentration with phenanthrene concentration for initial phenantherene concentration of10 mg/ml.

pseudomonas sp. The green background pigmentation of the medium indicates the production of pyocyanin by the organism.

MacConkey agar : MacConkey agar with dull gray, non – lactose fermenting colonies of *pseudomonas* sp. mixed with red lactose – utilizing colonies of *E. coli*.

Cytochrome oxidase test : Paper strip showing the blue color of a positive reaction (blue color), compared

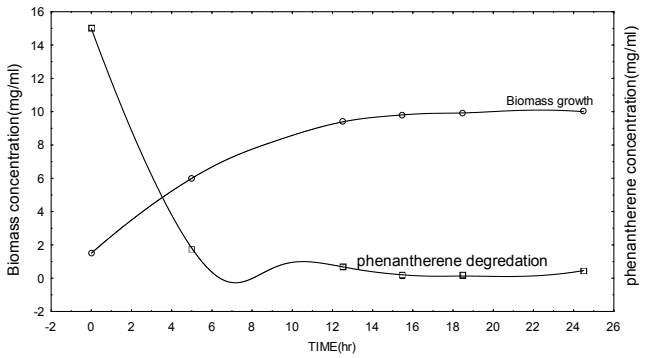

Fig. 7 : Growth concentration with phenanthrene concentration for initial phenantherene concentration of15 mg/ml.

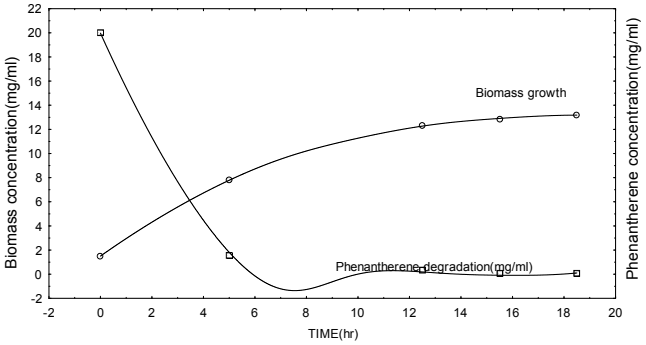

Fig. 8 : Growth concentration with phenanthrene concentration for initial phenantherene concentration of20 mg/ml.

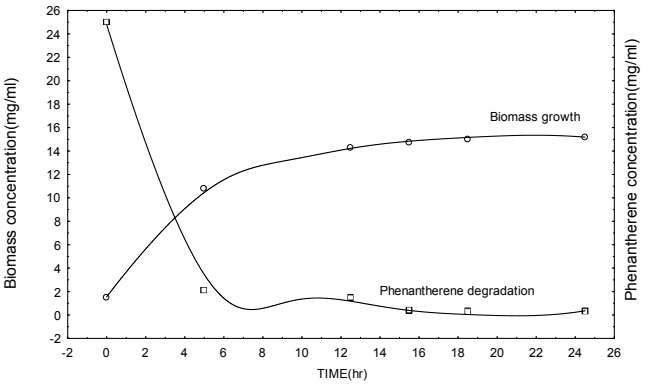

Fig. 9 : Growth concentration with phenanthrene concentration for initial phenantherene concentration of25 mg/ml.

to the negative control (blank). All strains of *Pseudomonas* sp. produce cytochrome oxidase.

Oxidative – fermentative (OF) : Media inoculated with *Pseudomonas* sp., illustrating acid production (yellow color) in the open tube but not in the medium overlayed with mineral oil. All strains of *pseudomonas* sp. utilize carbohydrates only oxidatively.

Motility : Semisolid motility medium inoculated with *pseudomonas* sp.. Most strains of Pseudomonas sp. are

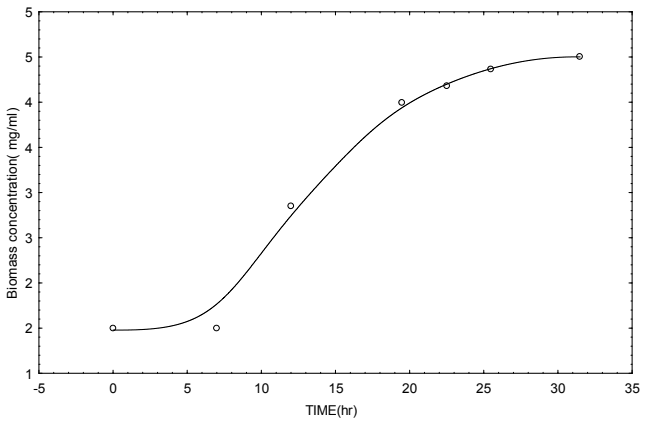

Fig. 10 : Growth concentration for initial phenantherene concentration of 5 mg/ml.

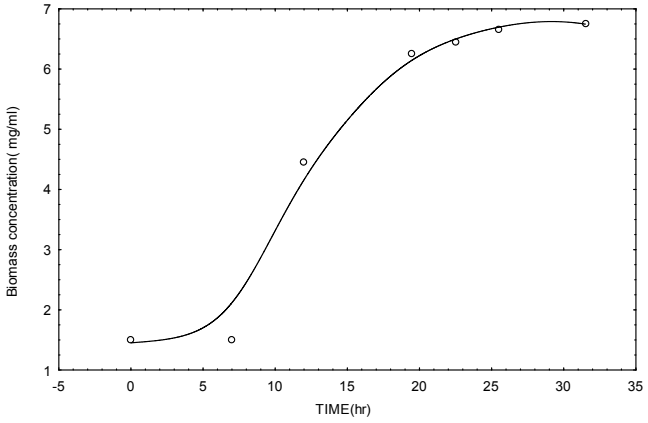

Fig. 11 : Cell growth concentration for initial phenantherene concentration of 10 mg/ml.

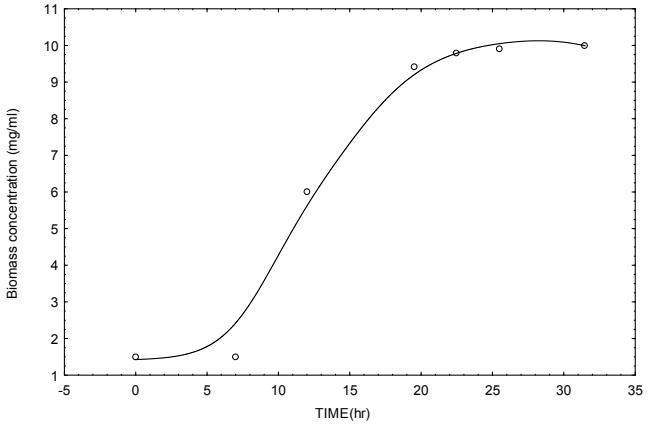

Fig. 12 : Growth behavior for initial phenantherene concentration of 15 mg/ml.

motile and produce a thick film of growth on the survace of semisolid media. Little growth or evidence of motility is present below the surface of the medium.

Fluorescent – denitrification (FN) : Medium inoculated with *Pseudomonas* sp. Most strains is *Pseudomonas* sp. produce nitrogen is indicated by the presence of gas bubbles in the denitrification medium.

Table 1 : The role of degrading phenantherene, this result used in section (Phenanthrene Degrading Order).

Time(hr)	Phenanthrene concentration (mg/ml)	
	Degraded	Remaining
0	0	25
6	22.89	2.11
12	23.88	1.12
18	24.61	0.39
24	24.67	0.33
30	24.67	0.33

Table 2 : Growth on phenantherene starting with CAo=10.

C_C mg/ml	C_A mg/ml	Time (hr)
1.5	10	0
4.46	1.95	5
6.26	0.824	12.5
6.45	0.072	15.5
6.65	0.026	18.5
6.75	0.026	24.5

Table 3 : Notation.

C_{Ao}	Initial substrate concentration (phenanthrene) in mg/ml
C_A	Substrate concentration (phenanthrene) in mg/ml
C_{Co}	Initial biomass concentration in mg/ml
C_C	Biomass concentration in mg/ml
K	Growth constant
r_C	Growth rate of Biomass in mg/ml.hr
C_M	Monod's constant

FN medium inoculated with *Pseudomonas* sp. Most strains produce fluorescent, which can be detected in the medium by observing bright blue fluorescence with a Wood's lamp.

Pseudosel agar : Pseudosel agar with colonies of *Pseudomonas* sp. This medium contains a strong detergent, cetrimide, and is selective for *Pseudomonas* sp. The production of pyocyanin is enhanced and medium was used for fluorescent pigment demonstration.

Flagellar strain of *Pseudomonas* sp. : Note the single polar flagellum characteristic of *Pseudomonas* sp. When examining with microscope (Microscope: Altay International CE BioLab-1007,Made in Japan).

Media : Nutrient agar made up according to the manufacturer's instructions. Liquid enrichment medium solution: KH_2PO_4 1.0 g, $Na_2HPO_4.12H_2O$ 1.25g, $(NH_4)_2SO_4$ 1.0g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.6H_2O$ 0.05g, $FeSO_4$. $7H_2O$ 0.005g, phenanthrene 0.05 g, dH_2O 1000 ml, pH 7.0(pH Meter: PSD 5, Orchidis Labortoire, Made in France). Dissolve the salts separately in dH_2O, combine the solutions and make up to 1L; adjust pH, dispense 20 ml aliquot into 100 ml Erlenmeyer flasks and autoclave. Dissolve 50 mg phenanthrene in 10 ml methanol and add 0.1 ml of this solution to 20 ml sterile liquid enrichment solution phenanthrene forms a finely divided precipitate (solubility of phenanthrene in water approximately 1 mg/L) (Zanaroli *et al*., 2010). 500 ml Erlenmeyer flasks containing 100 ml liquid enrichment medium. Inoculum5% of (2.1×10^6 cfu/ml) in screw capped test tubes containing 5 ml liquid enrichment medium containing 0.9% NaCl; 1% phenanthrene (or naphtha or fuel oil) solution in methanol; 0.1M potassium phosphate buffer pH 7.0.

Enrichment of Hydrocarbon Degrading Bacteria

Inoculating each of five Erlenmeyer flasks containing liquid enrichment medium with a small quantity of soil. The soil sample should have a distinct "oily" smell. The flasks are incubated with shaking (Shaker Incubator: Heraeus.Klasse 1.3.1.Made in Germany), at 38° C until the medium becomes turbid result of bacterial growth (2 -7 days). Carring out regular microscopically monitoring of the culture.

Transferring 1ml of the culture into fresh medium and examine the culture in the microscope as soon as it becomes turbid. Inoculate 1ml of the culture into fresh medium. Repeat the passage of the enrichment culture twice.

Results and Discussion

Colonial morphology

Based on the best cultivation unit and the most obeying colonial examination plate. Following colonial characteristics were taken from the plate :

Shape : circular; Edge: smooth; Elevation: low convex; Color: white; Size: (1.5 µm-4µm); Appearance: powdery; Odor: oily Smell (light).

In addition to the microscopic examination, the features referred to a species of *Pseudomonas* sp. bacteria according to Bergy's manual (1994). The next section represents a bacteriological process for identification to be confirmed from the species of the bacteria.

Isolation and Identification of Pseudomonas

For identification to ensure that the bacteria genus is *Pseudomonas*, the following consequence tests are done:

On MacConkey and Blood ager plates : The

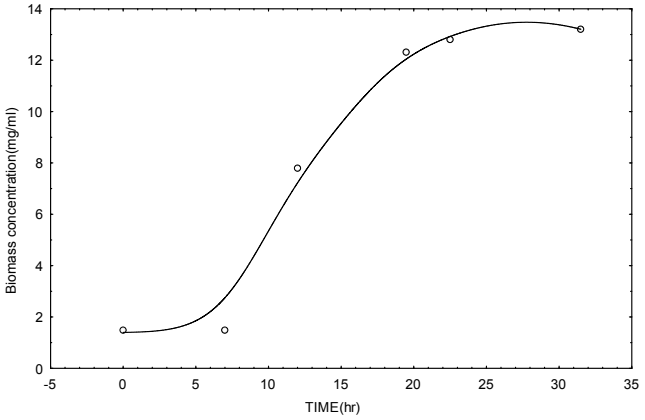

Fig. 13 : Growth behavior for initial phenantherene concentration of 20 mg/ml.

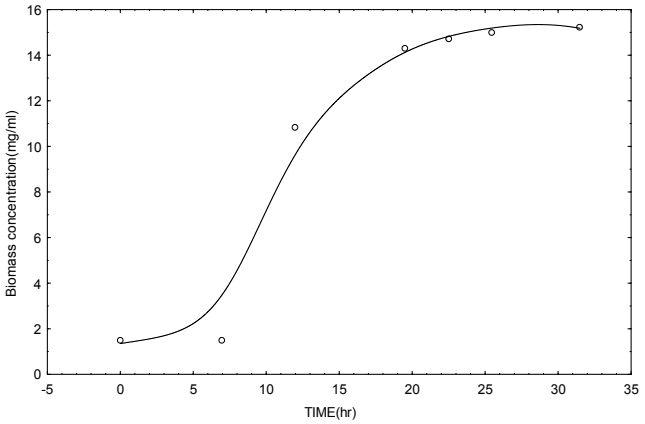

Fig. 14 : Growth behavior for initial phenantherene concentration of 25 mg/ml.

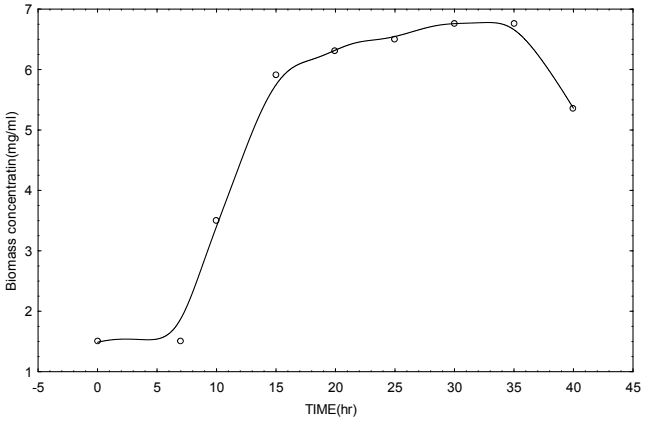

Fig. 15 : Growth concentration with phenanthrene concentration for limiting phenantherene concentration of 10 mg/ml for a prolonged period.

bacterial colonies were spreaded on the surface of plates by streaking method. Incubated for 24 hrs. Ability of such bacteria to grow on MacConkey agar is determined by inspecting by reflected light.

Cytochrome oxidase activity : By cytochrome

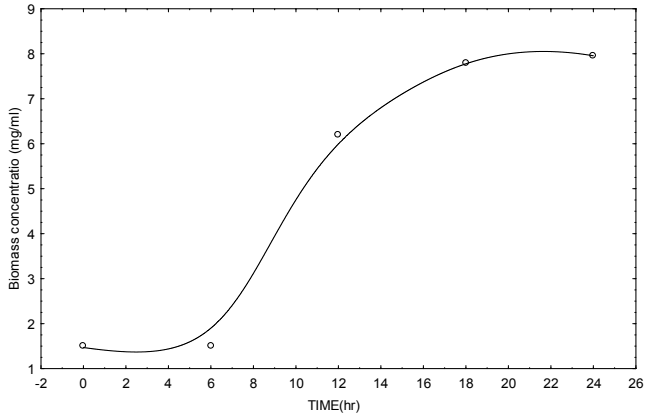

Fig. 16 : Biomass growth rate on heavy naphtha.

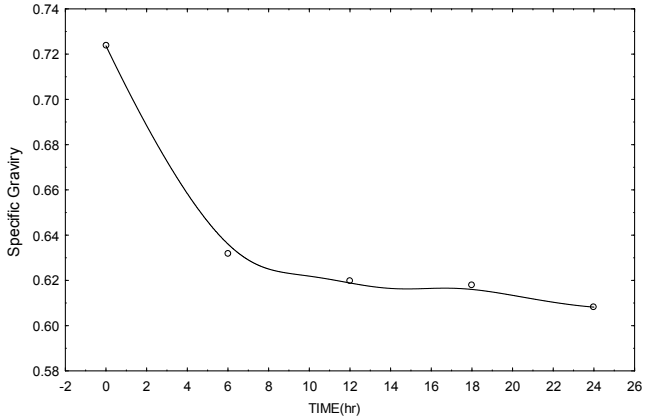

Fig. 17 : Specific gravity declination when growing bacteria on heavy naphtha.

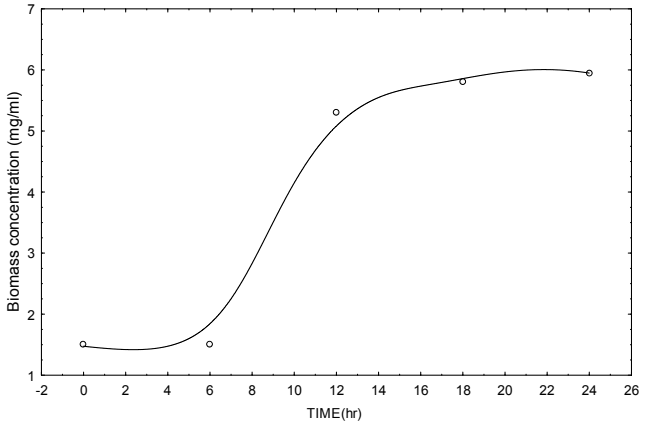

Fig. 18 : Biomass growth rate on fuel oil.

oxidase test paper strip showing the blue color of a positive reaction. Filter paper was impregnated with oxidase reagent and taking 2-3 colonies of such bacteria and emulsified on this filter paper. Blue color indicates a positive reaction illustrated.

Oxidative-Fermentative (OF) : Oxidative-Fermentative (OF) media inoculated with *Pseudomonas* species acid production (yellow color) in open tube was done.

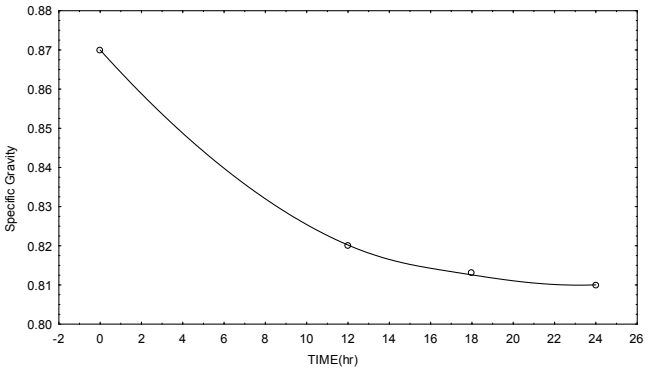

Fig. 19 : Specific gravity declination when growing bacteria on fuel oil.

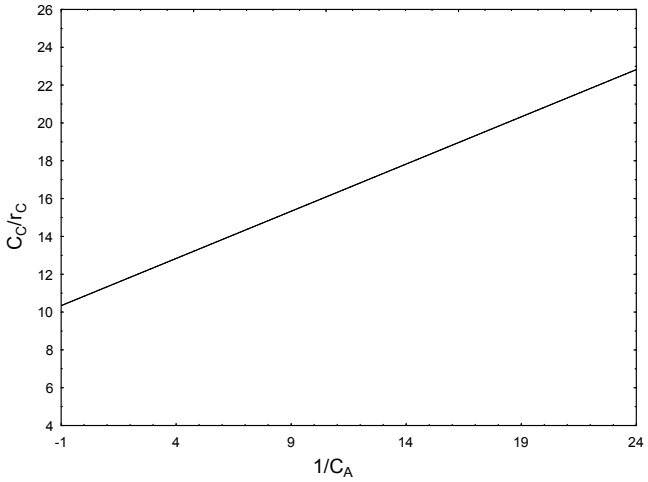

Fig. 20 : Monod's kinetic constants for initial phenantherene concentration of CAo=10 mg/ml.

Motility : On semi-solid motility medium inoculated with pseudomonas, most of pseudomonas strains are motile, produce a thick film of growth on the surface of semisolid media. Semi-solid agar used for the detector of motility by stab inoculating only, the upper 4 mm, the bubbles are the sign of growth, and grow on such medium is sign of motility, another feature leading to *Pseudomonas* sp. genus.

Nitrogen production : Most strains of pseudomonas produce nitrogen gas from nitrate, and/or nitrites. Free nitrogen is indicated by the presence of gas bubbles in the denitrification medium.

Pseudosel agar : This medium is highly selective to *Pseudomonas* sp. that appeared the blue-green pigment diffuses from the areas of bacterial growth, while the uninoculated medium remains clear.

Flagella persons : Flagellar strain of pseudomonas appeared clearly throughout microscopically observation. The fig. 1 below shows the single polar flagellum characteristic of pseudomonas species.

Effect of Operating Variables

The effect of pH

Fig. 2 represent the influence of pH value on the yield of microbial cells. The yield increases from pH value 5 and reaches a maximum at pH value of 7 for five different phenanetherene concentration media (5-25) mg/ml.

After that for the same sample, after a pH value of 7 the yield decreases when increasing the pH until it shows no response of life even after incubation for 10 days at pH value above 8.

The above results might suggest that the best pH value for life activities for all living microorganism is (7), afterward a pH value of 8 most microorganisms can't adapt with the environment.

Effect of temperature

Fig. 3 represent the observations for the effect of temperature on bacterial raw growth. The second temperature was chosen to be near the minimal extreme (25° C) (Hall, 1998) that was at (28°C), growth was retarded significantly to be initialized within 8 days .The other chosen temperature was near the maximal extreme (40°C) (Hall, 1998) was (38°C). The result was very encouraging , the growth was initiated after 2 days only.

The response of the bacterial growth to the temperature of incubation was almost a straight line configuration, descending whenever incubation temperature ascending, to conclude that this bacteria is of a thermophillic type. At temperature of 32°C, the growth was activated established metabolism within five days ,the activation noticed from the turbidity of the fresh media .this result agreed well with findings of Schmauder (1997), who recommended a temperature of 32°C for PAH-bacterial growth.

Configuration of the microbial fermentation kind

To perform any further test on the liquid enrichment media culture, it's necessary first to determine the kind (type) of microbial growth. This can be easily configured by observing the cell growth strategy with time for different initial substrate concentration (phenanthrene) which is used as inculcation for metabolism.

Fig. 4 shows the change of biomass concentration with time for different initial substrate concentrations. The trend for the five curves indicates that the curves have no intensity for interaction which eventually leads to configure the type of fermentation as a substrate – limiting kind.

This method to configure the type of fermentation

has been used and totally approved by Octave Livenspiel (1999) during his course of research on microbial fermentation kinds. The experimental data were given in table 1.

Compatibility with Monod's equation

As a basic principle bacteria generally undergoes Monod's Kinetic (Holt *et al*., 1994). Analytically this can be configured by observing the change of cell growth and substrate concentration with time, as suggested by Levenspiel (1999) and Kersters *et al*. (2005).

Figss 5 to 9 show the change of cell growth and substrate concentration with time. All these figures indicate that cell growth is directly proportional to time while substrate concentration decreases exponentially with time. This trend is approved by Levenspiel (1999) and Kersters *et al*. (2005). These results analytically confirmed that the bacteria undergoes Monod's Kinetics (Holt *et al*., 1994).

Cell Growth Behavior

The results are not enough for experts in biotechnology and environmentally scientists (Hall, 1998; Weiner and Matthews, 2003; Levenspiel and Levenspiel, 1972). Bequette and Bequette (1998) suggested that in order to decide that weather the growth is compatible or not with Monod's kinetics, the biomass growth with time should be examined too. Figs. 10-14 shows the behavior of cell growth with time for the five samples of cultures of different initial substrate (phenanthrene) concentration. The above mentioned figures show an ideal biomass behavior all with the same time interval of 48 hr. This behavior confirms that the growth is compatible with Monod's equation. The behavior is S shaped starting within 7 hours after inoculation. This period is called lag phase. After the 7 hours period the growth undergoes a log phase. And at the last two hours the growth experiences the stationary phase. The experiment duration wasn't long enough to undertake death phase. Nevertheless, the results shown in the figs. 10-14 confirms the Monod's kinetics according to Levenspiel and Levenspiel (1972) and Fogler (1999). The biology technique (Weiner and Matthews, 2003) didn't interact with the biochemical methodology of Levenspiel and Levenspiel (1972), but it was another form of confirmation for the microbial growth kinetics.

Effect of Limiting Substrate Concentration with Longer Time of Observation

When taking a sufficient amount of phenanthrene like an initial concentration of 10 mg/ml and monitoring under longer period the growth undergoes the complete cycle of its own. That can be realized in fig. 15, at which a perfect growth curve has been sketched in this growth curve, the four growth phases are now obvious. The first period is a lag phase where cells are getting used to the new environment and it took 7 hours. Afterward the growth phase started, when all the needed enzymes made up at the previous period, cells got not no other job but to response.

That's why in this phase a relative fast digestion for the substrate is witnessed, and that's what makes the growth exceeds logarithmic wise. Next to the growth phase the stationary phase is introduced gradually , an environment full of cells eventually leads to the depletion of food, and it's also a period for the cells to rest after the relatively fast and exhausting reproduction process occurred just before this phase. Most of the cells after the stationary phase got old with no more nutrients for cell reproduction, which leads to the dying phase, a natural cause of the accumulation of toxic materials (metabolism products for batch reaction like urea, CO_2 and others).

The influence of this bacterial growth on some petroleum products

This growth is now confidently obeys the Monod's kinetic when it's capable of cracking phenanthrene then can easily crack any petroleum product however complicated it is. Fig. 16 represents the cells growth rate when growing on heavy naphtha and fig. 17 shows the decrease in naphtha specific gravity as a result of the bacterial action. That easily explained by the digestion of some hydrocarbon molecules (especially aromatics) by the bacteria that reduced the specific gravity and eventually improved the product grade.

Another product is selected to be more complex like fuel oil, the results where as the previous product except for the gentle decline in the specific gravity of the product, that is explained by the complexity of the selected product that when it loses some carbon atoms it want be as effective as when dealing with a lighter product, that illustrated at figs. 18 & 19.

First Order Curve Fitting

The statistical software Statistica was used for estimating Monod's kinetics constants. The data taken for this estimation were the results for initial phenantherene concentration of (C_{Ao} =10 mg /ml) presented in table 1 and by employing method which includes the usage of the following equation :

The notation in table 3 $\frac{C_C}{r_C}\frac{1}{k}+\frac{C_M}{k}\frac{1}{C_A}$

After taking the necessary data of C_C, C_A and t from table 2 and with the aid of Statistica software and taking $r_C=dC_C/dt$. Then plotting C_C/r_C vs. $1/C_A$ as shown in fig. 20 is obtained, which may be represented the following equation :

$$\frac{C_C}{r_C} = 10.8382 + (0.4991797)\frac{1}{C_A}$$

Conclusion

In the scope of the present work a number points were been concluded like :

1. The most proper raw earth samples contaminated with oil are taken from Bajwan wells at north oil company-Kirkuk, for reasons of inoculation for a PAH degradation culture, and that's probably because they are older then other wells tested in this work.
2. Yields to configure that bacterial growth was accompanied by digesting phenanetherene is an evidence that phonon thereon is being used as the sole energy and carbon source.
3. The growth behaiviore observations showed that the growth was of a limiting–substrate growth type.
4. When experementing the bacteria on some petroleum cuts namly heavy naphtha and fuel oil,showed succesive biomass growth and significant specific gravity declination.

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