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Study of Degrading Some Petroleum Cuts by Bacteria

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ABSTRACT

Degradation of polycyclic aromatic hydrocarbon were studied by using the bacteria pseudomonas sp. isolated from soil contaminate with oil. The yield of microbial cells reaches a maximum at pH value of 7 for five different phenanetherene concentration media (5-25) mg/ml. At temperature of 32^{o}C, the growth was activated established metabolism within five days. Higher biomass concentration obtained with time of 22.5 min for initial substrate concentrations of 25 mg / ml. The change of cell growth and substrate concentration with time is directly proportional to time while substrate concentration decreases exponentially with time. These results analytically confirmed that the bacteria undergoes Monod's Kinetics. The order of degrading phenanthrene is obvious when a limiting substrate (5 or 10 mg/ml) is been used , the degradation processes are rather sluggish than those observed in (20 or 25 mg/ml). First order curve fitting was determined that the statistical software Statistica was used for estimating Monod's kinetics constants.

Key words: hydrocarbon degrading bacteria, *pseudomonas* sp., phenanthrene, pH, Temp., Monod's constants.

INTRODUCTION

Hydrocarbons are one of the most common types of environmental pollutants found in the soil and groundwater. With the vast amount of oil exploration, transport and storage that has occurred over the last century, most of the hydrocarbon contamination that exists today can be blamed on the spills and leaks of oil companies. Due to these spills and leaks, there have been more sites identified with petroleum hydrocarbon contamination than any other type of contamination. These sites vary from large oil spills to the much more common leaking underground storage

tank sites associated with many gas stations. With the large amount of pollution resulting from petroleum hydrocarbons, the cleanup of the contamination has become a topic of interest.

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic environmental pollutants consisting of two or more fused benzene rings. Anthropogenic inputs of PAHs from oil spills, ship traffic, urban runoff, wastewater and industrial discharge, as well as atmospheric fallout of vehicle exhaust and industrial stack emissions, have caused significant accumulation of PAHs in the marine environment. (Luo *et al*., 2009). The polycyclic aromatic hydrocarbons cracked by different species of bacteria to produce simpler uni- ring aromatic (toluene, xylene, benzene, methyl benzene …etc,) with different alkyls, depending on the species of bacteria and the site of cut at the polyaromatic hydrocarbon molecule (Schmauder,1999).

All polycyclic aromatic hydrocarbons (PAH'S) are difficult to be cracked, but phonantherene is considered to be the most diffecult to be digested according to the unique molecular structure containing the three- ringed PAH. Phenanthrene (toxic) as sole carbon and energy source (Header & Fuchsia ,1997).

Environmental bacteria are generally considered to be the most important organisms in the natural biodegradation of PAHs (Cerniglia, 1992). the potential of microorganisms pointed out as degrading agents of several compounds indicates biological treatment as being the most promising alternative for reducing the environmental impact of oil spills (Facundo *et al*. 2001, Robert *et al.* 2003).

Bioremediation offers a promising means to reclaim such contaminated soil (Bragg *et al.*, 1994). Bioremediation employs microorganisms capable of degrading toxic contaminants (Eriksson *et al*., 1995). Augmenting the contaminated site with an appropriate inoculum of microorganisms is a promising technique to enhance the biodegradation of hydrocarbons. Moreover, using an indigenous microorganism consortium ensure that the organisms have a higher tolerance to the toxicity of hydrocarbons and are resistant to variations in the environment (Hadibarata and Tachibana, 2009).

The present work is concerned with polycyclic aromatic hydrocarbon degrading bacteria "PAH – bacteria" using the biological method namely, the enriched liquid media method.

EXPERIMENTAL SECTION

Media *:*

Nutrient agar made up according to the manufacture'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/1) (4).

500 ml Erlenmeyer flasks containing 100 ml liquid enrichment medium. Inoculum 5% 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 phenanthrene 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^o C until the medium becomes turbid result of bacterial growth (2 -7 days). Carring out regular microscopical monitoring of the culture.

Transfering 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.

Isolation of phenanhrene degrading bacteria:

The isolatio was worked as in Fattal (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^oC 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^oC 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 28^0C. if necessary the streaking procedure is repeated. Prepare stock cultures from colonies of different appearance (color, consistency, morphology) by streaking onto agar slants. The isolates of bacteria was Identified by (Fattal , 2004) as a *pseudomonas sp.*

Growth of cultures on phenanthrene:

prepare a starting culture by inoculate cell material from an agar slope (2-7 days old) into inoculation medium and incubate at 28^oC with shaking for 24h. Harvest cells by centrifugation (10min,6000 rpm)(Centrifuge:MLW,Janetzki Type T5,Made in Germany) and resuspend the cell pellet in 5ml potassium phosphate buffer. Inoculate 10 test tubes, each containing 5ml enrichment medium, with 0.1 ml of the cell suspension. (The number of cells in the inoculum was 6.1×10^4 cells/ml)

Two test tubes serve as controls and are examined immediately as described below, and the remaining eight are incubated at 38^oC Every 2 days two test tubes are removed and examined as follows:-

Determination of cell number using a cell counting chamber(Thomas's chamber). - Determination of phenanthrene concentration. 5ml methanol is added to each test tube to bring the phenanthrene into solution. After mixing thoroughly the samples are allowed to stand for 10 min before centrifuging at 6000 rpm for 10min . (carefully remove 1.2 ml of the superment for the determination of phenanthrene by HPLC analysis. All the measurements are done in duplicate using a standard solution of phenathrene 50mg/1 phenanthrene dissolved in methanol) (Schmauder,1999).

Sample selection of soil inoculum :

The most ancient soil contaminate with oil was taken as a sample from the north oil company-Kirkuk-Iraq for inoculation.

Cell separation:

Harvest cell by centrifugation for (10 min) at (6000 rpm) appeared to get (6.1 x 10^4 cells/ml) calculated by the means of Thomas Chamber.

After 2 days and 8 hours (56 hr's) most of the liquid enrichment medium was consumed to make a total count of bacterial cells remaining in the phenantherene solution. Harvest cells by centrifugation again. To gain fresh cells ready for another inoculation.

The growth in Still cultures:

Cultures that are liquid enrichment media cultures will act as a small experimental batch bioreactor. Five flasks of 250 ml were filled with (100ml) of liquid enrichment medium with phenantherene concentrations , that were respectively (5,10,15,20 and 25 mg / ml). Each flask was inoculated with (2%) of same starter of bacteria. The cultures were incubated at (38^oC) for (2 days) , samples were transferred in a screw test tube every 6 hours, freezed, and stored to determine the total count of cells and degraded phenantherene.

Determination of phenantherene concentration:

All measurements were done in duplicate using a standard solution of (10mg /ml) solution of phenantherene. Separation and detection conditions were according to (Schmauder,1999).

RESULTS AND DISCUSSION

Effect of pH

Fig.1 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.

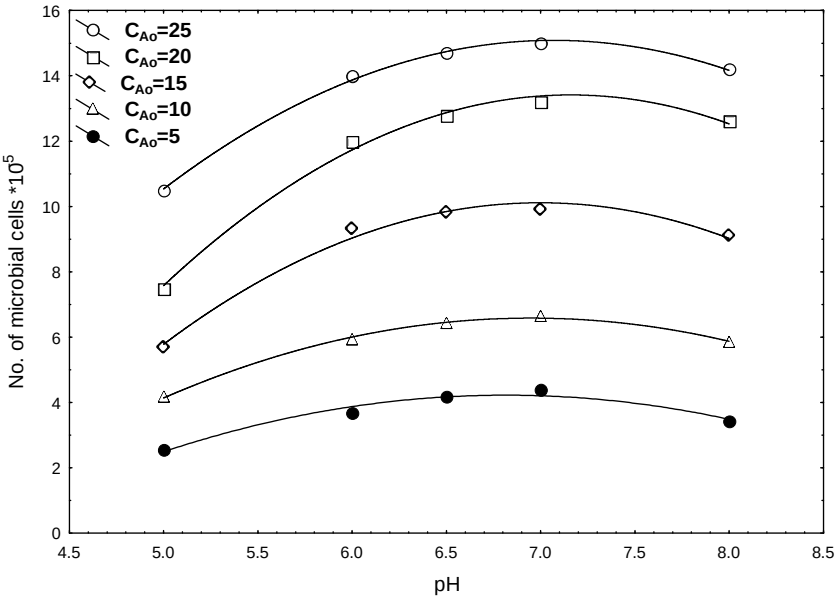

Fig.1 : Effect of pH value on the yield of number of cells produced for different initial phenanthrene concentrations (5-25) mg/ml.

Effect of temperature

Fig.2 represent the observations for the effec of temperature on bacerial raw growth. The second temperature was chosen to be near the minimal extreme 25 ^{o}C (Schmauder,1999), that was at (28^{o}C) , growth was retarded significantly to be initialized within 8 days .The other chosen temperature was near the maximal extreme 40^{o} C (Schmauder,1999) , was (38^{o}C).The result was very encouraging , the growth was initiated after (2) days only.

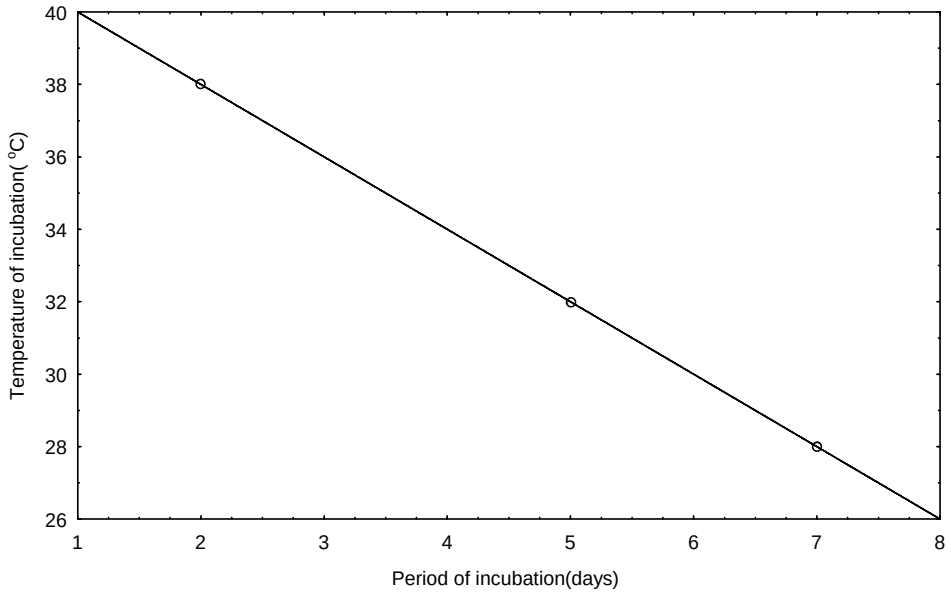

Fig.2 : Effect of temperature on the initial growth activation for phenanthrene cultivation

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^{o}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,1999) who recommended atemperature of 32^{o}C for PAH-bacerial 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 statigy with time for different initial substrate concentration(phenanthrene) which is used as a substrate for metabolism as been discussed before. Fig. 3 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 lead 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,1998), during his course of research on microbial fermentation kinds.

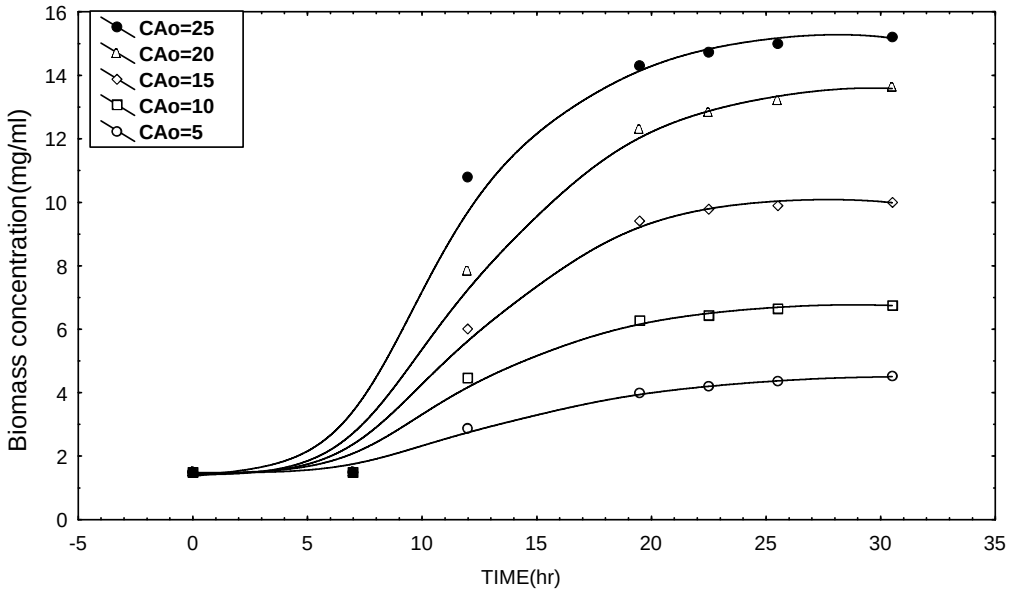

Fig 3 : Growth concentration for different phenanthrene concentrations virsus time , shows the intensity of fermentation kind to act as substratre-limiting.

Compatibility with Monod's equation: -

As a basic principle bacteria generally undergoes Monod's Kinetic. Analytically this can be configured by observing the change of cell growth and substrate concentration with time,as suggested (Octave,1998). Figs. 4 to 8 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 (Octave,1998). These results analytically confirmed that the bacteria undergoes Monod's Kinetics (Octave,1998).

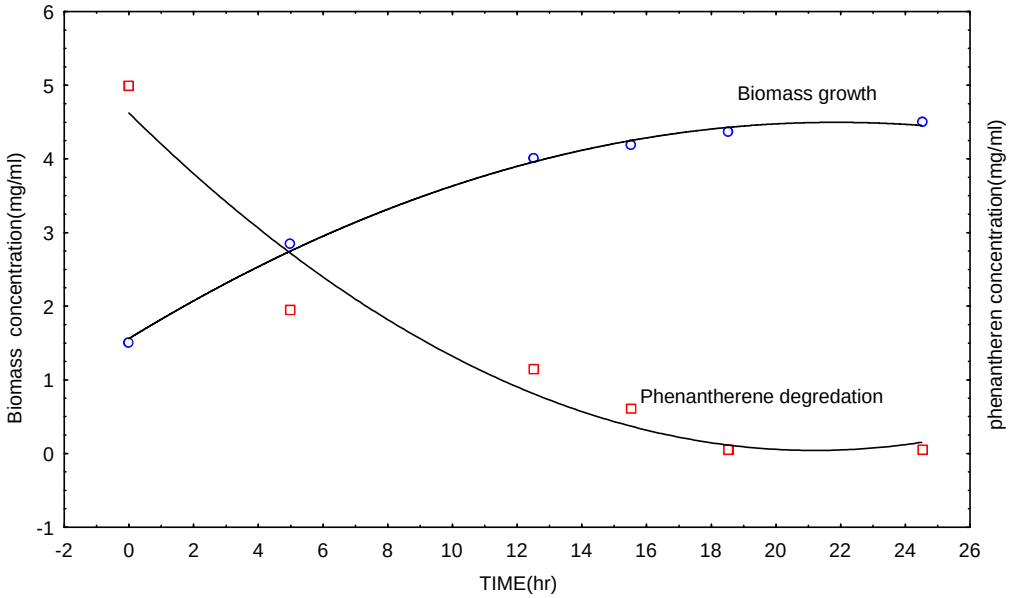

Fig.4 : Growth concentration with phenanthrene concentration or initial phenantherene concentration of 5 mg/ml

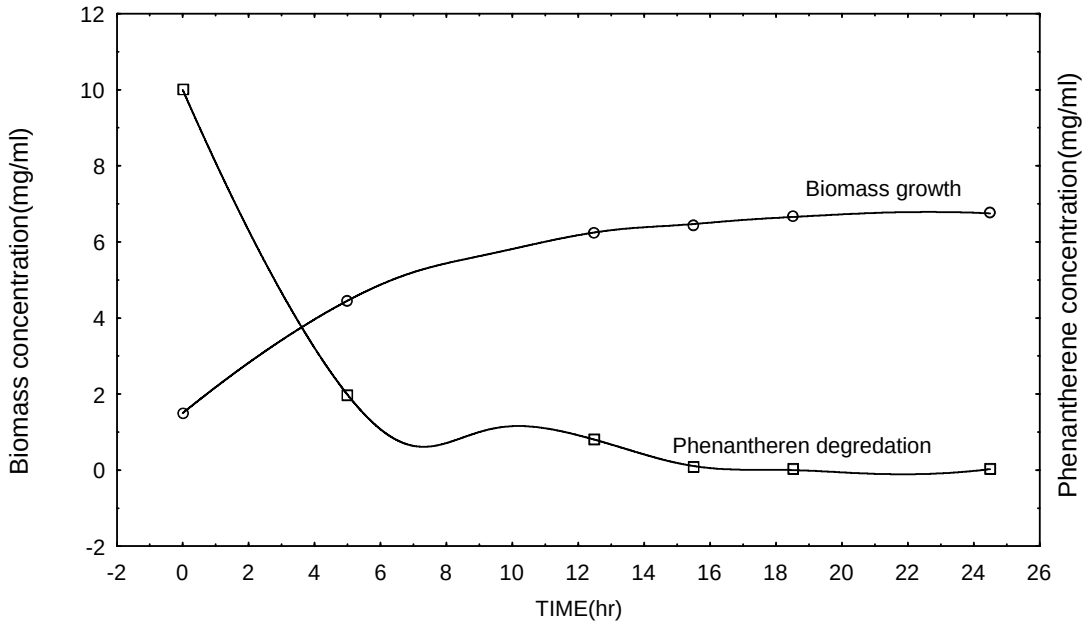

Fig.5 : Growth concentration with phenanthrene concentration for initial phenantherene concentration of 10 mg/ml

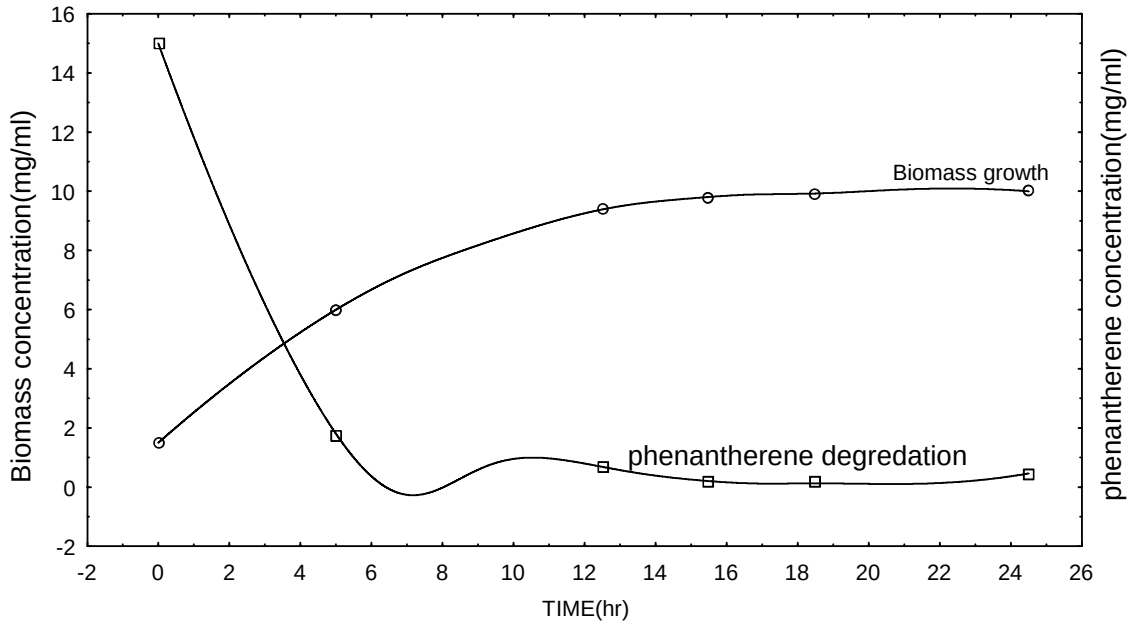

Fig. 6 : Growth concentration with phenanthrene concentration for initial phenantherene concentration of 15 mg/ml

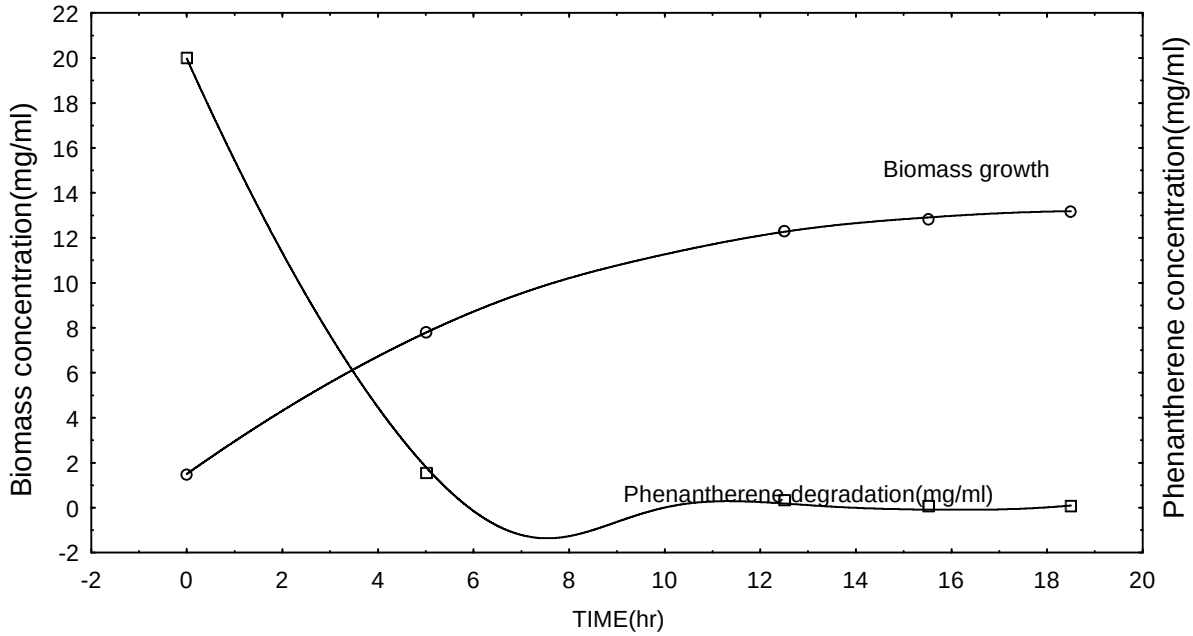

Fig. 7 : Growth concentration with phenanthrene .concentration for initial phenantherene concentration of 20 mg/ml

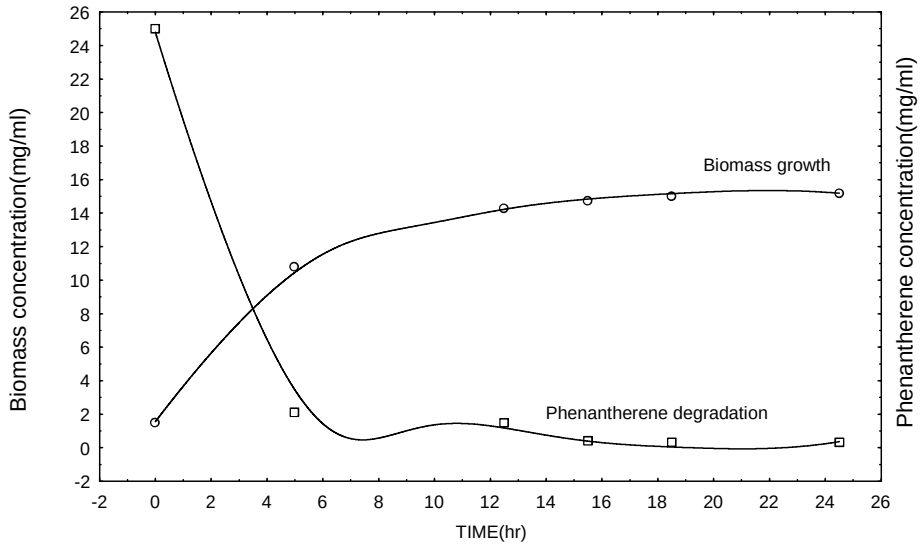

Fig. 8 : Growth concentration with phenanthrene concentration for initial phenantherene concentration of 25 mg/ml

Phenanthrene Degrading Order:

Fig. 9 to 13 represent the order of degrading phenanthrene involved in the studied bioreaction. It's obvious when a limiting substrate (for example 5 or 10 mg/ml)is been used as shown in figures 9 and 10 the degradation processes are rather sluggish than those observed in figures 11 to 13.

This may due to the fact that when there is a shortage of food in the environment the bacteria will not be comfortable to establish an active form of metabolism but only assure the presence of the species and basic metabolisms.

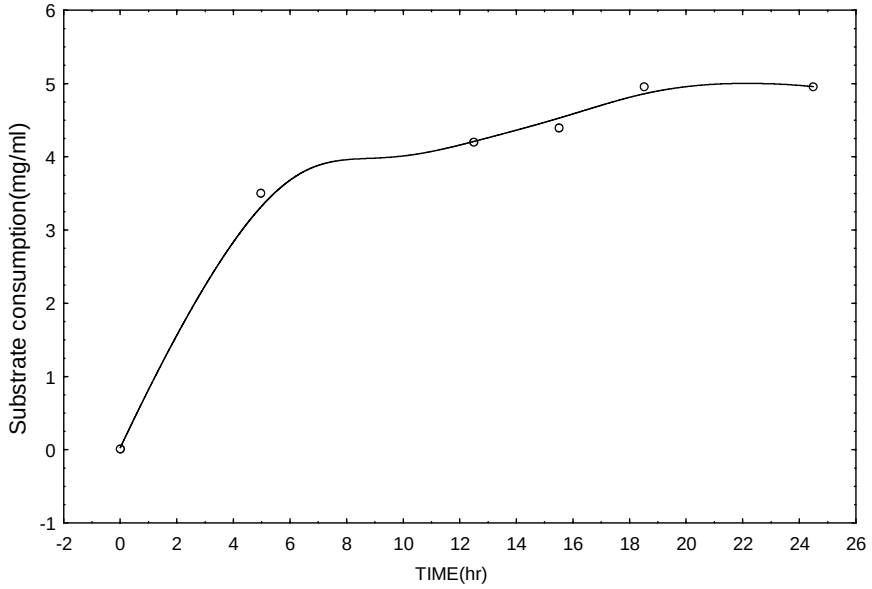

Fig. 9 : Phenantherene concentration consumed at the culture for an initial concentration of 5 mg/ml

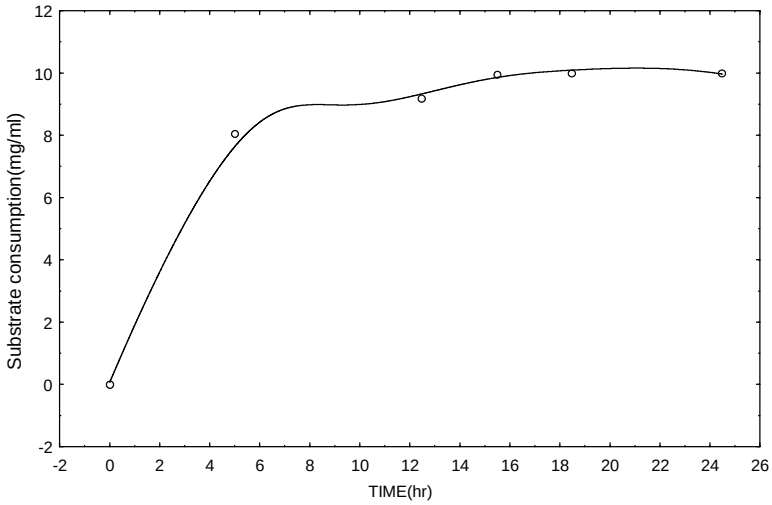

Fig. 10 : Phenantherene concentration consumed at the culture for an initial concentration of 10 mg/ml

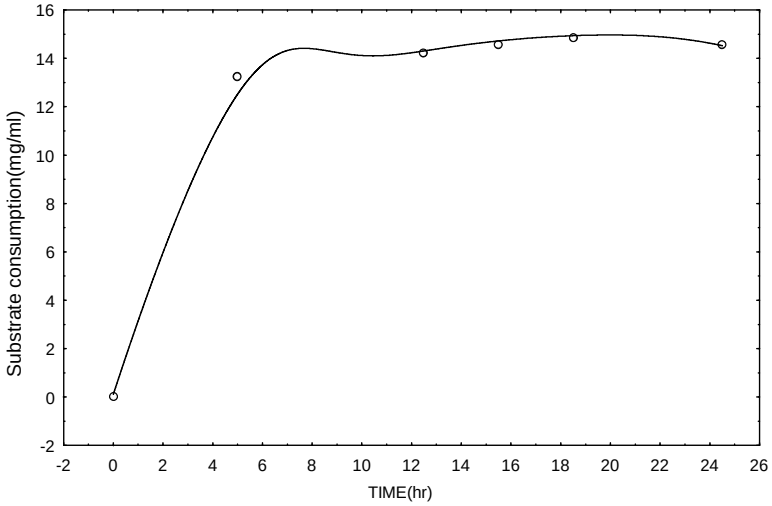

Fig.11: Phenantherene concentration consumed at the culture for an initial concentration of 15 mg/ml

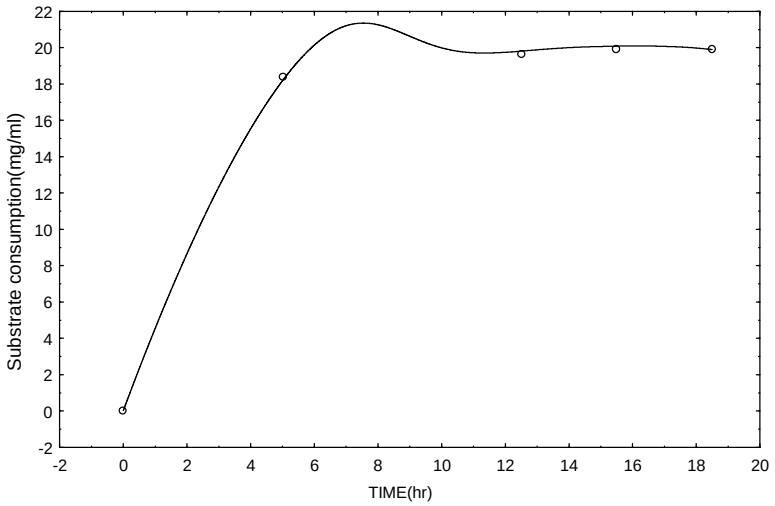

Fig.12 : Phenantherene concentration consumed at the culture for an initial concentration of 20 mg/ml

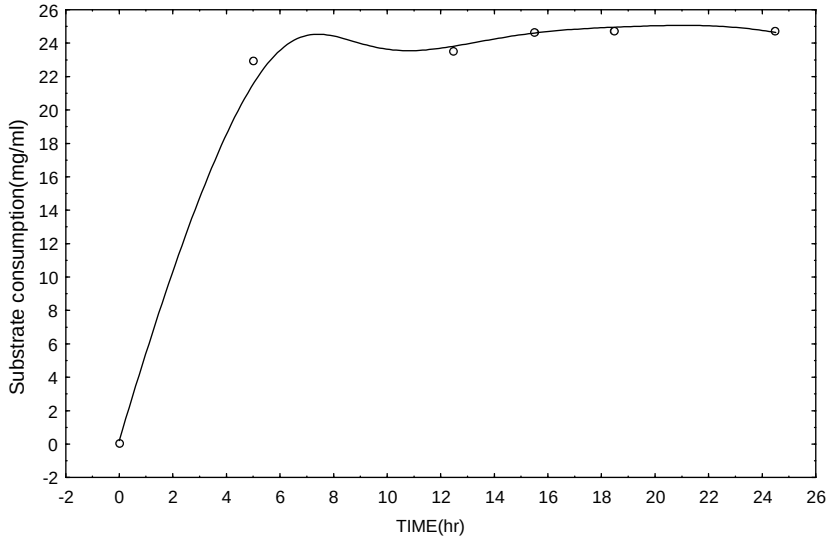

Fig. 13 : Phenantherene concentration consumed at the culture for an initial concentration of 25 mg/ml

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 appendix 1, and by employing method which includs the usage of the folowing equation:

$$\frac{C_C}{r_C} = \frac{1}{k} + \frac{C_M}{k}\frac{1}{C_A}$$

After taking the nessesary data of C_C,C_A and t from table (appendix 2),and with the aid of Statistica software and taking $rC=dC_C/dt$. then ploting C_C/r_C vs. $1/C_A$ as shown in figure 14 is obtained ,which may be represented the following equation:

The notation in Appendix-3

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

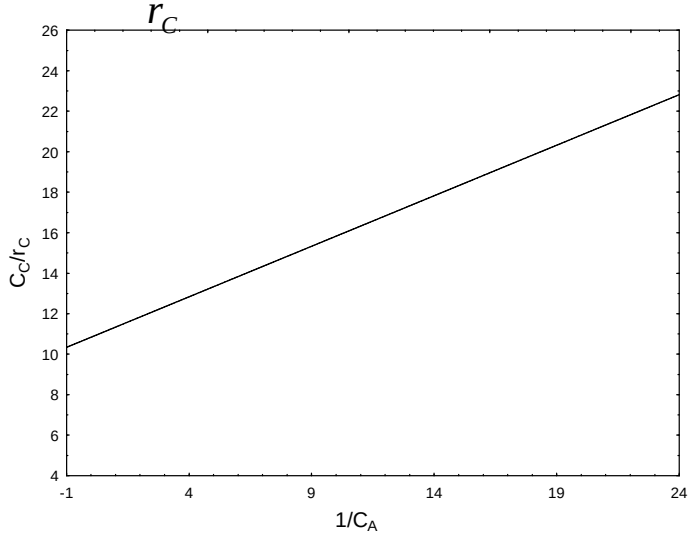

Fig. 14 : Monod's kinetic conistants for initial phenantherene concentration of CAo=10 mg/ml

CONCLUSION

The present work represent a combination of biological lab work and biochemical reactor features. 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- Kirknk, 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. Due to determintain of how biomass increased while phenantherene concentration decreased through out experimental observation. 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 most proper temperature for this bioreaction is (38^oC) .
4. The bioreaction rate increased with increasing the incubation temperature (with in the biorcation limitation).

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Appendices

Appendix 1 :The role of degrading phenantheren. The presented result were been used in section (Phenanthrene Degrading Order)

Time(hr)	Phenanthrene concentartion (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

Appendix 2: growth on phenantherene starting with CAo=10

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

	Appendix 3: Notation
C_{Ao}	Initial substrate concentration (phenethrene) in mg/ml
C_A	Substrate concentration (phenethrene) 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