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Research Article

ANALYSIS OF DIESEL SPILL BIOREMEDIATION BY BACTERIAL ISOLATES IN BATCH CULTURE USING MONOD AND HALDANE KINETIC MODEL

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ABSTRACT

The unintended oil spills from oil tankers, transportation and storage facilities, leads to petroleum hydrocarbon pollution that causes adverse effects on the air, water and soil environment. Bioremediation using indigenous microorganisms has become a promising tool for effective mineralization of toxic contaminants into harmless end products. In this study, bioremediation of petroleum contaminated soil was carried out in a batch scale using *Staphylococcus, Pseudomonas, Micrococcus, Escherichia*, *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Salmonella* and *Klebsiella species,* all isolated from petroleum hydrocarbon contaminated soil. The effect of 1 to 10% v/v diesel concentrations on the growth and biodegradation rate of all selected nine isolates was studied and specific growth rates (μh^{-1}) were calculated. The initial rate of growth and biodegradation of nine isolates increased correspondingly with the diesel concentration up to 5% (v/v), but further increase in diesel concentration resulted in a slight decrease in the rate of biodegradation. Diesel is known to be an inhibitory substrate. Both Monod and Haldane kinetic models were useful for the evaluation of the growth kinetic parameters (μ_{max}, K_s, K_i). It was found that the Monod model was able to express the growth parameters up to 5% v/v diesel. However, Haldane model effortlessly fitted with the experimental data for increase in the diesel i.e. over 6 % v/v diesel. These results showed that the diesel oil degraders were able to utilize a considerably high concentration of diesel and could be very effective in diesel biodegradation for restoration of contaminated sites.

Keywords: Biodegradation, Kinetics, Specific Growth Rate, Monod Model, Haldane Model, Diesel Inhibition.

1. INTRODUCTION

Petroleum and its products include hydrocarbons such as alkanes, aromatics, resins and asphaltenes associated with other organic compounds containing sulfur, nitrogen and oxygen. These can be emitted out in the nature through various processes and can pose toxicological effect on both living and nonliving beings [1]. The threats of oil spill are very common due to transportation and leakages of pipelines at the storage site in the areas near the petrol pump. Physical and chemical methods such as skimming or using chemical dispersants can be the crucial options for oil spill cleanup; however both are expensive and restricted in success [2]. Many technologies have been invented in last two decades to degrade petroleum pollutants from the environment. As compared to physical and chemical methods, bioremediation is a sustainable technique for cleaning of oil polluted sites, in which hazardous oily materials can be easily mineralized to harmless end products at very low cost using indigenous microorganisms [3].

The major constraint of bioremediation process, however is the slower biodegradation rate of hydrocarbons in natural environment due to their low bioavailability. Kinetic study is therefore essential to determine the equilibrium constant, the speed of reaction and control of the process in pilot scale hydrocarbon bioremediation studies [4]. Studies of biodegradation kinetics in a natural environment are often empiric, reflecting only the basic level of knowledge about the microbial population and its activity in the given environment [5]. Different hydrocarbon components such as aliphatic, aromatic and polycyclic compounds have dissimilar degradation rate, lighter crude oils have faster biodegradability than heavier ones. Thus, to find out petroleum biodegradation, kinetics is complicated and difficult in most cases. Furthermore due to differences in experimental techniques or data analysis, variations in the biokinetic constants have been reported for the same conditions. Hence, there is still a lack of knowledge on the subject of hydrocarbon bioremediation kinetics [6]. In this present study, bioremediation of diesel oil contaminated soil was carried out in a laboratory using previously isolated nine

potent bacterial species from petroleum hydrocarbon residues contaminated soil at the experimental study site and further studying growth kinetics of these isolates using Monod-Haldane model.

2. MATERIAL AND METHODS

2.1. Organisms Cultivation conditions

The organisms used in this present study, *Staphylococcus, Pseudomonas, Micrococcus, Escherichia*, *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Salmonella* and *Klebsiella species* were isolated previously from petroleum oil contaminated soil. Each isolates were inoculated individually in the 250 ml of Erlenmeyer flasks containing 50 ml Bushnell Haas (BH) medium with 1% diesel oil as a carbon source, supplemented with 2 gl^{-1} Glucose as a trace nutrientand incubated it at 30±2°C temperature and at 7 pH and kept it on a rotary shaker incubator at agitation speed of 150 rpm aseptically for 10 days. After 10 days of incubation period, the culture of each isolates were taken for the further study [7].

2.2. Batch Experiments for Substrate Utilization

A batch experiment was performed anaerobically for the study of growth kinetics of all nine potent isolates for a period of 72 hrs. The flasks were operated under batch culture and samples were taken from the flask at predetermined time intervals of 4hrs throughout the study. The kinetics of substrate utilization and rate of degradation in the batch culture for each potent bacterial isolates and their consortium were studied in 250 ml Erlenmeyer flasks (for each individual bacterial isolate) containing 50 ml BH liquid medium and various concentrations (1 to 10 % v/v) of diesel as a substrate and 2 gL^{-1} glucose as growth supporting mineral and samples were taken from the flask at predetermined time intervals of 4hrs. The liquid medium was maintained at pH 7, 30°C and kept on 150 rpm in a rotary shaker incubator for 72 hrs. All the cultivations were performed in triplicates with total media volume of 250 ml and a control devoid of the bacterial isolates was prepared for each set of experiments. Cell Mass was calculated as colony forming unit ($CFUml^{-1}$). For this 10^{-8} cells/ mL of the isolates were spread on the BH solid media and viable cell was counted as a CFU. Specific growth rate of each isolates at each concentration of substrate was determined by using equation 1.

μ=slope = - $\{(InX_1-X_0)/(t_1-t_0)\}$ (Equation 1)

Where, μ = Specific growth rate, $X_1 - X_0$ = Difference in substrate concentration, t_1-t_0= Difference in time

For this, various concentrations of diesel 1 to 10 % (v/v) were taken. Although biodegradability of crude oil is usually explained by first order kinetics, to conduct an extensive investigation, several kinetic models for crude oil degradation have been evaluated. In a batch experiment, for modeling of crude oil and micro-organism concentration change with time, a simple bio-kinetic Monod and Haldane's models with two components were chosen. The mathematical expressions for Monod (Eq. 2) and Haldane's (Eq.3) kinetics are as follows:

$\mu = \{(\mu_{max}S)/(Ks+S)\}$ (Equation 2)

$\mu = \{(\mu_{max}S)/(Ks+S)+S^2/Ki\}$ (Equation 3)

Where, μ_{max} = Maximum specific growth rate, Ks =half substrate saturation, S = Substrate Concentration, Ki= inhibition coefficient

Specific growth rate (μ) values were plotted against substrate for getting maximum specific growth rate (μ max), half substrate saturation (Ks) and inhibition constant (Ki). Comparison of rate of degradation (%) of all the nine potent isolates was measured at 1to 10 % v/v of diesel by gravimetric method at an interval of 4hrs [8].

3. RESULTS AND DISCUSSION

Isolates *Staphylococcus, Pseudomonas, Micrococcus, Escherichia*, *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Salmonella* and *Klebsiella species* are indigenous organisms, isolated from the petroleum oil contaminated soil at the experimental site and these were found to be potent degraders of petroleum hydrocarbons [9]. To measure specific growth rate (μ Equation 1), the cell biomass ($CFUml^{-1}$) were calculated during the exponential growth phase, and a plot of the natural log of biomass versus time were achieved; the slope obtained equals the μ (Fig. 1). The results showed that specific growth rate increased progressively from lower 1 % up to 5% v/v of diesel concentration, and it gradually decreased beyond 6% to 10% v/v of diesel. However, *Pseudomonas spp* achieved maximum μ value (0.0691 h^{-1}), followed by *Micrococcus spp* (0.0302h^{-1}), *Staphylococcus spp* (0.0224 h^{-1}), *Escherichia spp* (0.0179 h^{-1}) and *Acinetobacter spp* (0.017 h^{-1}) at 5 % v/v of diesel concentration. Among the rest of the isolates *Salmonella spp* showed higher μ value (0.0277 h^{-1}) at 2% v/v while *Bacillus spp* (0.0144 h^{-1}), *Corynebacterium spp* (0.0235h^{-1}) and *Klebsiella spp* (0.0403 h^{-1}) displayed higher μ value at 4, 5, 3 % of diesel oil respectively.

It has been also observed that, isolate *Micrococcus spp* (83.03 %) exhibited higher percentage of increase of specific growth rate on 1 to 5 % (v/v) diesel oil followed

by *Staphylococcus spp* (53.42 %), *Pseudomonas spp* (32.8%), *Escherichia spp* (26.05 %), *Acinetobacter spp* (24.08 %), thus indicating the possibility that these five oil degraders probably developed tolerance, subsequently adapted in the stressful environment and thus improve developmental potential in such concentration of diesel oil. While the rest of the isolates viz., *Salmonella spp*, *Bacillus spp*, *Corynebacterium spp* and *Klebsiella spp* exhibited comparatively lower growth rate (i.e. up to 0 to 9%) on 1 to 5 % of diesel oil, thus indicating comparatively lower tolerance as these isolates were not able to tolerate the increase of diesel concentration beyond 5%.

The biodegradation potential data revealed that *Pseudomonas spp* (51.8%) had achieved highest capacity to degrade diesel oil followed by *Acinetobacter spp* (48.8%), *Escherichia spp* (44.2%), *Micrococcus spp* (42.2%), *Staphylococcus spp*(42%), *Bacillus spp* (36.4%), *Salmonella spp* (23.2%), *Corynebacterium spp* (20.6 %) on 5 % while *Klebsiella spp* (31 %) displayed higher biodegradation potential at 2% of diesel oil during 72 hrs of incubation time (Fig. 2). Though it appeared that the biodegradation potential of all the nine oil degraders decreased with increase in the concentration of the diesel in the growth medium, supporting the assumption that higher diesel concentration does not support the metabolic activity in the isolates.

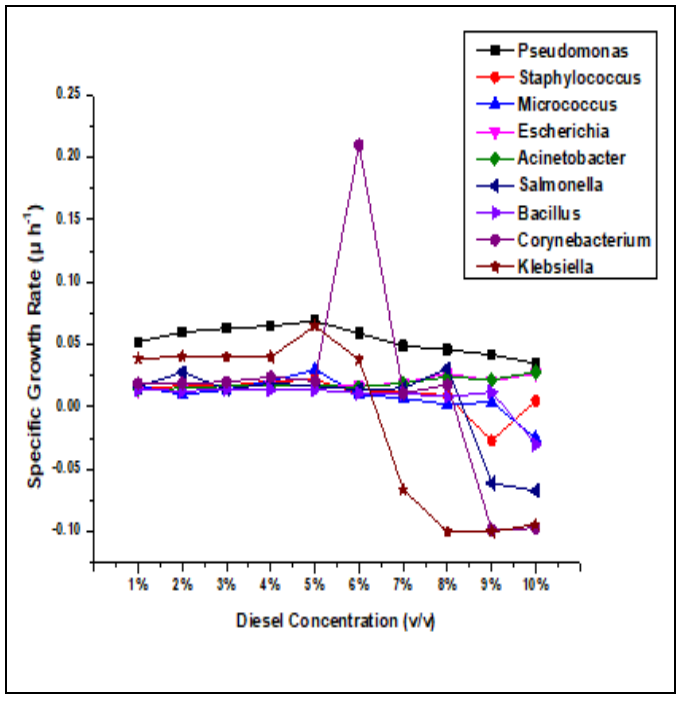

Fig. 1: Specific Growth Rate μ (h^{-1}) derived by all nine diesel biodegraders on 1 to 10 % (v/v) of diesel concentration

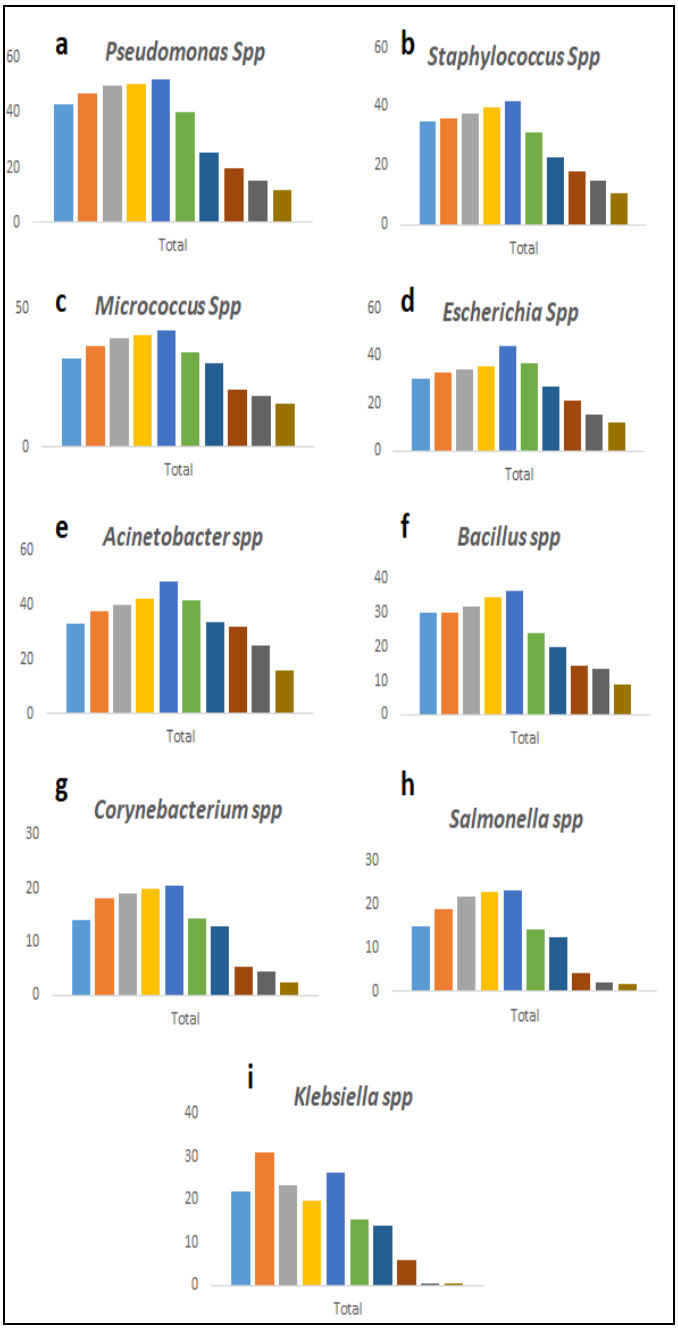

Fig. 2: Pivotal diagram showing sum of removal of diesel oil by selected nine oil degraders on 1 and 10 % (v/v) of diesel during 72 hrs of incubation period.

3.1. Evaluation of Monod's and Haldane's Kinetic Models

The data represented in the table 1 displays the biokinetic parameters such as (μ_{max}), (K_s) and (ki) derived from the Monod and Haldane models of growth kinetics. All the nine isolates achieved higher K_s values on various concentrations of the diesel as a substrate such as *Pseudomonas spp* achieved higher ks (11.17 gl^{-1}) with μ_{max}

($0.083h^{-1}$) on 4 % diesel while *Micrococcus spp* and *Escherichia spp* both had higher K_s (40.45, 41.54 gl^{-1} respectively) with μ_{max} (0.198, 0.327 h^{-1} respectively) on 2 % diesel whereas, *Staphylococcus spp* showed higher K_s(14.72 gl^{-1}) with μ_{max} (0.058 h^{-1}) on 3% diesel and *Acinetobacter spp* showed higher K_s (6.72 gl^{-1}) with μ_{max} (0.008 h^{-1}) on 5% diesel. Similarly, *Salmonella spp* and *Bacillus spp* both had achieved higher K_s (48.98 and 24. 33gl^{-1} respectively) with μ_{max} (0.32 and 0.147 h^{-1} respectively) on 2 % diesel while *Corynebacterium spp* and *Klebsiella spp* both displayed higher Ks (16.2 and 38.72gl^{-1} respectively) with μ_{max} (0.156 and 0.224 h^{-1} respectively) on 1 % diesel.

The high value of Ks obtained for concentrations of 2 and 4% v/v diesel indicate high utilization of hydrocarbons. In addition, it was observed that the value of Ks with μ_{max} found to decrease with the increase in the diesel concentration. All the potent isolates exhibited decrease in their Ks with μ_{max} values with respect to diesel concentration beyond 5 % i.e. from 6 to 10 % v/v. The results clearly explained the suitability of Monod model with the parameters especially during the exponential growth i.e. up to 5 % diesel whereby all nine isolates had grown exponentially so that the data obtained was well fitted in the derivation of the Monod model.

Perhaps, the growth in the isolates decreased when diesel concentration increased from 6 to 10 % and this decrease in growth was derived by the Haldane's kinetic model. At a high initial concentration of diesel the specific growth rate decreased, which is shown by substrate inhibition after a certain concentration. To fit the data and estimate the values of three bio-kinetic constants of the Haldane equation (Equation 3), based on the non-linear least square technique was used and presented through Origin 8.5. The values of inhibition constant (Ki) for 1 - 10%diesel concentration showed inhibitory effect on the growth of the test isolates (Table 1).

Table 1: Bio-kinetic Parameters (K_s, μ_{max} and K_i) derived by all nine diesel biodegraders at 1 to 10 % (v/v) diesel concentration

Isolates	Biokinetic Parameters	% of Diesel Concentration (v/v)				
		2	4	6	8	10
Pseudomonas	$\mu_{max}(h^{-1})$	0.047	0.083	0.352	0.302	0.02
	Ks (gl^{-1})	3.39	11.17	0.038	0.205	0.088
	Ki (gl^{-1})	-	-	0.257	0.341	45.27
Micrococcus	$\mu_{max}(h^{-1})$	0.198	0.04	0.197	0.009	0.126
	Ks (gl^{-1})	40.456	2.729	0.049	0.25	0.653
	Ki (gl^{-1})	-	-	0.129	1.281	0.142
Staphylococcus	$\mu_{max}(h^{-1})$	0.088	0.038	0.019	0.002	0.01
	Ks (gl^{-1})	8.17	5.32	0.189	0.165	0.192
	Ki (gl^{-1})	-	-	6.493	1.082	2.292
Escherichia	$\mu_{max}(h^{-1})$	0.327	0.113	0.672	0.003	0.01
	Ks (gl^{-1})	41.54	10.41	0.086	0.166	0.213
	Ki (gl^{-1})	-	-	0.081	1.358	15.59
Acinetobacter	$\mu_{max}(h^{-1})$	0.004	0.028	0.117	0.006	0.006
	Ks (gl^{-1})	4.623	0.602	0.098	0.169	0.181
	Ki (gl^{-1})	-	-	0.361	1.441	1.395
Salmonella	$\mu_{max}(h^{-1})$	0.32	0.051	0.009	-	-
	Ks (gl^{-1})	48.98	6.31	0.191	-	-
	Ki (gl^{-1})	-	-	1.75	-	-
Bacillus	$\mu_{max}(h^{-1})$	0.147	0.019	0.023	0.03	0.055
	Ks (gl^{-1})	24.33	1.16	0.039	0.24	0.101
	Ki (gl^{-1})	-	-	2.75	2.32	0.175
Corynebacterium	$\mu_{max}(h^{-1})$	0.03	0.02	0.009	-	-
	Ks (gl^{-1})	4.03	4.87	0.135	-	-
	Ki (gl^{-1})	-	-	0.64	-	-
Klebsiella	$\mu_{max}(h^{-1})$	0.059	0.205	0.004	-	-
	Ks (gl^{-1})	10.86	27.53	0.13	-	-
	Ki (gl^{-1})	-	-	0.61	-	-

The results showed that isolates *Pseudomonas spp* and *Escherichia spp* attained higher Ki (45.27, 15.59gl^{-1} respectively) with lower μ_{max} (0.02, 0.01h^{-1}) on 10 % diesel v/v, *Micrococcus spp* and *Acinetobacter spp* showed higher Ki (1.142, 4.03 gl^{-1} respectively) with μ_{max} (0.009, 0.022h^{-1}respectively) on 7 % diesel v/v while *Staphylococcus spp* had higher Ki (6.493 gl^{-1}) with μ_{max} (0.019 h^{-1}) on 6 % diesel v/v and *Bacillus spp* displayed higher Ki (2.32gl^{-1}) with μ_{max} (0.03 h^{-1}) on 8 % diesel v/v. Rest of the isolates viz., *Salmonella spp*, *Corynebacterium spp* and *Klebsiella spp* were not able to grow on diesel concentration beyond 6 % v/v. The specific growth is determined by excluding the data for death phase. The decrease in specific growth rate with increase in substrate concentration is a result of self-inhibition which occurred because of excess substrate concentration [10]. The findings obtained by reserachers [11, 12] during the study of growth kinetics of hydrocarbon degrading *Bacillus spp* and *Acinetobacter spp* respectively were in acceptance to the present study. Similarly, the obtained specific growth rate is found to be well within the range as reported in the literature [13].

4. DISCUSSION

Microbial growth is described as an orderly increase in all chemical components in the presence of suitable medium, where three fundamental growth constants can be characterized such as total growth which measures the difference between initial and maximum bacterial density; exponential growth rate and lag time where growth slows down. Present study was carried out in the batch culture - a closed system that contains an initial limited amount of substrate i.e. Diesel. The inoculated potent bacterial oil degraders passed through a number of growth phases. During the exponential phase, nutrients were in excess and the microorganism grew at maximum specific growth rate μmax for the prevailing conditions. Also, the relation between the specific growth rate (μ) of a population of microorganisms and the substrate concentration (S) is a valuable tool in biotechnology. It has been seen that, specific growth rate (μ) of all the selected potent bacterial isolates were increased steadily till the concentration of diesel reached upto 5%.This indicated that organisms produced growth induced metabolites at this limited substrate concentration. Monod parameter ks value, noticed exceptionally larger than that of μ max for all the bacterial isolates. However, the present approach allowed a prediction that a larger value was to be expected, because the effective area-to-substrate of the cells would be much smaller than that for dissolved substrates. It also concluded that at such initial substrate concentration organisms had high affinity towards its growth conditions. Gonzo *et al.*, 2014; Smith *et al.*, 2002 reported the cases of such high values of Ks (2.4gL^{-1} and 11.06 mgL^{-1} respectively) than its μ max (0.085 h^{-1} and 0.408 h^{-1} respectively) during the study of substrate utilization. Due to low solubility of diesel considerable amount of biodegradation was seen and diesel oil removed during exponential phase of bacterial growth. However, substrate was not degraded completely. A certain amount of diesel persisted in the culture broth. As it was assumed that the difference in substrate concentration between the bulk oil and adjacent cell was the driving force of transport, there would be no more mass transfer thus no more degradation of substrate when the substrate concentration in bulk oil reached the substrate concentration in cell. The validity of the model developed by assuming direct contact of microorganisms with oil surface could be elucidated from the experiment. It was clearly seen from the R^2 obtained for the reciprocal plots of μ verses s drown that the experimental values were well fitted in the Monod model.

5. CONCLUSION

The biodegradation of Diesel oil for a concentrations ranging from 10 to 50 mg/dm^3 was successfully achieved. The kinetics of substrate utilization and bacterial growth of using diesel as the sole carbon and energy source were determined to contribute to improving the biological process for oil polluted soil treatment. The use of *Staphylococcus, Pseudomonas, Micrococcus, Escherichia* and *Acinetobacter species* to degrade diesel has been shown to result in increased substrate utilization rate. The removal of oil efficiency of these potent oil degraders was verified at exponential growth phase within a period of 72 hrs and Monod bio-kinetic parameters (μ, Ks and μ max) were derived using linear equation. Monod kinetic model was able to predict the microbial growth in the batch culture at lower initial substrate concentration indeed. The above parameters are essentially used for biodegradation process understanding, bioremediation speed measurement and improvement of effective clean-up for petroleum contaminated environment.

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