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Applied Growth Kinetic Models for Crude Oil Spill Bioremediation in a Batch Scale Bioreactor

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Abstract

Hydrocarbon pollution occurs mainly by accidental oil spills, deliberate discharge of ballast waters from oil tankers and bilge waste discharges; causing site pollution and serious adverse effects on the environment. As compared to physical and chemical methods, such as booms, skimmers, adsorbents, chemical surfactants, oxidants, etc., bioremediation is a sustainable technique for restoration of oil-polluted sites, in which highly hazardous oily materials can be easily mineralized to harmless end products at very low cost using indigenous microorganisms. In this study, bioremediation experiments of artificially crude oil contaminated water was carried out in a batch scale bioreactor using the mixed culture of *Ochrobactrum pseudintermedium* sp. C1 and *Bacillus cereus* sp. K1. The effect of initial crude oil concentrations (1 to 10% v/v) on the growth and biodegradation rate were studied. The initial rate of growth and biodegradation increased with initial crude oil concentration up to 4% (v/v), but further increase in oil concentration resulted in a gradual decrease in the rate of biodegradation due to substrate inhibition. Both Monod and Haldane kinetic models were applied to evaluate the growth kinetic parameters (μ_{max}, K_s, K_i, S_{max}, $Y_{x/s}$). It was found that the Monod model was unable to present the growth parameters over the defined concentration range. However, Haldane model perfectly fitted with the experimental data and the following kinetic parameters were obtained: μ_{max}=0.085 hr^{-1}; K_s=32722.83 mg/dm^3; K_i=53205.64 mg/dm^3; S_{max}=41725.76 mg/dm^3. These results showed that the mixed culture was able to utilize a considerably high concentration of crude oil and could be very effective in crude oil biodegradation for restoration of highly oil contaminated sites.

Keywords: Biodegradation; Haldane model; Kinetics; Monod model; Total petroleum hydrocarbons

Introduction

Oil spilling accidents are very common in the areas near the seashore because of shipping of oil tankers and bursting of oil supply pipelines. It is estimated that more than 2 million tons of oil enters marine environments from ships and other sea-based activities annually [1]. Recently in India, Mumbai-Uran oil spill through pipeline bursting witnessed about 600 metric tons spillage of crude oil which caused a wide spread mortality of sea animals [2]. Physical skimming or using chemical dispersants are the primary response options for oil spill cleanup; however both are costly and limited in effectiveness [3]. Expectations for innovative and sound technologies for the removal of petroleum contaminants have therefore increased in last two decades.

Crude oil is a complex mixture of many petroleum hydrocarbons, like alkanes, aromatics, resins and asphaltenes associated with other organic compounds containing sulfur, nitrogen and oxygen. Some fractions of oil can cause chronic sub-acute toxicological effect (reduced growth and reproduction, poor health, low recruitment rates), which can alter population dynamics and disrupt tropic interactions and the structure of natural communities within ecosystems [4]. The technique of microbial remediation has been shown to be a viable treatment option to minimize the adverse effects caused by oil spillage since its first successful commercial application in 1972 at the Sun Oil pipeline spill site in Pennsylvania [5]. As compared to physical and chemical methods, such as booms, skimmers, adsorbents, chemical surfactants, oxidants, etc., bioremediation is a sustainable technique for restoration of oil polluted sites, in which highly hazardous oily materials can be easily mineralized to harmless end products at very low cost using indigenous microorganisms [6].

However, the major constraint of bioremediation process is the slower biodegradation rate of hydrocarbons in natural environment due to their low bioavailability. Kinetic study is essential to determine the equilibrium constant, the speed of reaction and control of the process in pilot scale hydrocarbon bioremediation studies. 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 [7]. Lighter crude oils (higher API gravity) normally have faster biodegradability than heavier ones. On the other hand, different crude oil components such as aliphatic, aromatic and polycyclic compounds have dissimilar degradation rates. Thus, prediction of 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 [8]. Hence, there is still a lack of knowledge on the subject of hydrocarbon bioremediation kinetics.

In this present study, bioremediation experiments of artificially crude oil contaminated water was carried out in a batch scale bioreactor using a mixed culture of *Ochrobactrum pseudintermedium* sp. C1 and *Bacillus cereus* sp. K1 [9,10]. Kinetics of bioremediation process can be evaluated in two ways: (1) The first concerns with the factors influencing the amount of transformed compounds with time and (2) the other approach seeks the types of curves describing the transformation and determines which of them fits the degradation of the given compounds

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by the microbial culture [11]. The second approach has been adopted in this study to evaluate, model and analyze degradation kinetics for in-situ bioremediation of crude oil in a controlled bioreactor system.

Materials and Methods

Chemicals

Crude oil having the specifications represented in Table 1 was procured from IOCL Oil Refinery, Haldia, India. N-hexane of liquid chromatography grade purchased from E. Merck Co. (Germany) was used. Other chemicals and solvents were of AR grade and purchased from local suppliers. Bushnell-Haas (BH) media and Nutrient agar media of Hi-Media Laboratories Pvt. Ltd were used for isolation, cultivation and maintenance of culture (Table 1).

Organism and cultivation conditions

The organisms used in this present study, Ochrobactrum pseudintermedium C1 and Bacillus cereus K1 were isolated previously from waste oil contaminated soil in our laboratory [12, 13]. Both the organisms were identified by 16*S rRNA* gene sequencing method from Bhat Biotech India Pvt. Ltd. Bangalore, India and submitted to NCBI GenBank database under the accession numbers KJ094035 and KJ922989 respectively [9,10]. For isolation of the microorganisms, Bushnell Haas (BH) media was used as enrichment medium with the following composition (g/dm^3): K_2HPO_4 (1.0), KH_2PO_4 (1.0), NH_4NO_3 (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), $FeCl_3 \cdot 6H_2O$ (0.05), $CaCl_2 \cdot 2H_2O$ (0.02). The pH of the medium was adjusted to 7.0 ± 0.2. Cultivations were performed in 250 ml Erlenmeyer flasks containing 50 ml BH medium at 37 ± 2°C incubation temperature and agitation speed of 150 rpm in a rotary shaker incubator (ORBITEK-LJE, Scigenics Biotech Pvt.ltd., Chennai, India) for 7 days. Nutrient agar media was used for maintenance of the culture. The composition of the nutrient agar used was as follows: beef extract 5.0 g, peptone 10.0 g, NaCl 5.0 g, agar 15.0 g in a liter of distilled water. The cultures were grown in this media for 16–18 h at 37°C and were used as inoculum at 10% (v/v) level throughout the study.

Batch experiments

A set of experiments were performed for a period of 72 h varying the initial concentration of crude oil in the range of 1-10% (v/v). The main components of the system are a fully mixed bench scale reactor (Eyela Co., Tokyo, Japan), constant temperature water circulator, monitoring device for temperature, dissolved oxygen and pH. The reactor with a total volume of 1.5 dm^3 was manufactured from glass having a water jacket for controlling the process temperature which was kept constant at 37 ± 2°C for all experiments. The air flow rate to the reactor and stirring speed were adjusted to 1.5 ml/min and 150 rpm, respectively. Dissolved oxygen concentration, pH and temperature were measured continuously throughout the experiments. The reactor was operated under batch operation and samples were taken from the reactor at predetermined time intervals using a peristaltic pump. For each initial substrate concentration, cell mass and the substrate concentration were determined at an interval of 4 h throughout the study. All the cultivations were performed in triplicates with total media volume of 500 ml and a control devoid of the bacterial isolates was prepared for each set of experiments.

Sl. No.	Properties
1.	Specific gravity – 0.880
2.	API gravity – 31.5
3.	Viscosity at 40˚C – 9.6 at 70˚C – 7.2
4.	Initial boiling point (IBP) – 65ºC
5.	Sediment – 0.08
6.	Pore point – -8˚C
7.	Water content – Nil
8.	Sulphur content – 2.6(wt%)
9.	Ash content – 3.2(wt%)
10.	Carbon residue – 0.35(wt%)

Table 1: Characterization of crude oil.

Analytical methods

The residual oil samples from inoculated and un-inoculated cultures were extracted using hexane and chlorofrom in succession according to Kumari et al. [6]. The organic phase was concentrated by evaporation of the solvent after drying over anhydrous Na_2SO_4 and analyzed by gas chromatography according to USEPA test methods [14]. 1µl of sample were injected for analysis by using a Thermo Scientific Trace 1300 series gas chromatograph equipped with flame ionization detector and TR-5 column (30×10^3 cm length; 0.032 cm id; and 1×10^{-3} cm film thickness). Nitrogen was used as carrier gas. The injector and detector temperatures were maintained at 300°C and 280°C respectively. The column was programmed at an initial temperature of 40°C; this was held for 2 min, then ramped at 15^{0}C/min to 300^{0}C/min and held for 10 min. The relative percent degradation of crude oil was calculated by the differences in summation of peak area of total petroleum hydrocarbons (TPH) present in the residual oil from test samples compared to that from un-inoculated control samples [15]. The biomass concentration in the culture broth was determined by dry weight method. In this method, the broth was centrifuged at 5,000 rpm for 20 min. The bacterial mass was then transferred to a pre-weighted aluminum cup and dried at 50°C overnight. The exact weight of the bacterial mass was determined by subtracting the weight of dry cup from that of the cup containing dry bacterial mass [16].

Growth kinetics

Although biodegradability of crude oil is usually explained by first order kinetics [17], to conduct an extensive investigation, several kinetic models for crude oil degradation have been evaluated. In a batch reactor, for modeling of crude oil and microorganism concentration change with time, a simple bio-kinetic Monod model with two components was chosen [18]. The mathematical expressions for Monod kinetics is as follows:

$$\mu = \frac{\mu_{max} S}{K_s + S} \quad (1)$$

Microorganism concentration change is given by:

$$\frac{dX}{dT} = \mu X \quad (2)$$

Substrate concentration change is given by:

$$\frac{dS}{dT} = -\frac{1}{Y_{X/S}} \frac{dX}{dT} \quad (3)$$

Where, $Y_{X/S}$ is the ratio of cell mass growth and substrate concentration used for cell growth. $Y_{X/S}$ can be expressed as:

$$Y_{X/S} = -\frac{dX}{dS} = \frac{X - X_0}{S_0 - S} \quad (4)$$

Where, X_0 and S_0 represent initial biomass and substrate concentration respectively.

However, the application of the rate equation at high crude oil concentration was unsatisfactory, which was due to high concentrations

of inhibitory compounds. In this case, Haldane's model was a well-fitted model for determination of the kinetic parameters, even at inhibitory levels of the substrate. The Haldane or Andrews's model has a similar form. As stated below:

$$\mu = \frac{\mu_{max} S}{K_s + S + \frac{S^2}{K_i}} \quad (5)$$

Where K_i is the inhibition coefficient (mg/dm^3).

In addition, at high substrate concentration, the cell growth was also inhibited. The cell growth rate was evaluated by the logistic equation which was a reasonable kinetic model for the prediction of the growth curve. The specific growth rate was predicted by the logistic model as given by Equation (6) [18]:

$$\mu = \mu_m \left(1 - \frac{x}{x_m}\right) \quad (6)$$

Where x_m is the maximum cell dry weight concentration (mg/dm3).

By substitution of Equation (6) into Equation (1) and performing integration, the following equation for the cell concentration is obtained [18]:

$$x = \frac{x_0 e^{\mu_m t}}{1 - \left(\frac{x_0}{x_m}\right)\left(1 - e^{\mu_m t}\right)} \quad (7)$$

The kinetic parameters have been determined both graphically and by non-linear regression analysis using STATISTICA v.10 (Statsoft, OK, USA) and MATLAB v 7.10 (R2010a).

Results and Discussion

Biodegradation of crude oil

Due to the simplicity of batch culture, experiments were conducted to examine the effect of the initial concentration of crude oil on the biodegradation rate of the mixed bacterial culture. The initial concentrations were varied from 8800 to 88000 mg/dm^3 (i.e. 1-10% (v/v)). Figure 1 depicts the TPH removal efficiency for several initial crude oil concentrations (4-10% v/v). Due to the presence of substrate inhibition and the toxicity of high TPH concentration, as the crude oil concentrations increase the removal efficiency decreases. The TPH removal efficiencies for concentrations 35200, 44000, 52800, 61600, 70400, 79200 and 88000 mg/dm^3 were 70.12%, 67.4%, 60.25%, 52.8%, 48.6%, 42.34% and 38.52%, respectively (Figure 1).

Figure 2 shows the TPH removal efficiency with respect to time for various initial concentrations of crude oil. There were quite similar trends of crude oil degradation for all of the oil concentrations. However, it was observed that biodegradation of crude oil increased up to concentration of 35200 mg/dm^3 (i.e. 4% v/v), after that TPH removal efficiencies were gradually decreased. In the exponential phase of crude oil degradation a dramatic reduction of TPH level was observed with initial concentration of 35200 mg/dm^3. A further increase in initial concentrations leads to a decrease in crude oil biodegradation rate. Thus bioremediation is more practical for this range of oil pollution. High concentrations of hydrocarbons can cause inhibition of biodegradation due to toxic effects, although the inhibitory concentration varies with oil composition. Hence, there is an optimum oil concentration range for bioremediation applications (Figure 2) [19].

Determination of kinetic parameters using Monod model

Using the biomass concentration values during the exponential growth phase, μ values were estimated from Equation 2. The biokinetic parameters in Monod's model, such as maximum specific growth rate (μ_{max}) and Monod's constant (K_s), were estimated by the Lineweaver–Burk plot ($1/\mu$ versus $1/S$) for several crude oil concentrations and were represented in Table 2. The high value of K_s for concentrations of 6%, 8% and 10% (v/v) was the result of high inhibition of hydrocarbons. In addition, it was observed that by increasing the oil concentration the value for maximum specific growth rate (μ_{max}) decreases (Table 2).

Figure 3 demonstrates the Monod kinetic model of substrate utilization, which is presented in Equation (3). The slope represents the biodegradation rate constant (h^{-1}). The rate constants for initial crude oil concentrations of 17600, 35200, 52800, 70400 and 88000 mg/dm^3 were 0.018, 0.022, 0.017, 0.012 and 0.009 h^{-1}, respectively. It is observed that as the crude oil concentration increased from the optimum concentration of 4% (v/v), the biodegradation rate constants were gradually decreased (Figure 3).

The biomass production rate was also linearly fitted according to Monod model, as shown in Figure 4. The specific growth rate can be obtained from the slope of the line drawn based on experimental data. Here also it was observed that specific growth reaches its maximum at a certain optimum oil concentration (4% v/v), after that the slope of the lines and values for μ slightly began to decrease (Figure 4).

Simulation with logistic model

The determination of kinetic parameters for Monod's model, having

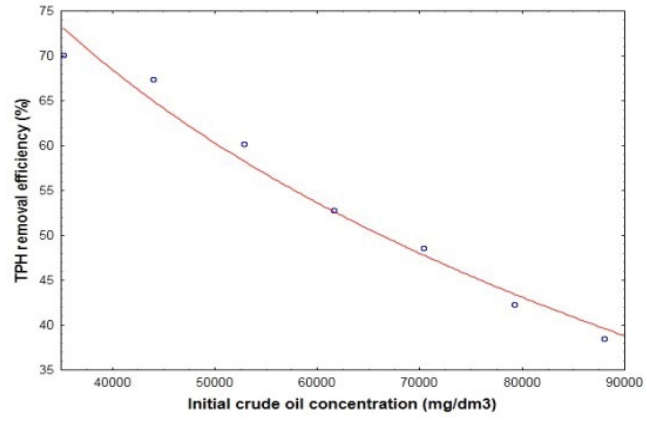

Figure 1: TPH removal efficiency with respect to initial crude oil concentration.

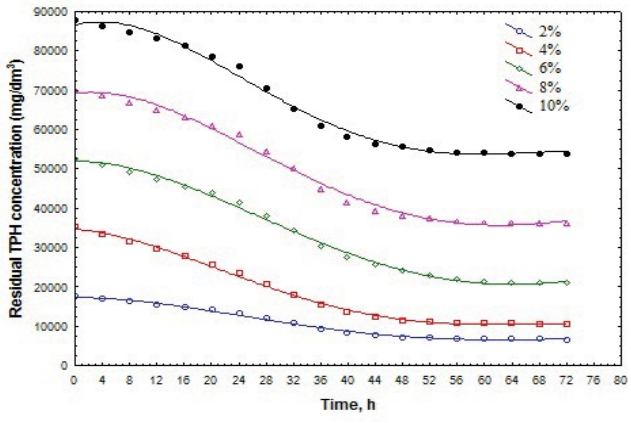

Figure 2: TPH concentration change with respect to time.

Initial crude oil concentration (v/v)	K_s(mg/dm^3)	μ_m (hr^{-1})	*Yx/s*
2%	1321.37	0.076	0.06
4%	1979.08	0.085	0.034
6%	25275.48	0.062	0.019
8%	38206.2	0.049	0.013
10%	56901.42	0.029	0.009

Table 2: Mono D kinetic parameters for crude oil biodegradation.

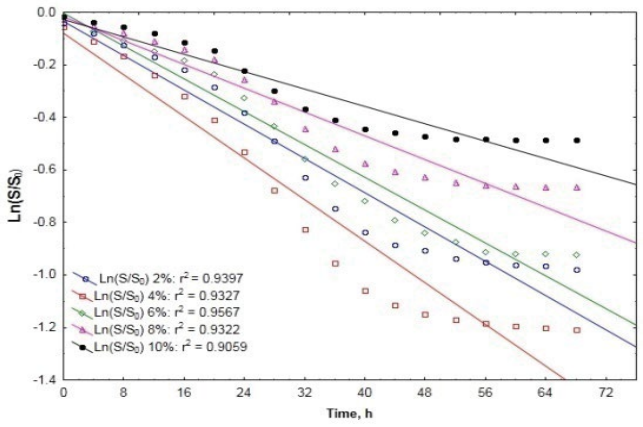

Figure 3: Logarithmic model for substrate utilization.

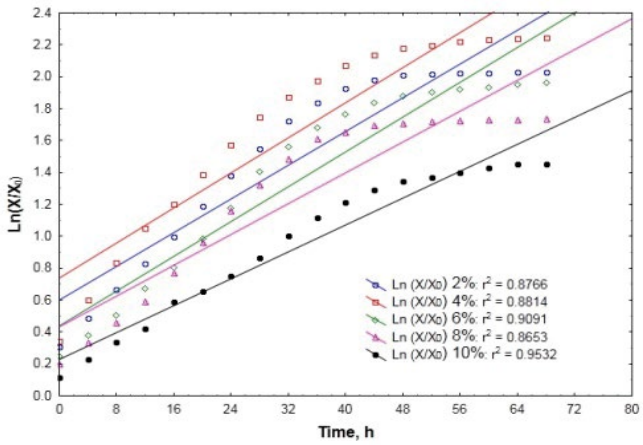

Figure 4: Rate of cell growth with respect to time.

two parameters in a linear model, is straight forward. Nevertheless, these values may not be correct values due to the presence of the inhibitory effect imposed by the high TPH concentration. Thus, the logistic equation is a promising model in order to evaluate kinetic parameters, while the inhibitory compounds are incorporated in this model. Figure 5 shows the cell biomass concentration for several initial concentrations ranging from 17600 to 88000 mg/dm^3. The growth curve for the organism on crude oil degradation was well fitted to the logistic model with several initial concentrations. The lag phase of cell growth at concentrations between 8800-35200 mg/dm^3 were found to be 12 to 16 h, however by increasing the concentrations from 35200 to 88000 mg/dm^3, the lag phases were prolonged up to 24-32 h. The lag phase may be reduced if the microbial culture is acclimated to crude oil prior to inoculation and transformation of the seed culture. Based on presented data at the initial stage, the rate of crude oil biodegradation was retarded due to inhibition created by the substrate. In addition, it can be predicted that if the incubation time was prolonged, most probably the residual TPH concentrations of 6% to 10% of crude oil should significantly decrease. The specific growth rates estimated from logistic model for initial crude oil concentrations of 17600, 35200, 52800, 70400 and 88000 mg/dm^3 were 0.087, 0.095, 0.072, 0.059 and 0.038 h^{-1}, respectively (Figure 5).

Simulation with Haldane model

One of the most useful kinetic models to evaluate the growth rate parameters is Haldane's kinetic model. The plot of specific growth rate with respect to initial substrate concentration defines the Haldane model, which is shown in Figure 6. The value of the specific growth rate increases along with the initial concentration of crude oil up to a certain maximum value. Then the specific growth rate decreases as the crude oil concentration progressively increases. At a high initial concentration of crude oil 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 biokinetic constants of the Haldane equation (Equation 5), MATLAB 10.0 computer program module, based on the non-linear least square technique was used. Of

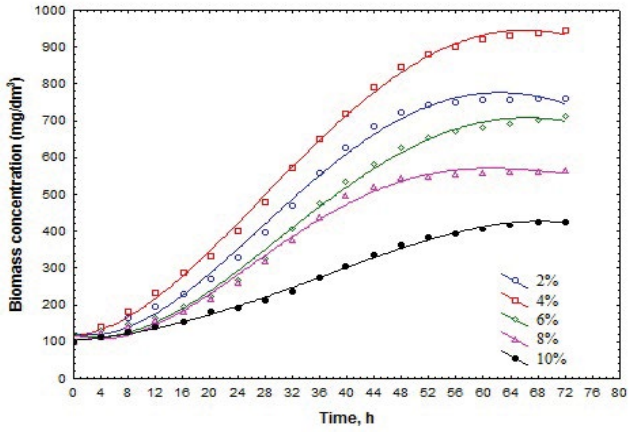

Figure 5: Simulated cell concentrations for several initial concentrations of crude oil fitted with logistic model.

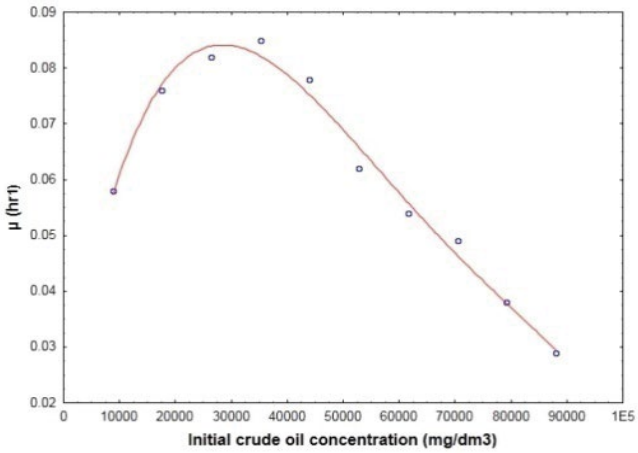

Figure 6: Haldane's growth kinetic model fitted to experimental data.

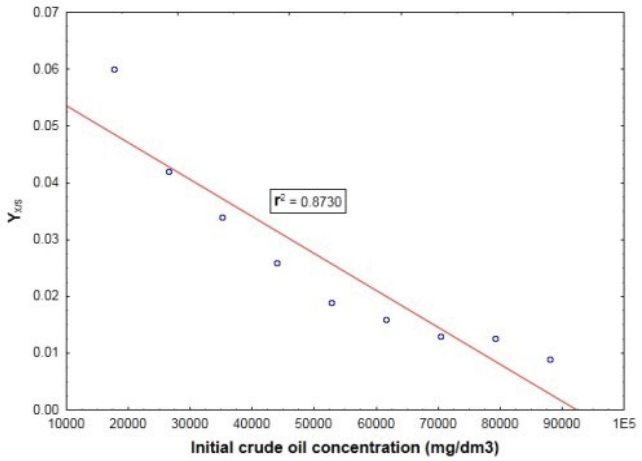

Figure 7: Yield coefficients for several initial concentrations of crude oil.

the three biokinetic constants, K_i, the inhibition constant, is a measure of sensitivity to inhibition by inhibitory substances. It should be noted that when K_i is very large the Haldane equation simplifies to the Monod equation. K_s, half-saturation constant are defined as the substrate concentration at which μ is equal to half μ_{max}. The smaller it is the lower the substrate concentration at which μ approaches μ_{max}. Also, K_s values show the affinity of microorganisms to substrate. However, if the substrate is inhibitory it can be shown that Equation 5 will go through a maximum value of substrate concentration S_{max} at which $d\mu/dS = 0$.

By putting $d\mu/dS = 0$ in (Equation 5), S_{max} can be found as:

$$S_{max} = \sqrt{K_s \times K_i} \quad (8)$$

Equation 8 reflects that the degree of inhibition is determined by K_s/K_i ratio, and not just by K_i alone. The larger K_s/K_i, indicates greater degree of inhibition [18]. Values of kinetic constants, μ_m, K_s, K_i, were obtained as 0.083 hr^{-1}, 32722.83 mg/dm^3 and 53205.64 mg/dm^3 respectively. Using these values S_{max} were computed as 41725.76 mg/dm^3. The higher K_s value indicates the ability of microorganisms to grow at relatively high substrate conditions in other words employed mixed culture showed a high affinity for crude oil. When the value of K_s/K_i ratio computed at this study is considered, the ratio value of 0.615 shows that the degree of inhibition is greater and the inhibition effect can be observed at relatively low crude oil concentrations. Thus, the growth kinetics for crude oil at high concentrations was well defined and fitted to Haldane's kinetic model (Figure 6).

Yield coefficients

To calculate the yield coefficients of crude oil, a linear regression was used to incorporate the experimental details. Figure 7 shows the plot used to determine the yield coefficient. The overall coefficient of correlation (R^2) for all of the initial concentrations was higher than 0.85. The yield coefficients observed from the plot for initial crude oil concentrations of 8800 to 88000 mg/dm^3 were 0.11, 0.06, 0.042, 0.034, 0.026, 0.019, 0.016, 0.013, 0.0126 and 0.009 respectively (Figure 7).

Conclusions

The biodegradation of crude oil for a concentration ranging from 8800 to 88000 mg/dm^3 was studied in this work. Biodegradation of crude oil was successfully achieved for the initial concentrations up to 35200 mg/dm^3. As the initial concentration of crude oil was increasing, the biodegradation rate of crude oil was dramatically decreased. A mathematical model was developed to predict the microbial growth rate. Due to the inhibition of hydrocarbon constituents of the crude oil, the Monod kinetic model was unable to predict the microbial growth in the batch culture. The Haldane and logistic kinetic models, which indicate the inhibitory effect of TPH, were reasonably well fitted with the experimental data. The values of the yield coefficients were also determined. The above parameters are necessarily used for biodegradation process understanding, bioremediation speed measurement and development of efficient clean up for a crude oil contaminated environment.

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