ISSN (Print) : 0974-6846
ISSN (Online) : 0974-5645
Indian Journal of Science and Technology, *Vol 12(13), DOI: 10.17485/ijst/2019/v12i13/142612, April 2019*

Biodegradation Kinetics of Polycyclic Aromatic Hydrocarbons by Pure Bacterial Culture: *Pseudomonas Stutzeri*

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Abstract

Objectives: To study the bio kinetics of naphthalene and anthracene biodegradation by using the pure culture of *Pseudomonas stutzeri,* under aerobic condition. **Methods:** The batch experiments was performed at various initial concentrations of naphthalene and anthracene ranging from 10 to 400 mg L^{-1} under aerobic conditions using an isolated bacterial culture. Biomass and degradation was measured with time for different initial liquid substrate concentrations of PAHs. The specific growth rates and degradation rate were also calculated. The kinetic parameters were estimated using Monod's, Haldane's, Linearized-Haldane's models for these two substrates and also determined the Root Mean Square Error (RMSE). **Findings:** The specific growth rate and degradation rate was found out based on the biomass growth and degradation value of naphthalene and antharacene for different initial concentration. The maximum specific growth rates (μ_{max}) were0.0076 and 0.00521 h^{-1} at naphthalene and, anthracene substrate concentration of 100 and 50, mg l^{-1} respectively. From these results, the maximum specific growth rate (μ_{max}) obtained from different models for naphthalene and anthracene in the range of .0081–0.0191 and 0.00542–0.00763 h^{-1}, respectively. The range of the values of Ks for naphthalene and anthracene indicates the ability of a microorganism to grow at low substrate levels, was from 78.65-206.88, 14.28–28.34, respectively. Many researchers have also reported degradation rate of naphthalene and anthracene compounds inhibited at comparatively low and medium concentration. The values of the endogenous coefficients obtained are 0.0049 and0.0044 h−1 for naphthalene and anthracene, respectively. The value obtained in the present study is very much in agreement with this value of decay coefficient. **Application:** It was conclude that the Haldane's model is best fitted to the PAHs degradation system. These models provided suitable prediction of the microorganism growth kinetic constants and interactions between PAHs substrates; based on the kinetic results help to design the bench scale reactor.

Keywords: Anthracene, Biodegradation, Bio Kinetics, Naphthalene, Polycyclic Aromatic Hydrocarbons (PAHs), *Pseudomonas*

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are a class of organic pollutants found in air, soil and water. These compounds and their derivatives enter the ecosystem via natural and anthropogenic sources. PAHs are products of incomplete combustion or pyrolysis offossil fuels, in part, from natural combustion such as forest fires and volcanic eruptions, but for the most part by human activities. In recent decades the major source of PAH pollution are industrial production, transportation, refuse burning, gasification and plastic waste incineration. PAHs are the most ubiquitous and persistent pollutants in the environment. These compounds are less soluble in water and quickly absorbed by the gastro-intestinal tract of mammals[1]. United States Environment Protection Agency has listed 16 PAHs as priority pollutants because of their toxicity, mutagenicity and carcinogenicity[2]. PAHs, some of which are known originators of mutagenic derivatives, are widely occurring in natural habitat such as soil, sediment, water, air and plants as a result of both natural and anthropogenic processes[3-9]. Since natural fires and petroleum formation have been occurring all through earth times, PAHs have undoubtedly been circulating through biogeochemical cycles for millions of years. Naphthalene (bi- cyclic) and anthracene (tri-cyclic) aromatic hydrocarbons are found in high concentrations biosphere.

Due to their properties, PAHs are characterised by their high durability in the environment, which allows them to accumulate in the soil for many years, and degrade with difficulty. However, PAHs released into the environment could be removed by several approaches and strategies including physical, chemical and biological strategies have been developed, optimised and utilised to remove PAHs contamination from polluted sites. Potential biodegradation Strains isolated from hydrocarbon-contaminated environments have been found as active as or even higher than those originating from non-contaminated soil. To date, several different bacterial genera belong to *Pseudomonas*, *Micrococcus*, *Sphingomonas*, *Bacillus, Mycobacterium* and *Cycloclasticus* have been characterized and reported as capable of degrading PAHs. The aim of the experiments is to be determined the PAHs degradation efficiency of the isolated microorganism and the growth kinetics parameters such as specific growth rate, $\mu(h^{-1})$; maximum specific growth rate, μ_{max} (h^{-1}) and half saturation constant, Ks $(mg.l^{-1})$, Ki = substrate inhibition constant, yield coefficient(Y).

2. Materials and Methods

2.1 Microorganism

The microorganism *Pseudomonas stutzeri* being an indigenous bacteria strain was isolated from crude oil exploration bore well sludge polluted area in Tamil Nadu, India. The microorganism was maintained on nutrient agar slant and stored at 4 °C ± 1°C for further use.

2.2 Culture Medium

The Mineral Salt Medium (MSM) used was composed of $(NH_4)_2SO_4$ -1 g l-1, KH_2PO_4 -0.2 g l-1, K_2HPO_4 -1.6 g l-1, $MgSO_4.7H_2O$ -0.2 g l-1, NaCl -0.1 g l-1, $FeSO_4$ -0.1 g l-1 and $CaCl_2.2H_2O$ -0.02 g l-1(10). Medium was prepared in deionized water and pH was adjusted to 7.0-7.5 using 0.4M HCl or 0.4M NaOHA.

2.3 Acclimatization of Culture and Inoculum Development

The acclimatization of isolate was performed separately in a MSM prepared using naphthalene and anthracene as a sole carbon source.

The bacterial strain isolated was slowly enriched and acclimatized in the MSM with a maximum substrate concentration of 25 mg L^{-1} naphthalene and 10 mg L^{-1} anthracene. The inoculated medium was incubated at 31±0.1°C for 48 h. Then the samples were analyzed regularly for degradation of naphthalene and anthracene and cell growth. From that harvested the cell used for further batch experiment.

2.4 Measurement of Cell Growth and Determination of Dry Weight of Cells

Free cell growth was determined by quantifying the optical density (OD at 660 nm) with a UV-visible spectrophotometer (Shimadzu UV-Min 1240, Japan) and reading from a standard calibration plot between OD660 and cell dry weight. A sample of culture broth (10 ml) was withdrawn from the bioreactor and centrifuged (Gallenkamp centrifuge) at 4000 rpm for 20 min in plastic centrifuge tubes. The supernatant was decanted into small bottles and stored at 4°C for subsequent PAHs estimation. The pellets was re-suspended in de-ionized water and re-centrifuged. The supernatant was decanted and pellets rinsed off from the tube into a pre-weighed 1.2 µm pore filter paper (Whatman GF/C). The filter paper was then dried in an oven at 105°C for 12 - 24 h, cooled in desiccators at room temperature and re-weighed until a constant dry weight was obtained. The difference between the pre-weighed filter paper and the final constant weight was used to estimate the dry weight of the cells.

2.5 Analysis of PAH in Mineral Salt Broth by Gas Chromatography

A known volume of the suspension was acidified to pH 2.0 and centrifuged at 20,000 rpm for 20 min and filtered through a 130 Whatman filter paper. The filtrate was extracted with dichloromethane. Extracted material was quantified in a gas chromatograph (Chemito GC model 7610) equipped with 5% phenyl polysiloxase-packed capillary column (BP-5) (30 m×0.25 mm×0.25 mm) used in FID mode. The injector and detector temp 280 °C and 290 °C and temperature program was 100 °C, ramp to 390 °C maintaining isotherm for 1 minute. A 0.2 µml aliquot was injected at split rate of 1:50. The percentage of naphthalene, anthracene degradation was calculated relative to PAH concentration in flask without inoculums (control) .The degradation or Removal efficiency was evaluated using the following equation (11).

Degradation (%) = [(Ac-Ai)/Ac]*100

Where, Ac- is the total area of peaks in each control sample,

Ai- is the total area of peaks in the appropriate abiotic control (with inoculums),

2.5 Batch Experiment of PAHs Degradation

The required volume of enrichment medium was prepared in a flask and dispensed in 150 mL volumes into 250 mL Erlenmeyer flasks before autoclaving. Thereafter, the following levels of naphthalene (dissolved as before) were added to each set of flasks: 50 mg l^{-1}, 100 mg l^{-1}, 200 mg l^{-1}, 300 mg l^{-1} and 400 mg l^{-1}. Three sets of flasks were inoculated with isolate, and the others unincluded (control).The Inoculated flasks were placed in a rotary shaker (120 rpm) then incubated at eight days. All the manipulations were conducted under sterile conditions. The residual PAHs were determined by gas chromatography. The ability of the isolates to grow and degradation on varying amounts of anthracene (25 mg l^{-1}, 50 mg l^{-1}, 100 mg l^{-1}, 150 mg l^{-1}, 200 mg l^{-1}). Thereafter, each set of triplicate flasks were inoculated with the isolate as applicable, and incubated as described before. Samples were also collected at 24 hourly intervals and measured for optical density and PAHs degradation.

2.6 Determinations of Kinetic Constants

The biological degradation is consummate through cleavage of benzene ring using the enzyme produced by the microorganism. The main aim and issues of these studies are to find out the growth kinetics constants of bacteria. The knowledge of the growth kinetics constants is necessary for the thoughtful of the efficiency of the microorganisms for the degradation and the operational parameters of the treatment units.

The specific growth rate of *Pseudomonas stutzeri* at different naphthalene and anthracene concentrations were calculated as per the following relationship[10-12].

$$dx / dt = \mu X \quad (1)$$

Where X is biomass growth (mg l^{-1}) at time t (h) and μ is the specific growth rate (h^{-1}), the above equation can be written as

$$In[X_2 / X_1] = \mu t \quad (2)$$

The specific growth rate (μ) in exponential phase is calculated using the following equation

$$\mu = \ln(X_2 / X_1) / (t_2 - t_1) \quad (3)$$

2.7 Mathematical Model equations

It is essential to estimate the affiliation between the specific growth rate, (μ) and the substrate concentration (S). Monod equation is a simple bio kinetic model, According to the model, PAHs is considered as non-inhibitory substrate. Monod's non-inhibitory kinetics equation is presented in the following equation:

$$\mu = (\mu_{max} \times S) / (Ks + S) \left[\text{Monod's equation}\right]$$

PAHs biodegradation by microbes has generally been known to be inhibited by PAHs itself. Hence, Monod equation is unable for describing inhibitory growth of microorganism at higher substrate concentrations. The following several kinetics models were reported to present the growth kinetics of inhibitory compounds. These models were fitted to the experimental data for selecting the best models. Haldane's, Linearized-Haldane's model, Andrews and Noack, inhibitory growth model also is selected due to its mathematical easiness and overall recognition for demonstrating the growth kinetics of inhibitory substrates. Above said inhibitory growth kinetics equations is as follows:

$$\mu = (\mu_{max} \times S / \left[Ks + S + \left(S^2 / Ki\right)\right]$$
$$\left[\text{Haldane's equation}\right]$$

$$\mu = \left(\mu_{max} \times S\right) / X \left[((1 + S) / Ki\right]$$
$$\left[\text{Linearized} - \text{Haldane's equation}\right]$$

Where,

μ_{max}= maximum specific growth rate (h^{-1})

Ks= half saturation constant (mg l^{-1})

Ki= substrate inhibition constant

S= Substrate concentration (mg l^{-1})

μ= predicted specific growth rate (h^{-1})

2.9 Error Analysis

In this study, with Root Mean Square Error (RMSE) between the substrate inhibition model predicted and experimental specific degradation rate at various PAHs concentrations constants were determined by decreasing the respective error function through the various quantity range studied.

3. Results and Discussion

3.1 Effects of Substrate Concentration

The batch aerobic reactor study at different initial concentrations of naphthalene (50,100,200,300 and 400 mg l^{-1}) and anthracene ranging from (25,50,100,150 and 200 mg l^{-1}) were carried out using a *Pseudomonas stutzeri* bacterial culture. Biomass was measured with time for different initial liquid substrate concentrations of PAHs.

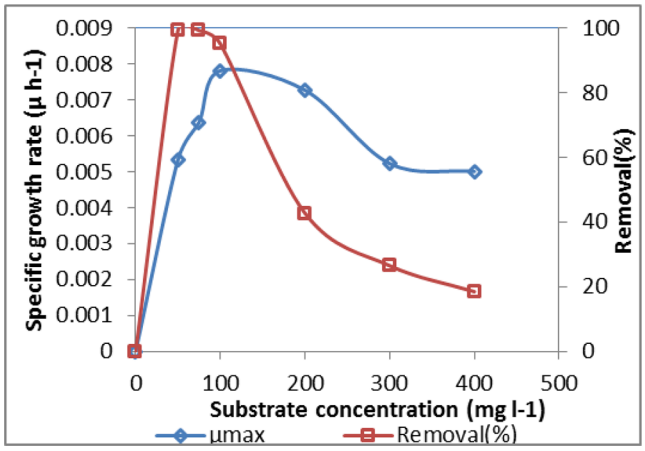

Figure 1. Effects of naphthalene concentration on specific growth rate (μ) and Removal rate.

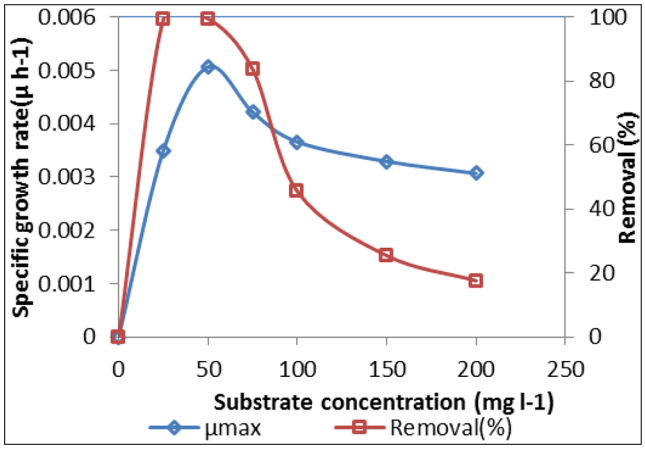

Figure 2. Effects of anthracene concentration on specific growth rate and Removal rate.

The specific growth rates were determined by using these data and above eq. (3). From this Figure 1 and 2 represent the specific growth rate and degradation rate of naphthalene and anthracene for different initial substrate concentrations using the strain *Pseudomonas stutzeri*. Figures show both specific growth rate (μ) and removal rate of substrate increase, with the increase in substrate concentration up to attain the certain maximum value. However, after attaining a maximum concentration, both the specific growth rate and removal rate started to decline with the increase in substrate concentration, that signifying the substrate inhibition. The results indicated that the effects were comparable for both specific growth rate (μ) and removal rate, but inhibition was more impact to specific growth rate than degradation rate. The naphthalene degradation time was lesser than the anthracene degradation. It appears that anthracene was more toxic and benzene ring than naphthalene. In this study revolved that the maximum specific growth rates (μ_{max}) were0.0076 and 0.00521 h^{-1} at naphthalene and, anthracene substrate concentration of 100 and 50, mg l^{-1} respectively.

Many researchers have also reported degradation rate of naphthalene and anthracene compounds inhibited at comparatively low concentration and medium concentration work[13-18].

3.2 Kinetic Constants of Bacterial Growth and Biodegradation of Naphthalene

Cell concentrations were measured with time for different initial substrate concentrations of naphthalene (100–400) mg l^{-1}. Figure 3 shows a linear plot of the Monod kinetic model, with reciprocal rate versus substrate concentration obtained for data from experiments conducted with 100 mgl^{-1} of naphthalene concentration. Similar plots were also arranged for other naphthalene concentration. The maximum specific growth rate (μ_{max}) based on Monod decreased with respect to the increase in initial concentration of naphthalene and consequently the specific naphthalene degradation rate

Table 1. Estimated bio kinetic parameters of *Pseudomonas stutzeri* in the presence of Naphthalene

Model	μ_{max} (h^{-1})	q_{max} (hr^{-1})	Ks (mgl^{-1})	Ki (mgl^{-1})	Y	RMSE
Monod's	0.0191	0.032	206.88	-	0.6	0.0017
Haldane	0.015	0.025	78.65	328.12	0.6	0.000315
Linearized-Haldane	0.0081	0.013	-	228.67	0.6	0.000804

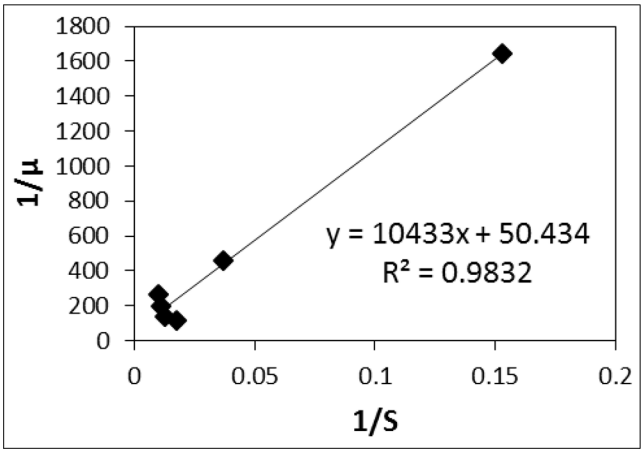

Figure 3. Lineweaver-Burk plot for naphthalene degradation by Pseudomonas stutzeri.

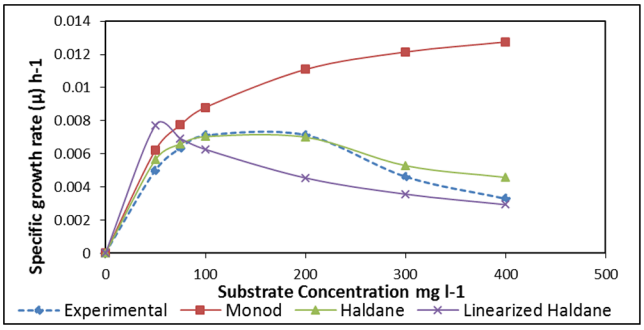

Figure 4. Experimental and model predicted profiles of specific growth rate of Pseudomonas stutzeri in the presence of Naphthalene.

(q_{max}) also decreased (Table 1). This further validates the substrate inhibition exhibited by naphthalene at higher concentrations. Hence bio kinetic parameters were evaluated based on Haldane model which incorporates substrate inhibition. Parameters evaluated based on Haldane model with non-linear fitting technique yielded best fit with the experimental values with lowest RMSE of (0.000315). The Monod model was solved graphically by Lineweaver-Burk plot. Figure 3 Illustrates the Lineweaver-Burk plot developed for estimation of μ_{max} and K_s in Monod's model. The Haldane-Andrews model constants μmax, Ks and Ki was solved using Solver in Microsoft Excel. The bio kinetic parameters estimated based on various models for *Pseudomonas stutzeri* in the occurrence of naphthalene are accessible in (Table 1). Transformations of the non-linear modeling equations to linear modeling equations, such as Lineweaver– Burk linear equation, are always considered as low accuracy simulations (18,19). Comparison of patterns of specific growth rate (μ) obtained from model prediction and experimental growth is presented in (Figure 4).

3.3 Kinetic Constants of Bacterial Growth and Biodegradation of Anthracene

The bio kinetic constants estimated for *Pseudomonas stutzeri* in the manifestation of anthracene is summarised in (Table 2). As noted in the earlier case transformations of the non-linear modelling equations to linear modelling equations, such as Line weaver– Burk plot (Figure 5), yielded low accuracy predictions with larger errors. Specific growth rates simulated by various models are compared with the observed values in (Figure 6). Non-linear Haldane model predicted with higher accuracy and lower as observed in the case of naphthalene. Inhibition constant of 163.8 mg l^{-1} was predicted by Haldane model indicate that anthracene is inhibitive to the organisms even in lower concentrations. This also explains the lower growth rates observed in experiments conducted with higher concentration of anthracene. In the case of anthracene the specific growth rate (μ) and substrate degradation rates were also lesser by 50 and 44 % then compared to naphthalene. This inference clearly indicates that *Pseudomonas stutzeri* had a low preference towards anthracene as a sole carbon source. The value of μ_{max} of *Corynebacterium sp.* and *Pseudomonas putida* was 0.0747hr-1 and 0.0663hr-1 respectively. The Monod's kinetics coefficient of anthracene evaluated with magnitude of 10.0mg l^{-1} for the action of *Corynebacterium sp.* was lesser compared with a amount of 13.03mg l^{-1} for the action of *Pseudomonas putida*[20-21]. Comparison of patterns of specific growth rate (μ) obtained from model

Table 2. Estimated bio kinetic parameters of Pseudomonas stutzeri in the presence of anthracene

Model	μ_{max} (h^{-1})	q_{max} (hr^{-1})	Ks (mgl^{-1})	Ki (mgl^{-1})	Y	RMSE
Monod's	0.0065	0.012	14.28	-	0.53	0.000681
Haldane	0.00763	0.014	28.34	163.8	0.53	0.000189
Linearized-Haldane	0.00542	0.010	-	118.88	0.53	0.000401

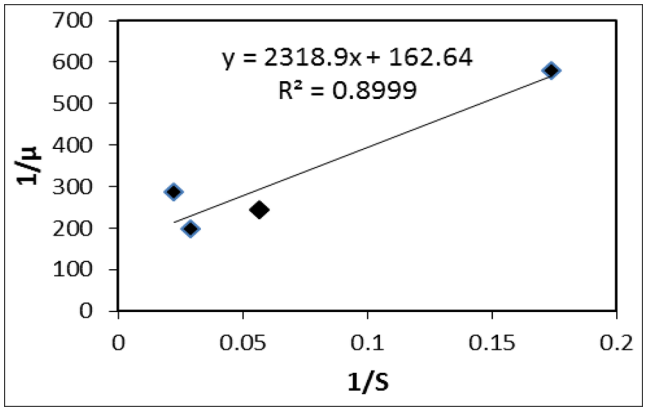

Figure 5. Lineweaver-Burk plot for anthracene degradation by Pseudomonas stutzeri.

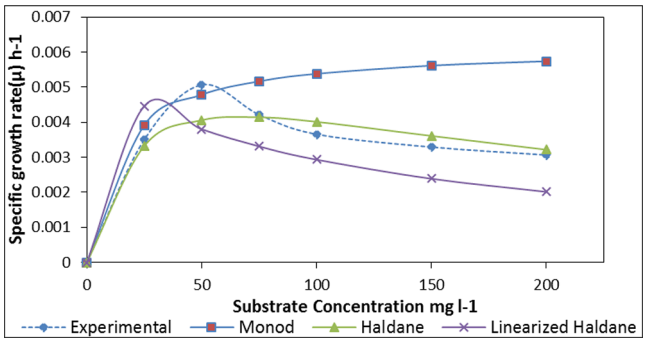

Figure 6. Experimental and model predicted specific growth rates of Pseudomonas stutzeri in the presence of anthracene.

prediction and experimental growth is presented in (Figure 6).

3.4 Choosing of the Suitable Growth Kinetics Model for Single Substrate

These model equations were solved using linear and nonlinear regression method. The kinetic parameters estimated from these three models for the two substrate naphthalene and anthracene are shown in (Table 1 and 2), along with RMSE between the substrate inhibition model forecast and investigational specific degradation rate at various concentrations. From results shown in table the maximum specific growth rate (μ_{max}) obtained from different models for naphthalene and anthracene in the range of .0081–0.0191 and 0.00542–0.00763 h^{-1}, respectively. The range of the values of Ks for naphthalene and anthracene, which indicates the ability of a microorganism to grow at low substrate levels, was from 78.65-206.88, 14.28–28.34, respectively. Monod kinetic parameters obtained from the sole-substrate biodegradation experiments of Naphthalene, Phenanthrene, Pyrene μ_{max} [h^{-1}] 0.23 0.037 0.8 × 10−3 *Ks* [mg l^{-1}] 23.75 0.8 0.11reported. For previous work[12-22] the half saturation Monod constants for PAHs are at low concentration and high concentrations also in these ranges were obtained. The Ki values for naphthalene and anthracene are shown in Tables. The high value of Ki indicates that the inhibition effect can be observed only in a high concentration range. Figure 3 shows the experimental specific degradation rate and the model predicted ones. From this figure it could be seen that among the four models tested, Haldane's model was found to fit the data quite accurately. From the table it is clear that Haldane's model yielded the least RMSE value of 0.000315 and 0.000189 with a correlation coefficient (R^2) value of 0.996 and 0.994. The kinetics models (Haldane's model) for single substrate was solved and obtain the model parameters. The following model equations and their regression coefficient were obtained

For naphthalene:

$$\mu\_ = [0.015 * S_N / (78.65 + S_N + S^2{}_N / 328.12)] \quad --------(R2 = 0.996) \tag{5}$$

For anthracene:

$$\mu\_ = \left[0.0076 * S_A / \left(28.34 + S_A + S^2{}_A / 163.8\right)\right] \quad ---------(R2 = 0.994) \tag{6}$$

where, S_N—substrate concentration of naphthalene
S_A—substrate concentration of anthracene

3.5 Endogenous or Decay Coefficient

The growth curve shows a decrease in cell growth after the complete degradation of substrate. During this decay phase some amount of the cell biomass becomes substrate for the rest of the active cell bio mass. This part of the growth curve in a batch reactor has been modelled by following equation.

$$dX / dt = -kdX$$

In order to determine the value of kd, the growth batch runs were not stopped, rather the measurement of cell density was continued further for another 5 days. The batch growth curves extended up to endogenous region. The data of this region were plotted as loge (Biomass)

versus time. The negative slope gives deterioration rate coefficient. The values of the endogenous coefficients obtained are 0.0049 and0.0044 h−1 for naphthalene and anthracene, respectively. In reported the value of decay coefficient as 0.005 h−1 for phenol degradation by a mixed culture.

Reported values of the decay rate coefficients 0.0056 and 0.0067 h−1 for phenol and catechol, respectively. In[23] proposed reported the decay rate coefficient ranges from 0.003 to 0.145 h-1 for combined wastewater of dye and starch. The value obtained in the present study is very much in agreement with this value of decay coefficient.

3.6 Yield Coefficient

To calculate the yield coefficient of naphthalene and, anthracene, the results of all the initial concentrations in shake-flask experiments were used. These shake-flask studies were carried out till the PAHs initially present was fully consumed. The value of biomass present just at the end of exponential phase was used in calculating the biomass produced as a result of consumption of substrate. Accordingly, the observed yield coefficient was then determined by performing linear regression on the following equation. Yield (g dry cell/substrate) for N and A can be calculated using the following equation.

$$Y_{X/S} = \left(X_M - X_0\right) / \left(C_S - C_0\right) \quad (4)$$

Where X_M and X_0 are the maximum and initial dry cell concentrations and C_S and C_0 are substrate concentration at the maximum cell concentration and initial substrate concentration, respectively

Yield coefficients for naphthalene and anthracene, was obtained from the slope of the graph plotted between (X-X_o) versus (S_0-S) respectively. As is evident from these graph, the value of the yield coefficient for naphthalene and anthracene 0.60 and 0.53 mg/mg respectively and also the respective coefficient of correlation R^2 is 0.99 and 0.986. The R^2 values are high within the range reported in the literature by. The yield coefficient values obtained from the sole-substrate biodegradation experiments of Naphthalene, Phenanthrene, Pyrene Y0.485 0.497 0.502. Present research the yield co efficient values are higher than the values obtained by other researchers.

4. Conclusion

The batch tests were conducted to examine the interaction of naphthalene and anthracene for single components by pure culture *Pseudomonas stutzeri* under aerobic condition. The time taken for biodegradation of, anthracene was longer than naphthalene. The effects of inhibition on naphthalene and anthracene are quite different. Naphthalene was faster degradation than anthracene, resulting in high biomass growth. Haldane's model gives a better fit with experimental kinetics data of naphthalene and anthracene. It was conclude that the Haldane's model is best fitted to the PAHs degradation system. These models provided suitable prediction of the microorganism growth kinetic constants and interactions between PAHs substrates.

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