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SCIENTIFIC PAPER

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BIODEGRADATION OF PHENANTHRENE IN AN ANAEROBIC BATCH REACTOR: GROWTH KINETICS

The purpose of the present research was to demonstrate the ability of mixed consortia of microorganisms to degrade high concentrations of phenanthrene (PHE) as the sole carbon source. Batch experiments were carried out by the induction of mineral salt medium containing PHE to the seed culture and monitoring PHE biodegradation. The microbial propagation was conducted using PHE concentrations in the range of 20 to 100 mg/l. The microbial growth on PHE was defined based on Monod and modified Logistic rate models. The kinetic studies revealed that maximum specific growth rates (μ_m) for PHE concentrations of 20, 50 and 100 mg/l were 0.12, 0.23 and 0.035 h^{-1}, respectively. The doubling times for microbial population in PHE concentrations of 20, 50 and 100 mg/l were 13, 15 and 17.5 h, respectively. Also, maximum cell dry weight (x_m) of 54.23 mg/l was achieved, while the inhibition coefficient was 0.023 h^{-1}. It was observed that the experimental data were well represented by the proposed models. It was also found that the biodegradation of PHE was successfully performed by the isolated strains.

Key words: PHE; biodegradation; mixed culture; kinetic model; logistic rate equation.

Waste materials and wastewaters are unavoidable facts of modern life. The ever increasing environmental contaminations created by various resources are a great threat to human and ecosystem health. Polycyclic aromatic hydrocarbons (PAHs) count for one of the major soil pollutants due to their high tendency to interact with non-aqueous phase liquids (NAPL) and soil colloids [1,2]. PAHs which constitute a class of hazardous aromatic compounds are comprised of two or more fused benzene rings in various arrangements [3]. Phenantherene (PHE) with three fused benzene ring is one of the PAHs whose high toxicity promote the research for its remediation from the soil and water resources [4].

Hydrophobic nature of PAHs has limited their solubility in an aqueous phase. It has been reported that the solubility of PHE in water is about 1.1-1.2 mg/l [5,6]. However, in the process of PHE biodegradation, the concentration of PHE is depleted with incubation time while more PHE may be soluble till the substrate is exhausted. The low solubility of PHE in the aqueous phase contributes to their persistence and for being highly residual in NAPL and the environment [7]. While PAHs are introduced into the soil environment, they are strongly adsorbed by soil. This phenomenon reduces their removal efficiency as their transfer into mobile phase is limited by low mass transfer [3]. Amongst various technologies applied to remove and degrade PAHs, biodegradation has been gratefully acknowledged as a preferred alternative to chemical methods which may be costly or generate secondary pollutants [7,8]. Microorganisms, especially bacteria, are able to produce surfactants or emulsifiers which may boost the stability of PHE emulsions. The low solubility of PHE in the aqueous phase may be enhanced in the presence of biosurfactants. The biosurfactants also improve the slow mass transfer of

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the substrate to the degrading microorganism and enhance the pollutant availability [2,9].

A variety of microbial species are able to utilize PAHs as a sole carbon and energy source and convert them to detoxicated compounds including CO_2 [7,10]. A survey of literature has revealed successful examples of PAHs' biodegradation. Several Gram-negative bacteria such as *Burkholderia* [11-13], *Comamonas* [14], *Pseudomonas* [3,7,15,16], *Sinorhizobium* [17,18], *Sphingomonas* [10,19-21], *Rhizobacteria* [1,22] and Gram-positive bacteria such as *Arthrobacter* [23,24], *Mycobacterium* [8,9,25,26] and *Corynebacterium* [19] have been successfully implemented for PHE biodegradation.

Although pure cultures have been extensively used for biodegradation of PHE, they may not represent the actual behavior of the environmental microbes. The use of a microbial consortium screened and isolated from the adapted environment for degradation of PHE is a reasonable and effective way to conduct the biodegradation. In the present research a consortium of microorganisms originating from the industrial zones with a closed drainage system was used to conduct the microbial PHE degradation. The isolated strains were cultured in a synthetic media in the presence of PHE and the culture was acclimated to PHE in order to enhance the bioremediation process. The microbial growth and utilization of PHE was monitored and a kinetic model based on biodegradation of PAH was developed.

EXPERIMENTAL

Strain and medium preparation

Mixed strains of microorganisms able to utilize PHE were screened from effluents of the local industrial zone. The mixed culture contained Gram positive and negative microorganisms but mostly dominated by Gram-positive organisms. Nutrient broth was used to screen the special strains. In order to maximize the cell growth on PAH, the media composition and growth parameters were optimized through a simulation process [27,28]. The propagation was carried out in basal media at adjusted pH of 7.2. Mineral salt medium (MSM) consisted of 7.0 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of $NaSO_4$, 0.5 g of NH_4Cl and 0.2 g $MgSO_4$ in a 1000 ml solution. A stock solution of trace metal and a mineral solution ware also prepared using 33mg $MnSO_4$, 36mg $CoCl_2{\cdot}6H_2O$, 10 mg $CuCl_2$, 20 mg $NiCl_2{\cdot}6H_2O$, 50 mg $ZnSO_4$ and 30 mg Na_2MoO_4 in 1000 ml distilled water. The trace metal solution (2.5 ml) was added to one liter of MSM. The isolated strains grown on PHE were anaerobically acclimated and adapted to a crucial environment for 14 days. The acclimated media was transferred to fresh media with a gradual increase in PHE concentration, while the pH was adjusted to 7.2. PHE has very low solubility in water, ethanol as an organic solvent was used to dissolve a high concentration of PHE (20-100 mg/l). A stock solution of PHE was prepared by dissolving PHE in the ethanol solution, and then it was added to MSM for a suitable concentration. No toxicity was created by ethanol when a defined amount of stock solution was used.

Degradation experiments

PHE degradation experiments were conducted in a 167 ml serum bottle containing 50 ml of MSM. The acclimated seed culture was prepared and harvested at a mid-exponential phase. The inoculation was performed in an anaerobic chamber (glove box), under the nitrogen gas flow. An inoculum of 3 ml seed culture was anaerobically transferred to MSM in a serum bottle containing 50 ppm PHE. The media were incubated at 25 °C on a rotary shaker (150 rpm).

Characterization of organisms and cell mass measurements

Since the existence of solid particles of PHE affected the measurement of the broth optical density, the cultures were filtered through glass wool (cotton wool) to remove solid PHE. The cell population was determined by turbidity of the media using spectrophotometer. A calibration curve was developed based on the cell dry weight with respect to optical density. The cell dry weight of the samples was determined based on calibration curve. The optical densities of the filtrates containing suspended cells were measured by spectrophotometer (Cecil, 1010, England) at wavelength of 600 nm (OD_{600nm}). The Gram stained slides for the isolated microorganisms were observed under microscope (Olympus B071, Japan). Transmission Electron Microscopy (TEM) (Philips CM12, Netherlands) was preformed for the isolated organisms.

Analysis of PHE

A 3 ml sample was acidified to pH value of 2 using 2 N sulfuric acid and extracted three times by half of the sample volume of ethyl acetate. Any possible intermediate product liberated from biodegradation of PHE such as 1-hydroxy-2-naphthonic acid (1H2NA) was also extracted by ethyl acetate. The extracts were pooled and dried using anhydrous Na_2SO_4; finally the solvent was removed by vacuum evaporation at 35 °C. In order to measure the total PHE concentration, 3 M sodium hydroxide was added

to dissolve cells to conduct the cell wall disruption, and then the extraction was carried out as stated above [24]. The PHE remained outside the cells. PHE was quantified by GC analysis using resorcinol as an internal standard. GC analyses were conducted with a gas chromatograph (Perkin Elmer Clarus 500, USA) equipped with a flame ionization detector (FID). Helium was used as the carrier and make-up gas for the FID. Metabolites were separated on a PTE-5 (Supelco, USA) capillary column with the length of 30 m, within the diameter of 0.25 mm and coated film thickness of 0.25 nm. The temperature programming was used. The oven temperature was initially set at 90 °C for 3 min and then the temperature was increased to 280 °C at a rate of 10 °C/min. The injector and detector temperatures were set at 300 °C.

RESULT AND DISCUSSION

Screening and isolation of microbial culture

Screening and isolation of microorganisms were carried out for the effluent stream obtained from a closed drainage system of the local industrial zone. The inoculum from industrial effluent was added to MSM in the presence of high concentration of phenol (1000 ppm). The microorganisms which were able to survive in the toxicated media were primarily screened. The second generation of screened organisms was acclimated with low concentration of PHE (20-50 ppm) in MSM. The isolation was conducted in broth and Petri-dishes to identify various colonies. The resistive and grow-able microbes were identified by Gram stain. The isolated strains of microorganisms as mixed cultures were dominated by Gram positive microorganisms. However, Gram-negative organisms were also present in the growth media. The TEM for the isolated organisms were performed. Figure 1 presents 9 plates of TEM for several distinct organisms in morphological status of bacillus, cocci and dicocci with magnification of 500, 1000 and 2000 folds. The TEM

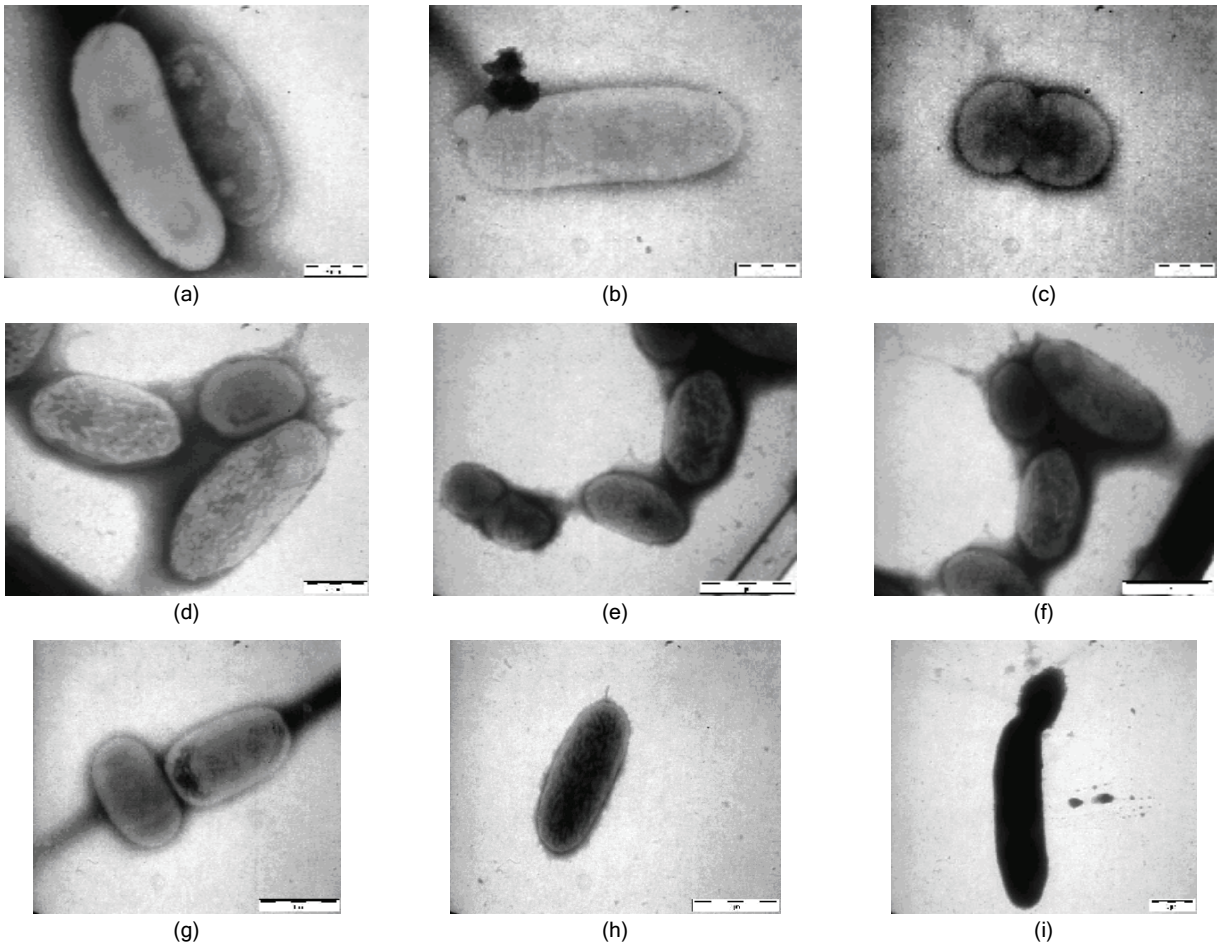

Figure 1. TEM of the mixed culture: a) two bacillus with magnification of 500 fold; b) single bacillus with magnification of 500 fold; c) Dicocci with magnification of 500 fold; d) three different types of bacillus with magnification of 1000 fold; e) Dicocci and bacillus with magnification of 1000 fold; f) a few types of bacillus with magnification of 500 fold; g) duplicated cocci with magnification of 1000 fold; h) single bacillus with magnification of 1000 fold; (i) Duplicating bacillus with magnification of 2000 fold.

images show the consortiums of organisms responsible for the biodegradation of PHE.

PHE biodegradation

Figure 2 shows the biodegradation of PHE and also a cell growth curve of the isolated mixed culture on PHE (as the sole carbon source) with respect to time at initial PHE concentrations of 20, 50 and 100 mg/l. It was observed that the PHE biodegradation was successfully achieved at all PHE concentrations. There were quite similar trends of PHE reduction with respect to incubation time for various initial concentrations. It was also concluded that the cell dry weight was increased while the PHE concentration was gradually increased. During the exponential stage of cultivation, a drastic reduction of PHE level was observed with initial PHE concentrations of 20 and 50 mg/l. The rapid reduction of PHE in the medium was due to the assimilation of PHE in the cells. The degradation rate at PHE concentration of 100 mg/l was slightly lower than that of 20 and 50 mg/l which was probably due to the toxicity created by high concentrations of PHE. A short lag phase of less than 12 h for biodegradation of 20 and 50 mg/l PHE was observed; then it was shifted to a linearly increasing trend. The lag phase for the PHE concentration of greater than 50mg/l was prolonged. The delay was most probably due to inhibition created by increasing the substrate concentration (100 mg/l).

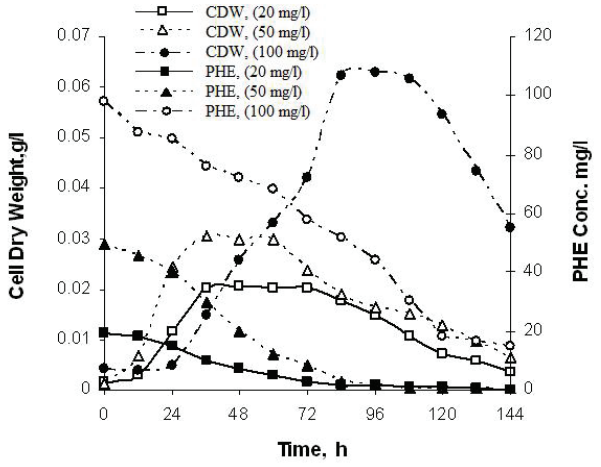

Figure 2. PHE biodegradation using mixed culture at various concentrations.

The variation of the medium pH along with PHE biodegradation was monitored and the obtained results are presented in Figure 3. The pH fluctuation was finally dropped to acidic region that was due to the production of acidic intermediate compounds resulted by the PHE biodegradation. It has been reported that the biodegradation of PHE leads to 1-hydroxy-2-naphthonic acid (1H2NA), which is an intermediate product accumulated in the medium [29]. The 1H2NA was extracted by ethyl acetate. The organic phase was changed to an orange color. The analysis of the intermediate was conducted by GC-FID (after esterification). The UV absorption spectroscopy showed that 1H2NA was the predominant product, but it was found that the concentration of 1H2NA was very low for a single strain of the isolated organism. After biodegradation of PHE in a mixed culture, the accumulation of 1H2NA resulted due to broad enzymatic capabilities of the mixed culture. As a result, the level of aromatics in the medium was dropped drastically. The biodegradation of PHE shifted the pH of the media from a neutral to acidic condition. Perhaps that was due to formation of organic acids which resulted in the consumption of metabolites in the presence of microorganisms in the media.

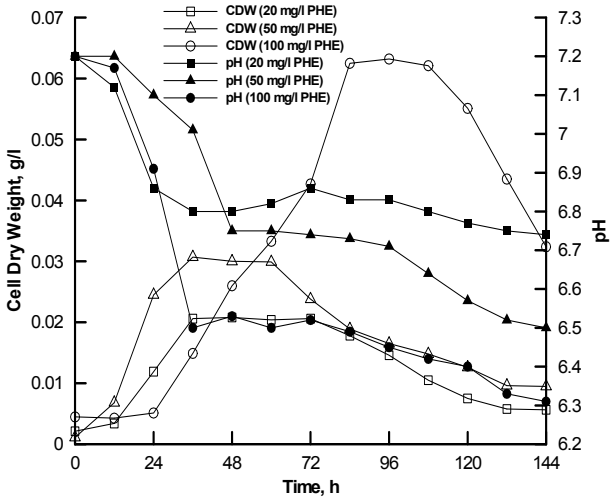

Figure 3. Monitoring pH in PHE biodegradation processes.

Substrate consumption rate

Aromatic substrates were consumed not only to provide the required energy but also for the cell growth and maintenance. Thus, as an alternative to describe the substrate utilization for microbial degradation, the first-order chemical reaction kinetic was proposed [27,28]. The expression for the substrate consumption with respect to time based on the first-order differential equation is described as follows:

$$-\frac{\mathrm{d}S}{\mathrm{d}t} = K_s S \tag{1}$$

where S is the substrate concentration (mg/l) and K_s is the first-order rate constant (h^{-1}). The expression for PHE concentration is obtained upon integration of Eq. (1) which yields:

$$S = S_0 \exp(-K_s t) \quad (2)$$

where S and S_0 are the instantaneous and initial PHE concentrations (mg/l), respectively. Taking the natural logarithm of both sides of Eq. (2) leads to a linear model stated as:

$$\ln\left(\frac{S}{S_0}\right) = -K_s t \quad (3)$$

The data presented in Figure 4 showed that the PHE biodegradation in the batch culture has followed the first-order kinetic model. The slopes of the linearly fitted data were 0.007, 0.013 and 0.019 for PHE concentration of 100, 50 and 20 mg/l, respectively. The rate constants gradually decreased while the PHE concentration was increased. That was probably the result of substrate inhibition at high PHE concentration. The data were well fitted to the first-order linear model with R^2 greater than 0.96. Other researchers have also reported that the biodegradation of PAHs follows the first-order kinetic model [30]. The obtained results revealed that the half life ($t_{1/2}$) of PHE was 36, 53 and 99 h at the initial concentrations of 20, 50 and 100 mg/l, respectively. The obtained kinetic parameters are summarized in Table 1.

Growth kinetics in batch bioreactor

The microbial cells growth was monitored as they were incubated in a batch culture containing fresh media. It is common to use cell dry weight as a measure for the cell concentration. The simplest relation which describes the exponential cell growth is unstructured model. This model demonstrates the cell as an entity in the solution which interacts with the

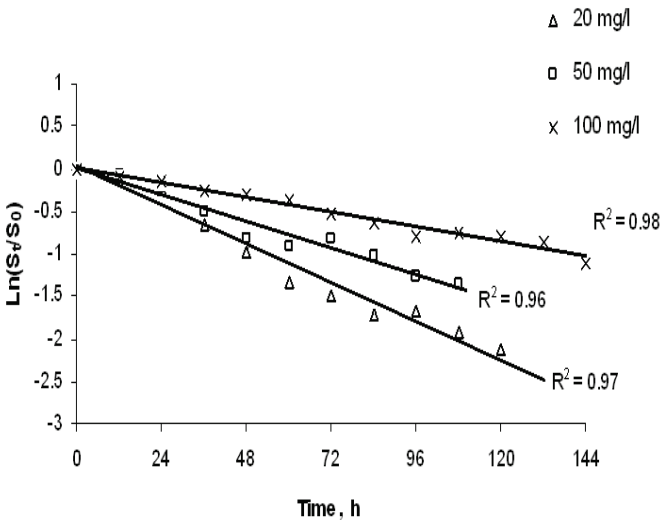

Figure 4. First-order PHE biodegradation rate model in a batch reactor.

Table 1. Kinetic parameters, rate model with and without substrate inhibitio

Parameter	PHE concentration, mg/l		
	20	50	100
Growth kinetics, logistic rate			
x_0 / mg l^{-1}	1.03	0.02	4.485
x_m / mg l^{-1}	6.18	1.05	54.23
μ_m / h^{-1}	0.12	0.23	0.035
k / h^{-1}	0.0125	0.012	0.023
R^2 / %	0.92	0.97	0.98
Substrate utilization rate			
K_s / h^{-1}	0.032	0.035	0.01
R^2 / %	0.99	0.99	0.92
Monod rate			
μ / h^{-1}	0.022	0.023	0.023
μ_m / h^{-1}	0.022	0.013	0.015
K_s / h^{-1}	1.88	0.35	15.39
R^2 / %	0.97	0.96	0.95

environment. One of the suitable and simple rate models is Malthus law which relates the cell growth to the cell density as stated below [31,32]:

$$\frac{dx}{dt} = \mu x \tag{4}$$

where x is the cell dry weight (mg/l), μ is the specific growth rate (h^{-1}) and t is the incubation time (h). This model predicts unlimited growth with respect to time. The Monod rate equation was applied for the case of PHE biodegradation using mixed culture:

$$\mu = \frac{\mu_m S}{K_s + S} \tag{5}$$

where μ_m is the maximum specific growth rate (h^{-1}), S is the substrate concentration (mg/l) and K_s is the Monod constant (mg/l).

Figure 5a shows a linear plot of the Monod kinetic model, with double reciprocal rate *versus* substrate concentration. The Lineweaver-Burk plot fitted in straight line with the experimental data. The values of Monod constants were 1.88 and 15.39 mg/l for 20 and 100 mg/l PHE, respectively. The data obtained for PHE concentration of 100 mg/l showed a competitive inhibition. Maximum specific growth rates (μ_m) of 0.021 and 0.015 h^{-1} were achieved for PHE concentrations of 20 and 100 mg/l, respectively. Thus, the Monod constant was affected by the initial PHE concentration. Figure 5b shows the Lineweaver-Burk plot for 50mg/l PHE. The microbial growth rate decreased while the concentration of PHE gradually increased. It was also realized that the specific growth rate (μ) for the mixed culture was almost constant and independent of PHE concentration. The specific growth rate (μ) of 0.022, 0.023 and 0.023 h^{-1} were obtained for PHE concentrations of 20, 50 and 100 mg/l, respectively. Similar results have been reported by Tian and his coworkers [33] for the specific growth rates of *Pseudomonas mendocina* under various PHE con-

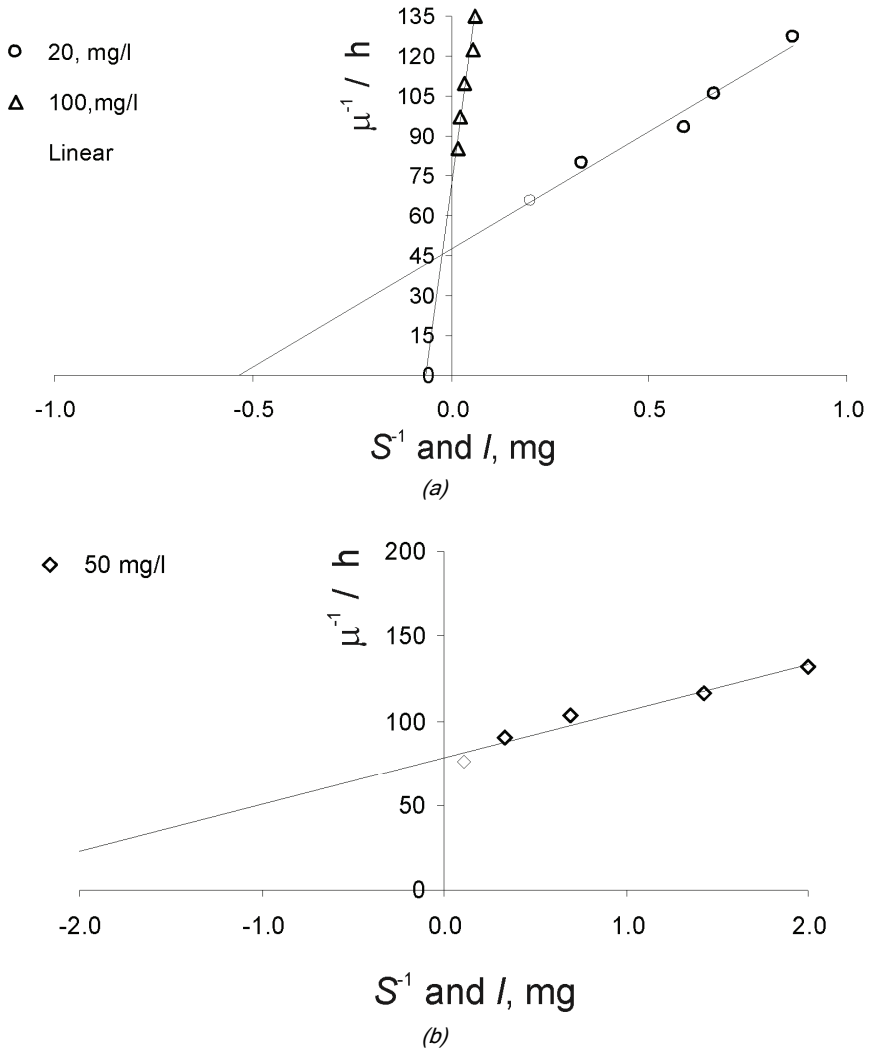

Figure 5. Linweaver-Burk plot for linearized Monod rate model.

centrations of 0.038, 0.035 and 0.033 h^{-1} for 20, 50 and 100 mg/l PHE, respectively. They have also indicated that the microbial growth rate was independent of PHE in the solid phase presented in the medium. In addition, the low solubility of PHE may cause a limited cell growth. Other researchers have also reported similar results for naphthalene degradation [34]. Table 1 summarizes the obtained kinetic parameters for the substrate consumption based on Monod equation.

So far, various equations have been proposed to support the microbial growth models. An example of these models is the Logistic equation which predicts lag phase, exponential growth rate and stationary cell population (x_m) [32]. It is useful to develop a specific model that accommodates PHE consumption of biomass and non-toxic metabolites production without any serious inhibition. To describe the above issues in a mathematical model, the Malthus' law which is similar to Eq. (4) was applied for the species successfully grown on PHE [31,32]:

$$\frac{dx_1}{dt} = \mu x_1 \tag{6}$$

To consider a linear decrease in the cell population, the second-order cell population inhibition is applied and the growth rate equation is presented as follows [32]:

$$\mu = \mu_m(1-\frac{x_1 x_2}{x_m^2}) \tag{7}$$

where x_1 is the growing cell concentration (mg/l) and x_2 presents the declining cell concentration as a result of either the toxic by-products or depletion of PHE. Substituting Eq. (7) into Eq. (6), gives:

$$\frac{dx_1}{dt} = \mu_m x_1(1-\frac{x_1 x_2}{x_m^2}) \tag{8}$$

The products which inhibit or promote the cell population in the bioreactor are obtained from the following equation:

$$\frac{dx_2}{dt} = kx_2 \tag{9}$$

where x_1 and x_2 are the mass of cell species (mg/l) and k is the decline or promotion constant (h^{-1}). This constant is positive when the cell population is inhibited by toxic chemical and negative when the cell population is promoted by nutrients. Integrating Eq. (9) considering the initial biomass concentration (x_0) as the integration constant yields an exponential growth for the biomass cell:

$$x_2 = x_{02}e^{kt} \tag{10}$$

Inserting Eq. (10) into Eq. (8) provides an ordinary differential equation with respect to biomass concentration:

$$-\frac{dx_1}{dt} + \mu_m x_1 = x_1^2 \frac{x_{02}\mu_m}{x_m^2} e^{kt} \tag{11}$$

Equation (11) is simplified by suitable substitutions of

$$u = \frac{1}{x_1}$$

and

$$-\frac{dx_1}{dt} = x_1^2 \frac{du}{dt}$$

thus the following equation is obtained for u as the new dependent variable:

$$\frac{du}{dt} + \mu_m u = \frac{x_{02}\mu_m}{x_m^2} e^{kt} \tag{12}$$

Equation (12) is a first-order linear differential equation which was solved for u to yield:

$$u = e^{-\int \mu_m dt}\left[\int e^{\int \mu_m dt}\left(\frac{x_{02}\mu_m}{x_m^2}\right)e^{kt}dt + C\right] \tag{13}$$

Then, the integration, gives:

$$u = \left(\frac{x_{02}}{x_m^2}\right)\left(\frac{\mu_m}{k+\mu_m}\right)e^{kt} + ce^{-\mu_m t} \tag{14}$$

The final substitution for biomass concentration in a mixed culture was obtained. This equation has the potential to predict the inhibition which is the resulted of the intermediates to compete with PHE consumption:

$$x = \frac{x_0 e^{\mu_m t}}{1-\left(\frac{x_0}{x_m}\right)^2\left(\frac{\mu_m}{k+\mu_m}\right)\left[1-e^{(k+\mu_m)t}\right]} \tag{15}$$

The growth curve of the organisms on high PHE concentration was quite well fitted with the logistic model as described in Figure 6. Also, Figure 7 presents the cell concentration profile based on the simulated model. Equation (15) was fairly fitted with the experimental data with R^2 value of above 0.93. The value of k in this equation is proportionality constant which is associated with the constant promotion or decline of the cell population in the batch culture. Maximum cell dry weight (x_m) of 54.21 mg/l was achieved while the inhibition value was 0.023 h^{-1}.

Growth kinetic parameters and regression analysis of the mixed culture are summarized in Table 1.

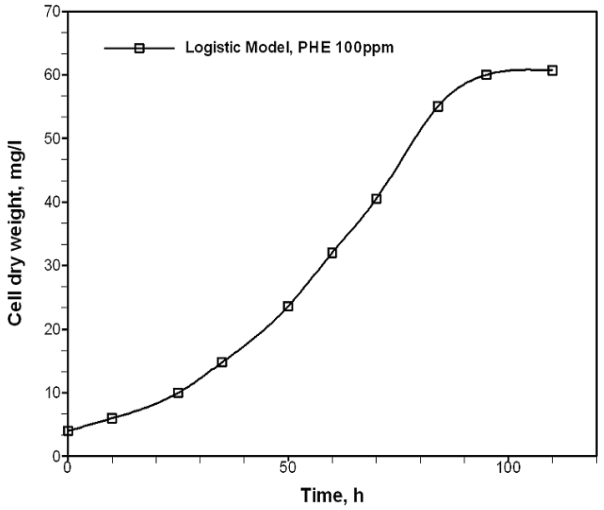

Figure 6. Simulated cell concentration for PHE fitted with Logistic model.

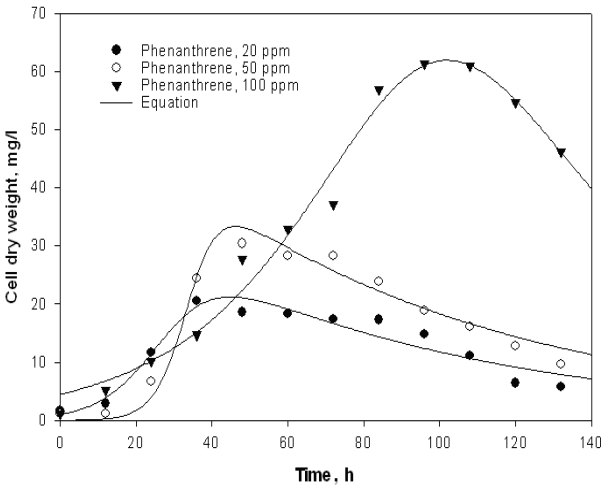

Figure 7. Simulated cell concentration with experimental resulted with second order inhibition model.

CONCLUSION

Biodegradation of PHE was successfully achieved by the isolated strains as a mixed culture which was consortia of microorganisms. Mathematical models were developed to predict a microbial growth rate and possible inhibitions. The effective constants were proposed based on the second-order inhibition model. The specific growth rate constant was obtained for various PHE concentrations. It was also concluded that the high PHE concentration resulted in a low reaction rate as the rate constant decreased while the PHE concentration increased. The obtained results revealed that pH changes in the media affected the PHE biodegradation as well as the substrate consumption rate.

Nomenclature

$Y_{x/s}$	Cell yield on substrate concentration (mg cell/ /mg PHE)
S	Substrate concentration at t time (mg/l)
S_0	Substrate concentration at t_0 time (mg/l)
K_s	First-order rate constant (h^{-1})
$t_{1/2}$	Half-life of PHE (h)
μ	Specific growth rate (h^{-1})
μ_m	Maximum specific growth rate (h-1)
x_0	Cell dry weight at t_0 time (mg/l)
x	Cell dry weight at t time (mg/l)
x_1	Growing cell (mg/l)
x_2	Declining cell (mg/l)
x_m	Stationary cell biomass (mg/l)
k	Cell decline or promotion constant (h^{-1})
k_s	Monod constant (h-1)
T	Time (h).

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NAUČNI RAD

BIODEGRADACIJA FENANTRENA U ANAEROBNOM ŠARŽNOM REAKTORU: KINETIKA RASTA

Cilj ovog istraživanja je bio da se pokaže sposobnost mešanih mikrobnih kultura da razgrade fenantren (PHE) kao jedinog izvora ugljenika. Šaržni eksperimenti su izvedeni zasejavanjem hranljive podloge sa mineralnim solima i PHE i praćenjem biodegradacije PHE. Gajenje mikroroganizama je sprovedenu u opsegu koncentracije PHE od 20 do 100 mg/l. Rast mikroba je definisan na osnovu Monod-ovog i modifikovanog logističkog modela. Ispitivanja kinetike su pokazala da su maksimalne vrednosti specifične brzine rasta (μ_m) za koncentracije PHE 20, 50 i 100 mg/l bile 0,12, 0,23 i 0,035 h^{-1}, respektivno. Vrednosti vremena dupliranja mikrobne populacije pri koncentracijama PHE 20, 50 i 100 mg/l bile su 13, 15 and 17,5 h, respektivno. Postignuta je maksimalna vrednost suve biomase (x_m) od 54,23 mg/l, dok je koeficijent inhibicije bio 0,023 h^{-1}. Uočeno je da se eksperimentalni podaci dobro slagžu sa predloženim modelima. Pronađeno je, takođe, da je biodegradacija PHE bila uspešna a izolovanim sojevima.

Ključne reči: fenantren; biodegradacija; mešana kultura; kinetički model; logistička jednačina brzine.