Kinetics model for growth of *Pseudomonas putida* F1 during benzene, toluene and phenol biodegradation

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Abstract

The effect of adaptation of *Pseudomonas putida* F1 ATCC 700007 (*Pp* F1) to the biodegradation of benzene (B), toluene (T) and phenol (P) was studied. The adaptation of microorganism to BTP decreased the biodegradation time from 24 to 6 h for benzene (90 mg/l) and toluene (90 mg/l), and from 90 to 18 h for phenol (50 mg/l). Andrews kinetics model for single substrate was solved to obtain maximum specific growth rates, half saturation and substrate inhibition constant. Cell growth using toluene ($\mu_{max,T} = 0.61$) and benzene ($\mu_{max,B} = 0.62$) as carbon sources were better and faster than the growth in phenol ($\mu_{max,P} = 0.051$). For the substrate mixtures, a sum kinetics model was used and the interaction parameters were determined. These models provided an excellent prediction of the growth kinetics and the interactions between these substrates. Toluene inhibited the utilization of benzene ($I_{T,B} = 5.16$) much more than benzene inhibits the utilization of toluene ($I_{B,T} = 0.49$). Benzene ($I_{B,P} = 0.27$) and toluene ($I_{T,P} = 0.14$) enhance the biodegradation of phenol, and phenol inhibits the biodegradation of benzene ($I_{P,B} = 1.08$) and toluene ($I_{P,T} = 1.03$).
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1. Introduction

Organic chemical mixtures are present in waste waters from industrial and municipal sources as well as in contaminated groundwater. Common examples of chemical mixtures that often become pollutants include gasoline and other petroleum fuels, pesticides and wood-treating substances [1]. The bioremediation of these substances has become an alternative to the traditional physical and chemical methods that can be costly and produce hazardous products [2]. Monoaromatic hydrocarbons such as benzene, toluene and phenol are obvious choices for mixture biodegradation studies. These molecules are produced in huge amounts and are used in fuels and as solvents and starting materials for the production of plastics, synthetic fibers and pesticides [3].

A number of substrate interactions have been observed during hydrocarbon degradation. Bauer and Capone [4] reported a stimulatory effect of benzene on the degradation of anthracene. Meyer [5] observed diauxic degradation of ben-zene in the presence of phenol. Goldsmith and Balderson [6] evaluated benzene, toluene and xylene (BTX) degradation kinetics in an enrichment culture. They obtained first-order kinetics parameters for individual compounds and demonstrated differences in the rates of degradation among substrates. Interactions between benzene and other aromatic hydrocarbons were evaluated by Arvin et al. [7]. These researchers concluded that benzene degradation was enhanced in the presence of either toluene or *o*-xylene. Further studies by Alvarez and Vogel [8] revealed interactions during BTX degradation by organisms from aquifer enrichments and by two pure culture organisms. These interactions were evaluated by comparing changes in the lag period and pseudo-zero order biodegradation rates.

Biodegradation of benzene, toluene and phenol as single and mixed substrates for *Pp* F1 was evaluated by Reardon et al. [1]. In this study, BTP were biodegraded at 45, 40 and 50 mg/l, respectively. These researchers investigated the interactions between these aromatic compounds and concluded that toluene and benzene enhanced the biodegradation of phenol, while phenol did not affect the biodegradation of benzene and toluene significantly and, there was no significant interaction between benzene and toluene.

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They used Monod kinetics to describe to specific growth rate in this low substrate concentration range.

The aim of this study was to investigate the possibility of biodegradation of benzene, toluene and phenol at high initial concentrations and to study the microorganism growth kinetics using Andrews models during biodegradation of benzene, toluene and phenol for single substrate and substrate mixtures as well as to determine the substrate interactions.

2. Materials and methods

2.1. Microorganism and media

Pseudomonas putida F1 strain ATCC 700007 was obtained from the American Type Culture Collection (Manassaa, VA). Initially microorganisms were grown aerobically at 30 °C and pH 7 in a medium containing; peptone (5 g/l) and meat extract (3 g/l) in 250 ml Erlenmeyer flasks. To eliminate the microbial adaptation period in the biodegradation experiments, *Pp* F1 was pre-adapted to 28 mg/l toluene and 30 mg/l benzene (there was no effect of the concentration of benzene and toluene on the adaptation). For the biodegradation experiments, a modified Hunter's mineral base (MSB) was used as the carbon free medium [9]. This medium was distributed in 16 ml aliquots in 160 ml serum bottles and sealed with butyl rubber stoppers and aluminum crimp caps (Kimple Glass Inc.).

2.2. Analytical methods

Biomass concentrations were determined by UV spectrophotometer (Shimadzu 1601) at 550 nm by measuring the absorbance of the microorganism solutions and correlated to biomass concentration. Benzene and toluene concentrations were determined by headspace analysis. Samples of the headspace gas (250 µl) were withdrawn from the serum bottles using a Hamilton 1725 RN gas-tight syringe (Hamilton Co.), and were analyzed using a CE Instruments GC 8000 TOP gas chromatography equipped with a flame ionization detector and a 0.75 mm × 30 m glass capillary column (ZB-Wax). The oven, injector and detector temperatures were fixed at 50, 250 and 200 °C, respectively. Aqueous samples of phenol were extracted with *n*-butanol (0.5 ml aqueous sample to 0.5 ml *n*-butanol). The *n*-butanol layer was removed and analyzed using the same GC. The liquid phase concentration of benzene and toluene were calculated as described in our previous study [10].

2.3. Biodegradation experiments

The biodegradation experiments of benzene, toluene and phenol were conducted in 160 ml serum bottles containing 16 ml MSB and benzene, toluene and/or phenol as the sole carbon and energy source. Experiments were carried out at pH 7 and 30 °C in an orbital shaker (Gerhardt) at 150 rpm.

The inoculum used here was taken from adapted cells. The biodegradation of these compounds was investigated individually and in mixture. Benzene and toluene were injected as pure stock with a 10 µl syringe. Phenol was added from an autoclaved MSB-phenol stock solution. Control experiments were also done without cells and the concentration of benzene and toluene remained unchanged.

3. Kinetics models

The specific growth rate in exponential phase is calculated using the following equation:

$$\mu = \frac{\ln(X_2/X_1)}{(t_2 - t_1)} \tag{1}$$

The Andrews model was used to describe the specific growth rate [11] for a single substrate:

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\mu_{max}S_L}{K_S + S_L + \frac{S_L^2}{K_i}} \tag{2}$$

In this expression, S_L is the liquid substrate concentration; t, the time; μ, the specific growth rate; X, the biomass concentration; μ_{max}, the maximum specific growth rate; K_S, the Monod half-saturation constant and K_i is the substrate inhibition constant. K_S is that value of the limiting nutrient concentration at which the specific growth rate is half of its maximum value.

For the substrate mixtures, the interactions parameters were determined using the sum kinetics model in Eq. (3).

$$\mu = \frac{\mu_{max,1}S_{1L}}{K_{S,1} + S_{1L} + \frac{S_{1L}^2}{K_{1i}} + I_{2,1}S_{2L}} \\ + \frac{\mu_{max,2}S_{2L}}{K_{S,2} + S_{2L} + \frac{S_{2L}^2}{K_{2i}} + S_{1L}I_{1,2}} \tag{3}$$

The interaction parameter $I_{i,j}$ indicates the degree to which substrate i affects the biodegradation of substrate j (large values indicate stronger inhibition) [12]. The specific growth rates used in this equation were determined from the data of biodegradation of BTP at different concentrations. The kinetics parameters (μ_m, K_S and K_i) in this equation were the same in the single substrate experiments.

Yield (g dry cell/substrate) for B, T and P can be calculated using the following equation.

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

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.

4. Results and discussion

4.1. The effect of adaptation on the biodegradation time

In order to investigate the adaptation procedure on the biodegradation of B, T and P, experiments were performed at 30 mg/l B or T. As seen in Fig. 1, 90 mg/l benzene or toluene was biodegraded in 24 h by non adapted cells, but similar concentration of benzene or toluene was degraded in 6 h by adapted cells (Fig. 1(a)). Similar results were obtained for the biodegradation of 15 and 30 mg/l benzene and toluene. As a result, to enhance the biodegradation of benzene and toluene, the cells must be adapted twice to benzene and toluene. There was no significant effect of the adaptation to phenol on phenol biodegradation, while using cells adapted to 30 mg/l toluene or benzene, phenol was degraded in 18 h (Fig. 1(b)).

There was no effect of the concentration of benzene or toluene on the biodegradation time of benzene and toluene. According to these results, cells were adapted to 30 mg/l benzene and 30 mg/l toluene as a mixture for two times. The same cells were used for the phenol biodegradation experiments. After that twice adapted cells were used in the experiments.

4.2. Determination of the growth kinetics model for single substrate

Cell concentrations were measured with time for different initial liquid substrate concentrations of benzene (3–187.7 mg/l), toluene (2.76–160.3 mg/l) and phenol (10–200 mg/l) [10,13]. The specific growth rates were calculated using

these data and Eq. (1). The kinetics model (Andrews model) for single substrate (Eq. (2)) was solved using Sigma Plot 2001 to obtain the model parameters. The following model equations and their regression coefficient were obtained for B, T and P:

$$\text{For benzene:} \quad \mu = \frac{0.62 S_{BL}}{1.65 + S_{BL} + \frac{S_{BL}^2}{180}},$$

$$(R^2 = 0.927) \tag{5}$$

$$\text{For toluene:} \quad \mu = \frac{0.61 S_{TL}}{6.47 + S_{TL} + \frac{S_{TL}^2}{88}},$$

$$(R^2 = 0.943) \tag{6}$$

$$\text{For phenol:} \quad \mu = \frac{0.051 S_{PL}}{18 + S_{PL} + \frac{S_{PL}^2}{430}},$$

$$(R^2 = 0.972) \tag{7}$$

The dependence of *P. putida* Fl specific growth rate on the concentration of benzene, toluene and phenol is shown in Fig. 2. The maximum specific growth rate (μ_{max}) obtained from different researchers for benzene, toluene and phenol (Table 1) were in the range of 0.335–0.73, 0.42–0.86 and 0.051–0.569 h^{-1}, respectively. The specific growth rate value of 0.62 for B and 0.61 for T fall into these ranges. Also, the value of P (0.051) falls near the low end of this range. The range of the values of K_S for BTP, which indicates the ability of a microorganism to grow at low substrate levels, were from 0.12 to 3.36, 3.98–13.8 and 5.27–32, respectively. For
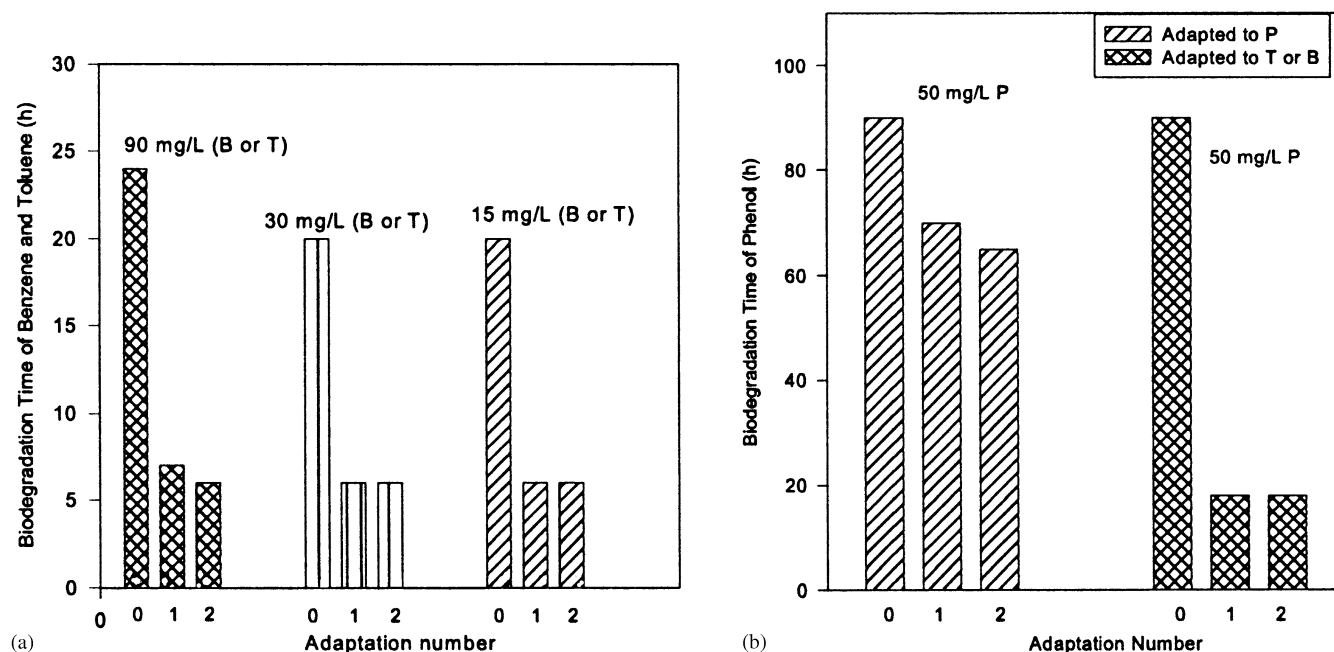

Fig. 1. The effect of the adaptation number on the biodegradation time (a) benzene and toluene, (b) phenol.
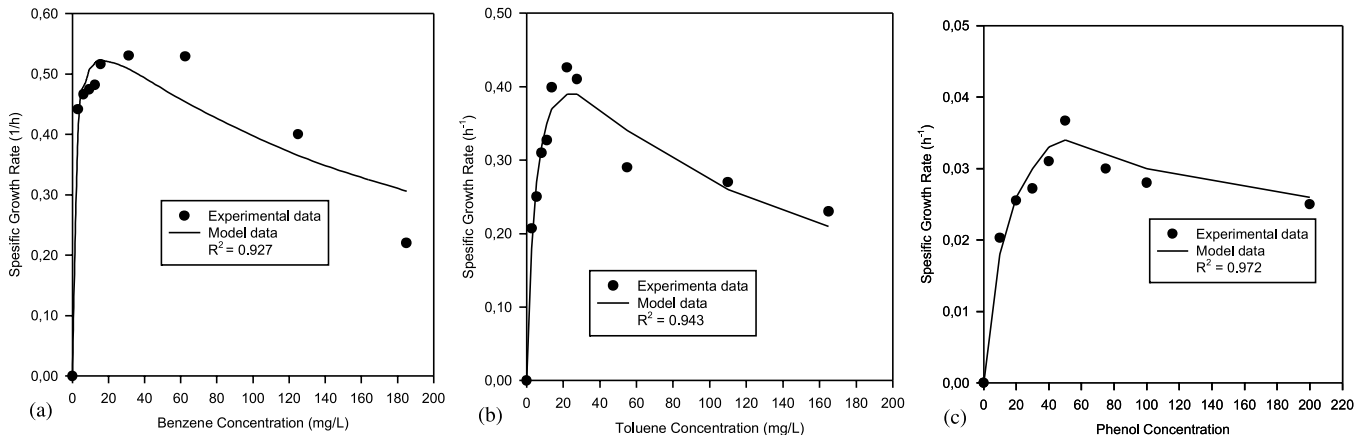
Fig. 2. Dependence of *Pp* F1 specific growth rate on the concentration of (a) benzene, (b) toluene and (c) phenol.

Table 1
Biodegradation model parameter values for benzene, toluene and phenol by *Pp* F1

Microorganism	μ_{max} (per h)			K_S (mg/l)			K_i (mg/l)			$Y_{X/S}$ (g/g)		
	B	T	P	B	T	P	B	T	P	B	T	P
Pp F1[a]	0.62	0.61	0.051	1.65	6.47	18	180	88	430	0.75	0.58	0.44
Pp F1[b]	0.73	0.86	0.11	0.12	13.8	32				1.2	1.28	0.8
Pp 54G[c]		0.42			3.98			42.78			0.9	
Pp ATCC 23973[d]		0.437			6			1980				
P. fragi B1[e]	0.335			3.17						1.04	1.22	
Pp O1[f]	0.44			3.36						0.65	0.64	
Pp NRRL β-14875[g]			0.569			18.54			99.37			0.52
Pp Q5[h]			0.119			5.27			377			0.55

[a] This study.
[b] [1].
[c] [14].
[d] [15].
[e] [16].
[f] [17].
[g] [18].
[h] [19].

previous work the half saturation Monod constants for B, T and P are also in these ranges. The K_i values for benzene, toluene and phenol are shown in Table 1. The high value of K_i indicates that the inhibition effect can be observed only in a high concentration range. In the literature, there are no data for K_i value of B and limited data for that of T and P.

$Y_{X/S}$ values were calculated using the maximum microorganism concentrations obtained for various total substrates concentrations (Fig. 3(a), (b), (c)). These values for B, T and P were defined as 0.75 ($R^2 = 0.9866$), 0.58 ($R^2 = 0.9546$) and 0.44 ($R^2 = 0.8721$), respectively (Table 1). These values are smaller than the values obtained by other researches due to high substrate concentrations.
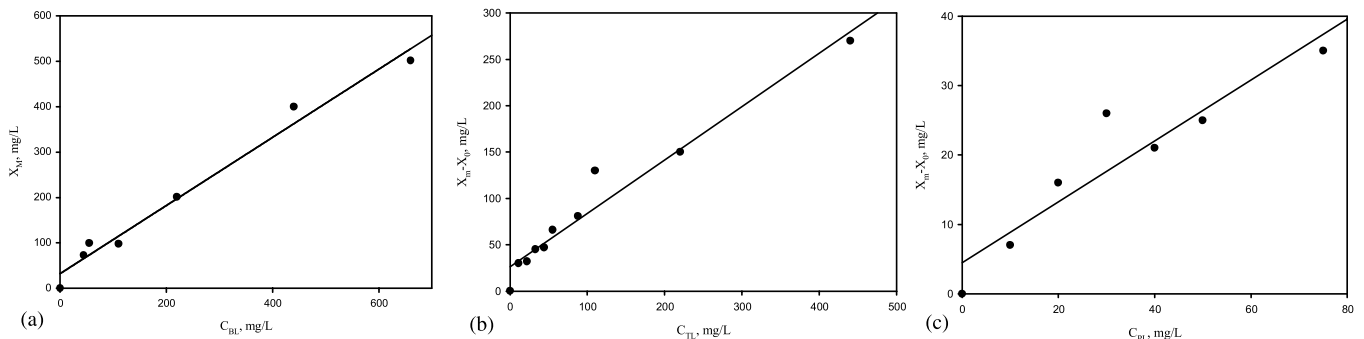

Fig. 3. Determination of $Y_{X/S}$ values of (a) benzene, (b) toluene and (c) phenol.

4.3. Determination of the interaction parameters during the biodegradation of substrate mixtures

Cell concentrations were measured with time for different initial liquid concentrations of benzene–toluene benzene–phenol and toluene–phenol mixtures [10,13]. The specific growth rates were calculated using these data and Eq. (1). The interaction parameters were determined using the model in Eq. (3). This model is similar to the model first proposed by Yoon et al. [12]. In the model proposed by Yoon et al. there was no substrate inhibition term (S^2/K_i). The aim of the present model is to determine the interactions between BTP under the substrate inhibition effects.

As mentioned earlier, the interaction parameter $I_{i,j}$ indicates the degree to which substrate i affects the biodegradation of substrate j and large values indicate stronger inhibition [12]. The kinetic parameters (μ_m, K_S and K_i) in this equation were the same of the single substrate experiments. This model was solved using the Marquartd Levenberg non linear regression program. The following model equations and their regression coefficient were obtained for binary mixtures of B–T, B–P and T–P.

For B–T:
$$\mu = \frac{0.62S_{BL}}{1.65 + S_{BL} + \frac{S_{BL}^2}{180} + 5.16S_{TL}}$$
$$+ \frac{0.61S_{TL}}{6.47 + S_{TL} + \frac{S_{TL}^2}{88} + 0.49S_{BL}},$$
$$(R^2 = 1.0) \tag{8}$$

For B–P:
$$\mu = \frac{0.62S_{BL}}{1.65 + S_{BL} + \frac{S_{BL}^2}{180} + 1.08S_{PL}}$$
$$+ \frac{0.051S_{PL}}{18 + S_{PL} + \frac{S_{PL}^2}{430} + 0.27S_{BL}},$$
$$(R^2 = 1.0) \tag{9}$$

For T–P:
$$\mu = \frac{0.61S_{TL}}{6.47 + S_{TL} + \frac{S_{TL}^2}{88} + 1.03S_{PL}}$$
$$+ \frac{0.051S_{PL}}{18 + S_{PL} + \frac{S_{PL}^2}{430} + 0.14S_{TL}},$$
$$(R^2 = 0.95) \tag{10}$$

The interaction parameters obtained from the sum kinetics (Eq. (3)) with interaction parameters are shown in Table 2. It can be seen that, $I_{B,T}$ is smaller than $I_{T,B}$, which indicates that toluene inhibits the utilization of benzene much

more than benzene inhibits the utilization of toluene. These results were confirmed by Oh et al. [17]. On the other hand, $I_{B,P}$ is smaller than $I_{P,B}$ and $I_{T,P}$ is smaller than $I_{P,T}$ which means that benzene and toluene enhance the biodegradation of phenol, and phenol inhibits the biodegradation of benzene and toluene. $I_{B,P}$ is bigger than $I_{T,P}$, which means that the presence of toluene increases the biodegradation times of phenol more than the presence of benzene, and that the presence of toluene enhances the biodegradation of phenol much more than that of benzene.

5. Conclusion

The purpose of this work was to study the effect of the adaptation of *Pp* Fl to benzene, toluene and phenol on the biodegradation of these compounds, and to investigate the interactions between these substrates during their aerobic biodegradation. In the batch biodegradation of these substrates conducted in this work, the biodegradation of BTP by adapted cells was more rapid than the non adapted cells. The cells were able to consume benzene, toluene and phenol completely. Microorganism growth kinetics was adjusted to the Andrews Kinetics model, which included inhibition terms. In order to investigate the interaction parameters of B, T, and P, sum kinetics were used. These models provided an excellent prediction of the microorganism growth kinetics and interactions between these substrates. Toluene and benzene were better substrates than phenol, resulting in faster growth. It was found that toluene and benzene enhanced the biodegradation of phenol; however, phenol inhibited the biodegradation of benzene and toluene.

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Table 2
Interaction parameters obtained by solving Eq. (3)

Parameter	$I_{B,T}$	$I_{T,B}$	$I_{B,P}$	$I_{P,B}$	$I_{T,P}$	$I_{P,T}$
Value (–)	0.49	5.16	0.27	1.08	0.14	1.03

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