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Multi-substrate growth kinetics of *Pseudomonas putida* for phenol removal

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Abstract The biodegradation of phenol by a pure culture of *Pseudomonas putida* was investigated in a continuously fed stirred-tank reactor, under aerobic conditions. The dilution rate was varied between 0.0174 h^{-1} and 0.278 h^{-1}, covering a wide range of dissolved oxygen and the inhibition region of phenol. Through non-linear analysis of the data, a dual-substrate growth kinetics, Haldane kinetics for phenol and Monod kinetics for oxygen, was derived with high correlation coefficients. Respective biokinetic parameters were evaluated as $\mu_m = 0.569\ h^{-1}$, $K_p = 18.539$ mg/l, $K_i = 99.374$ mg/l, $K_o = 0.048$ mg/l, $Y_{x/p} = 0.521$ g microorganism/g phenol and $Y_{x/o} = 0.338$ g microorganism/g oxygen, being in good agreement with other studies in the literature. Maintenance factors for both phenol and oxygen were calculated for the first time for *P. putida* while the saturation coefficient for oxygen, K_o, was genuinely evaluated from the constructed model, not imported or adapted from other studies as reported in the literature. All pertinent biokinetic parameters for *P. putida* have been calculated from continuous system data, which are most appropriate for use in continuous bioprocess applications.

Introduction

Phenol is a troublesome contaminant in surface waters and gives an objectionable taste to municipal drinking waters at even lower concentrations. Phenolic compounds are also common pollutants in many industrial wastewaters. Aqueous phenolic wastes have been treated for many years by activated-sludge processes, which are known to be sensitive to fluctuations in the phenolic load. However, phenol is not readily biodegradable and it is very toxic to most types of microorganism at sufficiently high concentrations. Phenol can inhibit the growth rate even of those species that have the metabolic capability of using it as a substrate for growth.

Pseudomonas putida, a gram-negative, polarly flagellated, unicellular bacterium, is known to be capable of using aromatic compounds as a sole source of carbon and energy. Optimum microbial growth conditions are 30 °C and pH 6.8 (Hill and Robinson 1975; Yang and Humphrey 1975).

In many literature studies, the microbial growth kinetics of *P. putida* was expressed in single-substrate models, either zero- or first-order, or Monod kinetics or substrate-inhibition models when the limiting substrate, phenol, inhibits growth. In these models of growth, oxygen was not considered as a limiting substrate assuming adequate aeration in the medium (Jennings et al. 1976; Shieh 1980; Mulcahy et al. 1981; Wang and Chi 1984). However, there are certain cases where dissolved oxygen concentration is solely responsible for controlling the growth and substrate consumption rate. For instance, growth in pellet form or in biofilms exerts an intensive mass-transfer resistance within a dense cell structure for all substrates, especially for the least soluble one, dissolved oxygen. Even in suspended growth, oxygen can be the only limiting substrate, because of high cell density or viscous medium where mass transfer is critically limited. Therefore, a realistic multisubstrate kinetic model with appropriate parameters must be constructed for application in such cases.

There are few multi-substrate models for *P. putida* in the literature (Tang and Fan 1987; Wisecarver and Fan 1989; Livingston and Chase 1989), but in none of them was the saturation constant for dissolved oxygen, K_o, directly calculated from original experimental data, rather than taken from other reports of similar cultures (Atkinson and Mavituna 1983; Wagner and Hempel 1988).

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In this work, a dual-substrate kinetic model for the growth of *P. putida* on phenol and dissolved oxygen was constructed through a non-linear analysis of continuous fermenter data. Data were obtained covering a wide range of dissolved oxygen and also the inhibition region of phenol, and all respective kinetic parameters were estimated from the same data set, which has not been reported yet.

Materials and methods

Microorganism

In continuous-culture runs, a pure culture of *P. putida* was used, strain NRRL (Northern Region Research Laboratory) β-14875, which is capable of degrading phenol. The strain was revived from freeze-dried samples and transferred to phenol-free liquid growth medium. Fresh cultures in exponential growth phase were prepared for inoculation from secondary or tertiary cultures adapted to phenol.

Growth medium

Constituents of the artificial growth medium were determined by reviewing the literature studies (Livingston and Chase 1989 and comprised (mg/l) phenol 500, glucose 10 000, yeast extract 1000, KH_2PO_4 420, K_2HPO_4 375, $(NH_4)_2SO_4$ 244, NaCl 15, $CaCl_2 \cdot 2H_2O$ 19.8, $MgSO_4 \cdot 7H_2O$ 61.4 and $FeCl_2 \cdot 4H_2O$ 3.2. Phenol was used only in continuous culture runs, where medium did not contain glucose and yeast extract. Glucose and yeast extract were used only in inoculum medium to supply carbon and energy, and to stimulate growth, where the medium did not contain phenol. To prevent precipitation of Ca^{2+} and Mg^{2+} ions in the growth medium, a stock solution of $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$ and NaCl salts was prepared and added in sufficient quantities to the solution of other medium compounds. Potassium monophosphate was used as the buffer and ferrous chloride was used to prevent precipitation during autoclaving. Neither trace elements nor vitamin compounds were included in the medium to simulate growth in a realistic wastewater. Moreover the effects of these components on growth kinetics parameters under steady conditions were regarded insignificant in recent studies (Livingston and Chase 1989; Zilli et al. 1993; Allsop et al. 1993)

Phenol-free growth medium, prepared for inoculum culture, was autoclaved at 121 °C for 15 min. In continuous culture runs, fresh growth medium was fed to the fermenter without sterilization.

Experimental set-up and procedure

Optimum growth conditions for *P. putida* were reported in the literature as 30 °C and pH 6.8 (Hill and Robinson 1975; Yang and Humphrey 1975). However, in this study 25 °C was chosen as the working temperature since most of the wastewater treatment systems operate around this temperature.

All continuous system runs were carried out in a New Brunswick (BioFlo II) fermenter with a working volume of 5 l in which temperature, pH, dissolved oxygen and agitation rate could be controlled. The sensitivities of control units for dissolved oxygen, pH and agitation were 0.1%, 0.01 unit and ±1 rpm respectively. pH was controlled at 6.8 by adding sterilized 0.1 M NaOH solution. The fermenter was aerated with a constant air flow rate of 1.5 standard l/min for each run. For setting-up the continuous system, a batch culture was initiated by the inoculation of 500 ml inoculum culture (10%, v/v) into the fermenter containing the growth medium with phenol. The agitation rate applied was 400 rpm so that microorganisms would grow without flocculation. When the culture entered the exponential growth phase, continuous pumping of fresh feed was started. In order to establish a steady state, the reactor was left to equilibrate over four or five retention times and the steady state was assumed if the absolute differences in consecutive measurements of microorganism and dissolved oxygen concentrations differed by less than 3%. Several dilution rates up to the wash-out point were applied and corresponding steady-state data were recorded. For a new steady state, the dilution rate was increased carefully by a gradual increase in the feed rate. A large perturbation was given to the feed rate for entry into the inhibition region of phenol, and this region was completely scanned by slowly decreasing the dilution rate.

Analysis

The microorganism concentration was determined using a standard dry-weight method (Tang and Fan 1987). Phenol concentration in the supernatant of 15-ml samples was measured by means of a Cecil 1100 chromatographic system. This system was equipped with a variable-wavelength monitor (CE 11220) operating at 254 nm and a Cecil HPLC pump system (CE 1100). Chromatograms were obtained using a Hichrom-S5OS1 column (Chromosorb), which has 8 nm pore size, 8 μm particle size and 250 mm × 4.6 mm column dimensions. Only one mobile phase, running at ambient temperature, was eluted and a 20 μl sample was injected. CH_3OH (BDH, Hipersolv for HPLC, England)/H_2O (Ultra pure) (40/60, v/v) was used as the mobile phase and the flow rate was adjusted to 1.0 ml/min (under 28 MPa pressure). Before the experiments, the mobile phase was filtered and sonicated in order to remove dissolved gases.

The oxygen-consumption rate was measured using a dynamic method suggested by Bandyopdhyay et al. (1967). In this method, the overhead volume of the continuous fermenter is flashed with nitrogen gas to remove the oxygen and, feeds of fresh broth and air are ceased. The linear drop of dissolved oxygen concentration in the reactor is then recorded against time and the quantity $-\mathrm{d}c/\mathrm{d}t$ yields the overall oxygen-consumption rate of the culture. Thus, the term $(-\mathrm{d}c/\mathrm{d}t)/X$ gives the specific oxygen-consumption rate, where c is the dissolved oxygen concentration (mg/l) and X is the concentration of microorganism (g/l).

Mathematical modeling

A mass balance for the microorganism over a continuously fed stirred-tank reactor (CFSTR) yields Eq. 1, if the nutrient feed does not include any microorganism (Bailey and Ollis 1986).

$$\mu = D = Q/V \tag{1}$$

where μ is the specific growth rate (h^{-1}), D is the dilution rate (h^{-1}), Q is the volumetric nutrient feed rate (l/h) and V is the reactor volume (l).

During the experimental study, steady-state concentrations of the microorganism, phenol and oxygen at several dilution rates were measured, and consumption rates of phenol and dissolved oxygen were calculated. These results were evaluated by a non-linear regression program, Systat (1993), to obtain maintenance and yield factors in Eqs. 2 and 3.

A mass balance for a single substrate in a CFSTR, at steady state, gives

$$Q(c_\mathrm{f} - c_\mathrm{s}) = \left(\frac{\mu X}{Y_{\mathrm{x/s}}} + m_\mathrm{s} X\right) V \tag{2}$$

where c_f is the substrate concentration in fresh feed (mg/l), c_s is the substrate concentration in the reactor at steady state (mg/l), $Y_{\mathrm{x/s}}$ is the yield factor for substrate (g microorganism produced/g substrate consumed) and m_s is the maintenance factor for the substrate (g substrate consumed g microorganism^{-1} h^{-1}).

The experimentally determined specific oxygen-consumption rate (OCR) can be related to oxygen balance over a CFSTR at steady state:

$$(\mathrm{OCR})XV = \left(\frac{\mu X}{Y_{x/o}} + m_o X\right)V \quad (3)$$

where m_o is the maintenance factor for oxygen (g O_2 consumed g microorganism^{-1} h^{-1}) and $Y_{x/o}$ is the yield factor for oxygen (g microorganism produced/g oxygen consumed).

Since the specific growth rate of *P. putida* in phenolic medium is limited by both phenol and oxygen interactively, a specific growth rate expression should be given in multiplicative form as,

$$\mu = \Omega\,(c_p) \times \emptyset(c_o) \quad (4)$$

Here, $\Omega(c_p)$ and $\emptyset(c_o)$ are individual single-substrate inhibition and growth models, respectively. C_p and C_o are phenol and oxygen concentrations (mg/l) in the reactor, respectively, at steady-state. For single-substrate inhibition models, several models proposed by Edwards (1970), Andrews (1968), Aiba (1968), Haldane (1925) and Luong (1987) were utilized, while for single-substrate models Monod (1949), Tessier (1942), Moser (Stainer et al. 1976) and Contois (1959) were referred to. All possible combinations of these models are made in Eq. 4 to find the best-fit model through non-linear regression. Since the non-linear regression is sensitive to initial guesses, the search for the values of the biokinetic constants was constrained within a predetermined range. This range was determined using literature values for the culture, *P. putida*, and was 0–1 h^{-1} for μ, 0–500 mg/l for K_p, 0–7 mg/l for K_o and 0–500 mg/l for K_i, where K_p and K_o are the saturation coefficients (mg/l) for phenol and O_2 respectively, and K_i is the inhibition coefficient (Dinopoulou et al. 1988; Beyenal and Tanyolaç 1996). Within this range, a comparison of goodness of fit for combined equations was done according to the method previously used by Luong (1987).

Results

The variation of specific growth rate with steady-state phenol concentration in the reactor is given in Fig. 1. In this figure, the specific growth rate first increases to approximately 40 mg/l phenol and then starts to decrease at higher phenol concentrations because of substrate inhibition. Even at small concentrations, phenol has a profound inhibitory effect on the specific growth rate of the culture.

For the determination of the dual-substrate model in Eq. 4, the literature models of growth and inhibition for a single-substrate were investigated using all combinations in multiplicative form. Biokinetic parameters of the dual-substrate growth kinetics expression were estimated using a non-linear regression program, Systat (1993). Regression results were evaluated for each combination by comparing their least-squared errors, (LSE = |model value – experimental value|^2), and also regression coefficients, as done previously by Luong (1987). The models with higher LSE values or unrealistic biokinetic coefficients – out of the predicted range – were discarded. The best-fit model was obtained as Haldane kinetics for phenol and Monod kinetics for oxygen in multiplicative form:

$$\mu = \mu_m \frac{c_p}{K_p + c_p + c_p^2/K_i} \cdot \frac{c_o}{K_o + c_o} \quad (5)$$

where, maximum specific growth rate, $\mu_m = 0.569$ h^{-1}, $K_p = 18.539$ mg/l, $K_i = 99.374$ mg/l and $K_o = 0.048$ mg/l ($R = 0.998$, LSE $= 0.999 \times 10^{-3}$).

In Fig. 2, the accordance of experimental and theoretical specific growth rates obtained is compared. With a regression coefficient of 0.998, Haldane–Monod dual kinetics defines phenol- and oxygen-limited growth rate of *P. putida* very well.

Steady-state reactor phenol and dissolved-oxygen concentrations along with oxygen-uptake rates are presented in Table 1. Equation 2 was solved for phenol through non-linear analysis using the same program (Systat) and, maintenance and yield factors related to phenol were found to be $m_p \approx 0$ g phenol g microorganism^{-1} h^{-1} and $Y_{x/p} = 0.521$ g microorganism/g phenol ($R = 0.979$, LSE $= 3.72 \times 10^{-4}$) respectively. Since the oxygen consumption rate was obtained for each run, Eq. 3 was solved again with Systat and maintenance and yield factors related to oxygen were found to be $m_o \approx 0$ g oxygen g microorganism^{-1} h^{-1}

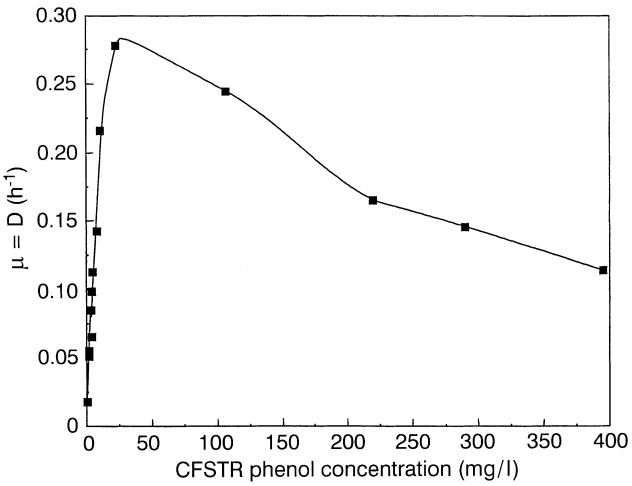

Fig. 1 The variation of specific growth rate with phenol concentration in a continuously fed stirred-tank reactor

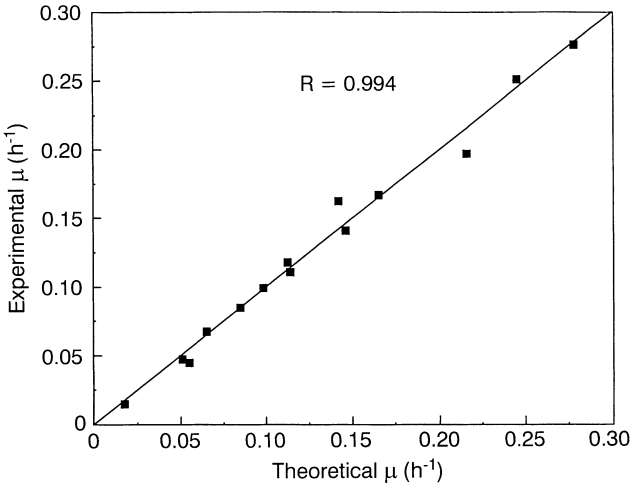

Fig. 2 The comparison of experimental and theoretical specific growth rates

Table 1 Continuously fed stirred-tank reactor steady-state data

$D = \mu$ (h^{-1})	X (mg/l)	c_p (mg/l)	c_o (mg/l)	OCR (g oxygen g microorganism^{-1} h^{-1})
0.0174	250	0.5	7.05	0.2677
0.0507	246	1.7	6.70	0.7914
0.0549	410	1.8	0.40	0.5137
0.065	238	4	0.10	1.0431
0.0847	215	3.3	5.02	1.5076
0.0983	243	4	4.13	1.5460
0.1124	240	5	4.13	1.7850
0.1139	51	395	1.88	1.8371
0.1423	298	8	1.73	1.8090
0.1460	105	290	4.65	2.2484
0.1655	87	220	1.50	4.1014
0.2158	250	11	1.60	3.2508
0.2450	190	106	5.00	3.9120
0.2780	228	23	4.00	4.4784

Table 2 Comparison of biokinetic parameters in dual-substrate growth models from other studies with those from this work. *NA* not available

Study	μ_m (h^{-1})	K_p (mg/l)	K_i (mg/l)	K_o (mg/l)	m_p (g phenol g microorganism^{-1} h^{-1})	m_o (g oxygen g microorganism^{-1} h^{-1})	$Y_{x/p}$ (g microorganism g phenol^{-1})	$Y_{x/o}$ (g microorganism g oxygen^{-1})
Our work (1997)	0.539	18.539	99.374	0.048	≈0	≈0	0.521	0.338
Livingston and Chase (1989)	0.418	2.9	370	0.26[a]	NA	NA	0.6	0.465
Tang and Fan (1987)	0.365	10.948	113	0.1[b]	NA	NA	0.496	0.354
Worden and Donaldson (1987)	0.230	10[c]	60	0.1[b]	NA	NA	0.4	0.286

[a] Adapted from Wagner and Hempel (1988)
[b] From Atkinson and Mavituna (1983)
[c] From Tang and Fan (1987)

and $Y_{x/o} = 0.338$ g microorganism/g oxygen respectively ($R = 1$, LSE $= 8.18 \times 10^{-4}$).

Discussion

In this study, a dual-substrate growth kinetics for *P. putida* was derived through non-linear analysis of continuous fermenter data and respective biokinetic parameters were evaluated with high correlation coefficients. The results agree reasonably well with the values observed in other studies.

Table 2 compares the biokinetic parameters of dual-substrate growth models from other studies with this work. In all studies except ours, maintenance factors for phenol and oxygen were not evaluated and the saturation constant for oxygen, K_o, was either assumed or imported from other reports for different substrates. Livingston and Chase (1989) made use of a mixed culture comprised of *Acinetobacter* and *Pseudomonas* species in batch runs to derive biokinetic parameters. In their model, they adapted the saturation constant for oxygen, K_o, from the work of Wagner and Hempel (1988); however, the degraded carbon source was naphthalene-2-sulphonate, not phenol. Tang and Fan (1987) again worked with a mixed culture in which *P. putida* was dominant and derived the parameters from batch runs; however, K_o was assumed to be 0.1 mg/l, a common value proposed for bacteria by Atkinson and Mavituna (1983). Moreover, they did not encounter oxygen-limiting conditions in their experiments, thus the contribution of K_o to the overall model was not clearly verified. On the other hand, Worden and Donaldson (1987) worked with a mixed culture under oxygen-limiting conditions in their semi-continuous fluidized-bed and stirred-tank system; however they imported K_p and K_o from Tang and Fan (1987) and Atkinson and Mavituna (1983) respectively, and the remaining parameters were chosen to provide agreement between the model predictions and experimental results.

All models from the literature contained unoriginal biokinetic parameters and these parameters were derived from insufficient batch data, which could not be appropriate for continuous system applications such as wastewater treatment and fluidized-bed biofilm reactor bioprocesses. However, our model made use of continuous data covering a wide range of dissolved oxygen and also the inhibition region of phenol, and all the respective kinetic parameters were estimated from the same data set.

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