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Cometabolism of p-nitrophenol by phenol-oxidizing *Ralstonia eutropha*: the involved kinetics

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ABSTRACT

A phenol-oxidizing *Ralstonia eutropha* was examined for its potentiality to degrade p-nitrophenol (PNP) and phenol in a cometabolic fashion in a trickle bed reactor (TBR) using Kissiris as a packing material. In explaining the kinetic behavior of the cells, responses of the freely suspended cells were also recorded. The kinetic parameters pertinent to growth, cell decay, product toxicity, competitive inhibition between substrates, and enhancement in cometabolic transformation as a result of the presence of the growth substrate, which are all inherent to the cell behavior, were determined experimentally, analyzed quantitatively, and modeled mathematically. By considering the total degradation time, including the length of lag phase period, phenol degradation was under adverse effect of PNP presence in the system, while phenol had an enhancing effect on the formation of biomass and degradation of PNP when it was used along with variable levels of PNP. The inhibition coefficient of PNP on phenol degradation (K_{ic}) and that of phenol on PNP transformation (K_{is}) were determined to be 2.36 mg_c L^{-1} and 86.02 mg_s L^{-1}, respectively. This indicates that PNP imposed much larger competitive inhibition to phenol utilization by the cells than the converse. Predicted values of the proposed model for simultaneous utilization of PNP and phenol by the test bacterium were in good agreement with the experimental data. Cell immobilization could increase cell tolerance to higher concentrations of both substrates. However, the values of specific degradation rates of the substrates were lower in TBR as a result of excessively higher biomass formed as compared with the content of the cells in shake flasks studies.

Keywords: Phenol; p-nitrophenol; Cometabolism; Mathematical modeling; *Ralstonia eutropha*; Trickle bed reactor

1. Introduction

The use of phenol as a raw material for the synthesis of various types of chemicals including petrochemicals, agrochemicals, pharmaceuticals, etc. has generated considerable attentions on the toxic effects of this organic compound as the consequence of its presence in the environments. There are several ways for the production of phenol such as coal tar distillation process, oxidation of methyl-ethylbenzene, oxidation of toluene, and heating of mono-chlorobenzene using sodium hydroxide under high pressure [1]. The capacity for global production of phenol is presently 8 million metric tons with 5.5 million metric tons production in 2012 by China, USA, Japan, Taiwan, and South Korea as the top five manufacturing countries [2,3]. Although derivatives of phenolic compounds from natural sources (also present in industries such as paper and pulp, olive oil production, etc.)

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are structurally diverse, but their concentrations are much lower than the amount of phenol produced through human activities [4]. Preference of bio-based techniques compared with different types of waste treatment (chemical/physical methods) has been extensively discussed. Advantages of bio-treatment processes are eco-friendliness and economic feasibility. The presence of p-nitrophenol (PNP) in industries is also considerable (e.g., manufacturing of drugs, dyes, plastics, explosives, fungicide, etc. with 10 mg L^{-1} as the acceptable concentration in natural waters) [5]. Structural resonance of both phenol and PNP has made their degradation harder. Furthermore, similarity in structures of phenol and PNP may increase the chance of some oxidoreductases enzymes being functional and inducible in catabolic degradation of these two compounds. This point has been addressed by Reardon et al. [6], where researchers actually conducted extensive experiments to study biodegradation kinetics of phenol alone and as mixture with benzene and toluene by *Pseudomonas putida*. In these studies, the main concept was the saturation kinetics with single or double substrates. The data for kinetics may be treated considering no interaction between substrates or having unspecified type of interaction [7]. Considering the inhibitory nature of phenol (benzene or toluene), more attention was on the growth inhibitory function of one substrate on the utilization of the other substrate, and inhibition in enzymatic reactions was used in these cases as the basis for defining the mathematical models (competitive, noncompetitive, and uncompetitive inhibition) [6,7].

Direction toward modeling for the kinetics of cometabolism began early from the biodegradation of organic compounds not supporting growth through the extensive studies of Schmidt et al. and the others [8–13]. Cometabolism is the transformation of nongrowth substrate by microbial cells that have maintain their growth by consumption of growth substrate. The definition also covers the transformation by the resting cells in the absence of growth substrate [14,15]. Saturation kinetics (Michaelis–Menten equation 'enzymatic reactions' and Monod equation 'growth substrate consumption'), pseudo-first-order model (as the extension of the saturation kinetics), coenzymes participation in enzymatic reactions (coenzymes regeneration should be considered in the oxidoreductases enzymes), and growth/enzyme inhibition (toxicity expressed by the formed intermediates and products) are among the most widely used approaches in the literature regarding cometabolism kinetics [16,17]. The concept of the current work was based on previous studies conducted in our laboratory focusing on *Ralstonia eutropha* performance in response to phenol with or without PNP [18–21]. Simultaneous biodegradation of these two compounds by the test bacterium was mathematically described in the present work by considering the modeling approach undertaken for cometabolism kinetics by Criddle [14] and Chang and Criddle [15], where the fate of the following events were monitored with the use of relevant parameters: the cell growth (Y_m), endogenous cell decay (b), cell response to toxic intermediates (T_c), and response of the cell to the competition, which exists between the growth- and cometabolic-substrates (K_{ic}, K_{is}). The utilization of the nongrowth substrate by the microbe was under positive influence of the growth substrate consumption, and the relevant term (T_y) was also included in the model of interest (the details of model description are given in the "Materials and methods" section) [14,15]. Nevertheless, the parameters Y_m, b, T_c, and T_y were not included in the model in our previous work.

There are some advantages in using immobilized cells against free cells. The first one is greater tolerance of the cells in the form of biomass film to higher concentrations of organic pollutant as the growth substrate, which implies that the cells have greater possibility to be distributed evenly in a particular matrix (i.e., integrity of the biomass is physically kept almost constant). The other point is the lower frequency of biomass washout occurrence in the test system. Thus, operational flexibility would be increased. Selecting a suitable reactor is essential for assessing the advantages of immobilized cells over the free cells.

Based on previous studies conducted in our laboratory focusing on the cometabolism of the PNP and phenol, it was of interest to assess the behavior of Kissiris-immobilized *R. eutropha* in response to periodic flow of liquid containing cometabolic substrate. The kinetics experiments were directed toward the use of trickle bed reactor (TBR), in which the cell behavior was monitored under aerobic conditions (TBR was operated countercurrently using Kissiris pieces as the packing material).

2. Materials and methods

2.1. Chemicals

Phenol, which is used as the growth substrate; PNP, as the cometabolic substrate; and all other reagents used for the growth and cultivation of microorganism in the present study were of analytical grade and purchased from the local suppliers.

2.2. Microorganism and culture conditions

The bacterium *R. eutropha* was purchased from the Iranian Research Organization for Science and Technology (IROST) and was maintained according to the instruction given by Persian Type Culture Collection (PTCC 1615). The nutrient medium (NM) used for the bacterium cultivation contained the following ingredients (g L^{-1}): glucose, 3; yeast extract, 2; peptone, 3; KH_2PO_4, 1; K_2HPO_4, 1; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.05; and agar at the amount of 15 g L^{-1} was used for medium solidification. Previously prepared bacterial culture as inoculum for the cultivation of NM broth, in which the culture at the level of 10% v/v was added to 100 mL of the broth using a 250-mL conical flask, was incubated at 30°C in a shaker incubator (150 rpm) for 24 h. The pH of the medium was adjusted to 7 using 2 N NaOH. The prepared culture medium was sterilized in an autoclave at 121°C for 20 min. A separate procedure had been conducted previously with glucose and phenol as the growth medium in the absence of yeast extract and peptone in order to have phenol-oxidizing *R. eutropha*, which was able to use phenol as the sole carbon source. Acclimation to phenol was completed when the bacterium was able to use phenol as the only carbon source [21]. This culture (10% v/v) was then inoculated into 90 mL of the mineral salts solution (MSS) in the presence of 200 mg_s L^{-1} phenol in a 250-mL conical flask and incubated at 30°C in a shaker incubator (150 rpm) for 24 h.

This phenol-grown *R. eutropha* was used in all experiments of the present study. MSS contains (g L^{-1}) 1 KH_2PO_4, 1 K_2HPO_4, 1 $(NH_4)_2SO_4$, and 0.05 $MgSO_4{\cdot}7H_2O$.

2.3. Construction and inoculation of immobilized bacteria column (TBR arrangement)

The entire height of glass column was 65 cm where the top and bottom of the column had height of 15 cm, while the cylindrical shaped part of the reactor had height of 50 cm with inside diameter of 5 cm. This main body of the reactor was filled with Kissiris pieces (having spherical shape and diameter of 1 cm) up to 45 cm (90% volume). Continuous aeration to the system at the flow rate of 0.5 vvm using an aquarium pump was through a heat-resistant perforated plate placed at the bottom of the Kissiris packing. Placing a perforated plate at the top of the column caused the test liquid (100 mL culture medium plus 900 mL MSS containing phenol at 200 mg_s L^{-1} concentration) to trickle to the microorganism bed at the flow rate of 6.5 mL min^{-1} using a peristaltic pump (Fig. 1). The whole test system including the glass column, perforated plates, Kissiris pieces, valves, and all the tubing connections were sterilized in an autoclave (at 121°C for 20 min). Additional note was on the immobilization of *R. eutropha* on Kissiris pieces, and this was done via recycling the test liquid for 60 d considering replacement of the liquid every 4 d while addition of phenol to the system was carried out every day at 200 mg_s L^{-1}. All the experiments were performed at 30°C and pH ≈ 7. The pH was almost constant during the operation of the reactor.

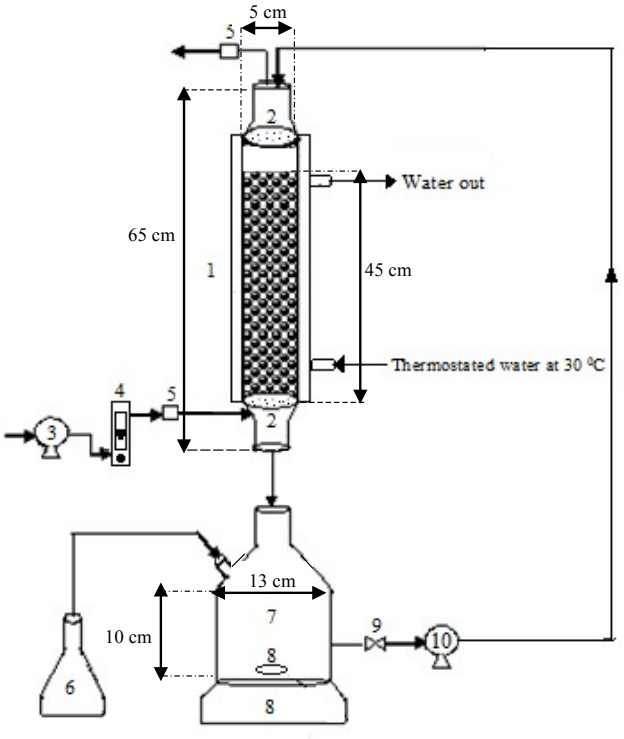

Fig. 1. Arrangement and dimensions of TBR containing Kissiris-immobilized *Ralstonia eutropha* used in the present study. Note: 1 – glass column reactor filled with Kissiris pieces; 2 – plastic perforated plate; 3 – aquarium air pump; 4 – flowmeter; 5 – air filter; 6 and 7 – the glass vessels with an appropriate port for feeding the test liquid to the column reactor; 8 – stirrer and magnetic bar; 9 – valve for taking sample; and 10 – peristaltic pump.

2.4. Model description

Specific rates of growth substrate utilization (q_s) and cometabolic substrate consumption (q_c) are presented in the following equations:

$$q_s = -\frac{1}{X}\frac{dS}{dt} = k_s\left(\frac{S}{K_s\left(1+\frac{C}{K_{ic}}\right)+S}\right) \tag{1}$$

$$q_c = -\frac{1}{X}\frac{dC}{dt} = \left(T_y q_s + k_c\right)\left(\frac{C}{K_c\left(1+\frac{S}{K_{is}}\right)+C}\right) \tag{2}$$

where X and S are the concentrations of the biomass and growth substrate, respectively. The cometabolic substrate concentration is shown as C, and further note on Eq. (1) is on the significance of k_s as the maximum specific rate of growth substrate utilization. The half-saturation coefficient, K_s, is the cell affinity toward the growth substrate. While K_{ic} expresses the extent of inhibitory action of the cometabolic substrate on growth substrate utilization by microorganism. Since there exists a competition between the growth and cometabolic substrates for occupying the catalytic site of a particular enzyme, K_{is} in Eq. (2) can be defined equivalently as K_{ic}. The use of $(1 + C/K_{ic})$ in Eq. (1) and $(1 + S/K_{is})$ in Eq. (2) is actually based on competitive inhibition in enzymatic reactions regarding the substrate being inhibited by an inhibitor. The terms k_c and K_c in Eq. (2) are the maximum specific rate of the cometabolic substrate utilization and half-saturation coefficient of cometabolic substrate, respectively. The term T_y also gives an estimation of theoretical transformation yield. This term indicates cell capability in consuming growth substrate along with the cometabolic substrate (biodegradation rate enhancement in terms of cometabolic substrate utilization by the cell).

The specific growth rate, shown as μ, is defined as the following equation:

$$\mu = \frac{1}{X}\frac{dX}{dt} = Y_m q_s - b - \frac{q_c}{T_c} \tag{3}$$

Coefficient of Y_m as the maximum or true growth yield (mass of bacteria generated per mass of growth substrate consumed) demonstrates the effect of utilization of phenol on the specific growth rate. Representation of cell growth decline or cell decay endogenously is indicated by b coefficient (Eq. (3)). The key point in T_c term is to recognize the difference between cell maintenance in the presence of growth substrate in the absence or presence of cometabolic substrate. In other words, cell sensitivities to cometabolic substrate

exert attention on the cell for maintaining itself in response to toxic products formed during transformation of cometabolic substrate [14,15].

Considering Eqs. (1)–(3), Runge–Kutta numerical method via application of Matlab R2014a software was employed in performing the analysis of experimentally obtained data. In explaining the described model, then, conceptually derived equations and their parameters should be evaluated experimentally (see below for the experiments' details).

2.5. Experimental scheme

Two sets of experiments in total number of 48 test runs were conducted for shake flask and TBR studies as shown in Table 1.

2.5.1. Shake flask studies

In each shake flask experiment, a 10 mL of culture solution of *R. eutropha* and 90 mL of MSS (see 'Microorganism and culture conditions' section) were poured into each of 21 conical flasks with 250 mL volumetric size. Then, different concentrations of either phenol or PNP with or without each of these two substrates were added to the flasks (Table 1). The cultivation of these solutions was then carried out in a shaking incubator (WIS-20, Wisecube®, witeg Labortechnik GmbH, Germany), at 30°C and 150 rpm. The effects of phenol or PNP biodegradation on the other substrate were studied using these experiments. Sampling analysis of the contents of phenol, PNP, and biomass were made over a certain period during the treatment time (see 'Analytical methods' section).

Five independent experiments were conducted to measure the maximum specific rate of utilization of phenol substrate (k_s), the half-saturation coefficient of phenol substrate (K_s), endogenous decay constant (b), and true growth yield (Y_m) (A_1–A_5 in Table 1). The values of q_s for the five different cases were obtained by drawing a plot of phenol concentration vs. time. While by considering Eq. (1) (neglecting '$1 + C/K_{ic}$' term), k_s and K_s values were determined using nonlinear regression analysis of data (see section 'Data fitting procedure'):

$$q_s = -\frac{1}{X}\frac{dS}{dt} = k_s\left(\frac{S}{K_s + S}\right) \tag{4}$$

The observed growth yield (Y) also was determined as the result of drawing the curve of biomass concentration vs. time and considering phenol utilization over treatment time (mass of bacteria generated per mass of growth substrate consumed). Considering Eq. (3) in the absence of cometabolic substrate ($q_c = 0$) and when utilization of phenol as the growth substrate was complete ($Y_m\ q_s = 0$), the first-order endogenous decay constant would readily be determined using a semi-logarithmic plot of active cell content vs. time:

$$\ln\left(\frac{X}{X_0}\right) = -bt \tag{5}$$

Further note is toward determination of Y_m in the absence of PNP with consideration of high concentration of phenol as the growth substrate:

$$Y = Y_m\left(\frac{\mu}{\mu + b}\right) \approx Y_m\left(\frac{k_s}{k_s + \frac{b}{Y_m}}\right) \tag{6}$$

The next independent experiments were six runs from B_1 to B_6, according to the descriptions of the concentrations given in Table 1, by which the maximum specific rate of utilization of cometabolic substrate (k_c), the half-saturation coefficient of cometabolic substrate (K_c), and theoretical transformation capacity (T_c) were calculated. By plotting PNP concentration vs. time and obtaining values of q_c for six different cases, the values of k_c and K_c parameters were measured by considering Eq. (2) (neglecting the terms '$1 + S/K_{is}$' and '$T_y q_s$') and employing nonlinear regression method:

$$q_c = -\frac{1}{X}\frac{dC}{dt} = \left(k_c\right)\left(\frac{C}{K_c + C}\right) \tag{7}$$

Theoretical transformation capacity can be determined considering Eq. (8) (in the absence of phenol as the growth substrate and by adding a high initial concentration of cometabolic substrate 'PNP'):

Table 1
Experimental plan for phenol and PNP degradation by *Ralstonia eutropha*

Serial number	Initial concentration of substrates ($mg_s\ L^{-1}/mg_c\ L^{-1}$)		Study in	
	Phenol	PNP	Shake flasks	TBR
A_1, A_2, A_3, A_4, A_5	25, 50, 75, 100, 115	–	√	
B_1, B_2, B_3, B_4, B_5, B_6	–	2, 4, 6, 8, 10, 12	√	
C_1, C_2, C_3, C_4, C_5	25, 50, 75, 100, 115	14	√	
D_1, D_2, D_3, D_4, D_5	115	2, 4, 6, 8, 10, 12	√	
E_1, E_2, E_3, E_4, E_5	100, 200, 300, 400, 500, 600	–		√
F_1, F_2, F_3, F_4, F_5, F_6	–	2.5, 5, 7.5, 10, 12.5, 15		√
G_1, G_2, G_3, G_4, G_5, G_6, G_7, G_8	75, 150, 225, 300, 375, 450, 525, 600	20		√
H_1, H_2, H_3, H_4, H_5, H_6, H_7, H_8	600	2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20		√

$$\left(T_c\right)_{\text{obs}} = -\frac{q_c}{\mu} = \frac{1}{\frac{b}{k_c} + \frac{1}{T_c}} \tag{8}$$

where b and k_c are known values. Definition below is used for determination of the observed transformation capacity ($(T_c)_{\text{obs}}$):

$$\left(T_c\right)_{\text{obs}} = -\frac{q_c}{\mu} = \frac{C_0 - C_\infty}{X_0} \tag{9}$$

where 0 and ∞ subscripts are representatives of initial and ultimate concentrations, respectively.

Further flask experiments were directed toward five treatments of C_1–C_5 in order to determine the value of the cometabolic substrate's inhibition coefficient (K_{ic}). Obtaining the value of the half-saturation coefficient for phenol substrate in the presence of PNP ($(K_s)_{\text{obs}}$) became possible using nonlinear regression method through the consideration of five different initial phenol concentrations, into which a constant high concentration of cometabolic substrate (≈14 $\text{mg}_c\ \text{L}^{-1}$ of PNP) has been added to each flask. Then, the following equation was used for the determination of K_{ic}:

$$\left(K_s\right)_{\text{obs}} = K_s\left(1 + \frac{C}{K_{ic}}\right) \tag{10}$$

The other five flask experiments, which were separately performed, were D_1–D_5, in which PNP at five different initial concentrations was poured into each flask containing a constant high concentration of phenol (≈115 $\text{mg}_s\ \text{L}^{-1}$). Then, the determination of half-saturation coefficient of cometabolic substrate in the presence of growth substrate ($(K_c)_{\text{obs}}$) became possible with the application of nonlinear regression. After that, growth substrate's inhibition coefficient (K_{is}) was calculated using the following equation:

$$\left(K_c\right)_{\text{obs}} = K_c\left(1 + \frac{S}{K_{is}}\right) \tag{11}$$

By considering Eq. (2), it can be concluded that the term '$T_y q_s + k_c$' is equaled to the maximum specific rate of utilization of cometabolic substrate in the presence of growth substrate ($(k_c)_{\text{obs}}$). Therefore, theoretical transformation yield (T_y) was determined through the use of the following equation:

$$T_y = \frac{\left(k_c\right)_{\text{obs}} - k_c}{q_s} \tag{12}$$

Of course, one should bear in mind that the value of q_s in Eq. (12) is the average of specific rates of utilization of phenol obtained for each initial concentration of the growth substrate in experiments C_1–C_5 (Table 1).

2.5.2. TBR studies

The reactor studies were conducted according to the experiments with serial numbers of E, F, G, and H, and the progress of biodegradation was evaluated based on regularly recorded decrease of phenol and PNP concentrations (with the same procedure used for shake flasks experiments). The biomass measurements (determination of dry cell weight [DCW]) were done only at the final state. Therefore, the kinetic parameters of cell growth and decay were not determined for immobilized cells and modeling of phenol and PNP degradation in TBR was simultaneously performed by solving Eqs. (1) and (2). Control experiments without the bacterial culture were also conducted to evaluate substrates potential removal by adsorption or ventilation processes.

2.6. Data fitting procedure

Fitting the experimental data on the Monod-like model (saturation kinetics), that is nonlinear in its parameters, was performed by Graphpad Prism 5 software using Levenberg–Marquardt nonlinear regression technique. This work requires an initial estimate of the parameters of the interest. The program, then, automatically calculates the lowest values of the sum squares of the differences between the values predicted by the model and the experimental data (residual) after several iterations. Sum squares of the residuals (SSR) were minimized by considering linear descent and Gauss–Newton methods. The coefficient of determination (R^2) gives the proportion of the total variability of data explained by the model ($R^2 = 1 - SSR/SStotal$) and shows extent of closeness of the experimental data to the described kinetic model. Standard error of the estimate ($S_{y.x}$) is a measure of the accuracy of predictions made with a regression curve ($S_{y.x} = \sqrt{(SS/df)}$ where df shows degrees of freedom). The values of R^2 and $S_{y.x}$, which are calculated and reported by Graphpad Prism 5 software, represent the goodness of fit for the result of each parameter determined according to the kinetic model used in the current study [22]. In evaluating the developed model and the obtained parameters, a dynamic feature of the cometabolism process was modeled at different initial concentrations of the substrates using Runge–Kutta numerical method through the application of Matlab R2014a software.

2.7. Analytical methods

During the experiments, a suitable volume of sample was withdrawn at regular time intervals, and this continued until completion of the growth substrate degradation (phenol) while the cometabolic substrate (PNP) was not completely degraded in the treatment time (see the relevant figures in the 'Results and discussion' section). The cell density was measured by obtaining the absorbance (optical density [OD]) at 600 nm using spectrophotometer (JASCO V-550 UV-Vis). Conversion of the OD to DCW was done with the use of a calibration curve and plotting OD_{600} vs. DCW (mg L^{-1}). After analysis of the cell density, the samples were centrifuged at 8,000 rpm for 15 min. The resulting supernatant was analyzed for residual phenol and PNP concentrations. By considering the Folin-Ciocalteu reagent, phenol concentration was determined spectrophotometrically by reading absorbance at 750 nm following the application of the treatment on the supernatant sample according to the details given elsewhere [23]. PNP concentration was measured with the use of spectrophotometer and obtaining absorbance values

at 400 nm. Exactly 1 mL of the supernatant (as mentioned above) was mixed and homogenized with 5 mL 0.1 N NaOH solution. The mixture was then stored at room temperature for 5 min, and the absorbance of the obtained solution was read at 400 nm wavelength [24].

The Kissiris pieces with the biomass were taken out from the reactor at the final state of degradation for the measurement of Kissiris-attached biomass dry weight in the TBR. The pieces were washed with distilled water, dried at 40°C, and 500 mbar in a vacuum oven (H. Jürgens and Co., GmbH and Co., D2800 Bremen, Germany) for 20 h, and weighed. The attached biomass was removed by heating the Kissiris pieces in NaOH solution (0.5 N), and the biomass-free pieces of Kissiris were washed using distilled water. The weight of biomass in the TBR was determined by obtaining the difference between dried Kissiris and dried bare pieces [25].

Control experiments without the bacterial culture were also conducted, and no considerable changes of the initial concentrations of the substrates were observed thus indicating that abiotic loss of the substrate in the present study was negligible.

The Kissiris-immobilized *R. eutropha* in TBR was analyzed morphologically at the end of the experimental work using scanning electron microscope (Philips XL30). The samples were washed with distilled water and dried in the vacuum oven (at 40°C and 500 mbar for 20 h). These samples were then coated with gold film in BAL-TEC Sputter Coater SCD 050 device. Micrographs were taken on the SEM instrument at magnification of 5,000× at 25.0 kV.

3. Results and discussion

3.1. Evaluation of kinetic parameters in terms of cell behavior

The systematic approach that commenced with the consideration of only growth or cometabolic substrate was employed in this study. Eight different kinetic parameters were experimentally measured. Subsequently, with the presence of both substrates in the test medium, three other kinetic parameters were determined (see below for the details). The values of all these kinetic parameters in shake flask and TBR studies are summarized in Table 2 and compared with the values determined in some other studies in the realm of cometabolism.

3.2. Cell response to either phenol or PNP as the single substrate under noninhibitory conditions

Degradation of phenol at different initial concentrations (shown in Table 1 as experiments A and B) was indicative of varied lag phases, where at ≈25 $mg_s\ L^{-1}$ no lag phase was observed (as shown in Fig. 2(a)). The corresponding biomass at initial phenol concentration of ≈25 $mg_s\ L^{-1}$ was low, although at the end of the exponential phase of the growth, the yields of biomass growth were greater for higher initial phenol concentrations (e.g., ≈148 $mg_{cell}\ L^{-1}$ biomass content for phenol concentration at ≈115 $mg_s\ L^{-1}$) (Fig. 2(c)). It seems that more time was needed for the production of necessary enzymes responsible for the degradation of higher levels of phenol (i.e., enough amounts of enzymes should be produced). Similar findings about the consumption of phenol, as the growth-limiting substrate, by *Pseudomonas sp.* DSM 548 and 36 bacterial strains isolated from the activated sludge (sludge from steel mill sewage treatment) have been reported [26,27].

The results of nonlinear regression from fitting the model (Eq. (4)) on the experimental data presented in Figs. 2(a) and (c) were as follows: k_s and K_s were 0.304 $mg_s\ mg_{cell}^{-1}\ h^{-1}$ and 36.11 $mg_s\ L^{-1}$, respectively (goodness of fit: $R^2 = 0.99$, $S_{y.x} = 8.9 \times 10^{-3}$). The value of true growth yield (Y_m) and the first-order endogenous decay constant (b) were calculated (Eqs. (5) and (6)) with the use of data from phenol degradation at different initial concentrations along with the formed biomass concentrations.

In the case of PNP, as the cometabolic substrate in the present study, relatively low amount of PNP was consumed (Fig. 2(b)), while considerable fluctuations in the biomass vs. time curve have been observed (Fig. 2(d)). Capability of *R. eutropha* to survive in the presence of PNP without phenol was fascinating. Operating metabolism of this bacterium for PNP is not constitutive, and loss of controlling enzymes in degrading PNP may occur very rapidly, as has been stated elsewhere on using phenol and glucose by *P. putida* ATCC 17484 [28]. The *R. eutropha* behavior may also be interpreted in the same way as vinyl chloride (VC) cometabolism by an ethane-grown *Pseudomonas* sp. [29]. Cell resources have been gained by the test bacterium upon its prior growth on phenol, and when the cells were exposed to PNP, accessibility of the resources by the cells is very possible. This could provide survival atmosphere for the cells in the expense of using PNP (Fig. 2(b)). An example of the absence of typical cell growth behavior, such as the findings shown in Fig. 2(d), is the induction of certain enzymatic pathways in the presence of some substrate without the involved substrate being used [30].

Determination of k_c and K_c values were carried out using Eq. (7) in similar way to k_s and K_s determination as described above. Kinetic parameters of PNP utilization were obtained as $k_c = 0.00404\ mg_c\ mg_{cell}^{-1}\ h^{-1}$ and $K_c = 9.54\ mg_c\ L^{-1}$ (goodness of fit: $R^2 = 0.99$, $S_{y.x} = 9.8 \times 10^{-5}$). The observed transformation capacity ($(T_c)_{obs}$), as the mass of cometabolic substrate per cell biomass, was measured using Eq. (9) and the details given in Figs. 2(b) and (d) (PNP at ≈ 12 $mg_c\ L^{-1}$). Additional note was on measuring theoretical transformation capacity (T_c), which was determined using Eq. (8) and by substituting the values of b, k_c, and $(T_c)_{obs}$. The value of T_c in the present study was determined as 0.334 $mg_c\ mg_{cell}^{-1}$. This quantity was in the range of 0.0003–0.5 $mg_c\ mg_{cell}^{-1}$, which was summarized by Alvarez-Cohen and Speitel Jr. [17] for cometabolism by phenol degradation. Also, the results of abiotic tests revealed that there was no considerable changes of the initial concentrations of the substrates indicating that abiotic loss of the substrate in the present study was negligible.

3.3. Simultaneous utilization of phenol and PNP substrates by freely suspended cells

Simultaneous oxidations of phenol and PNP under aerobic conditions are coenzyme-dependent bioprocesses, where performance of the controlling enzyme(s) may be explained in terms of whole cell functionality (the cell as the biocatalyst) [31]. In developing a suitable model for

Table 2
Values of kinetic parameters estimated according to the model described in the present study

Growth substrate ($mg_s\ L^{-1}$)	Cometabolic substrate ($mg_c\ L^{-1}$)	Microorganism	k_s ($mg_s\ mg_{cell}^{-1}\ h^{-1}$)	K_s ($mg_s\ L^{-1}$)	k_c ($mg_c\ mg_{cell}^{-1}\ h^{-1}$)	K_c ($mg_c\ L^{-1}$)	K_{is} ($mg_s\ L^{-1}$)	K_{ic} ($mg_c\ L^{-1}$)	T_y ($mg_c\ mg_s^{-1}$)	T_c ($mg_c\ mg_{cell}^{-1}$)	Y_m ($mg_{cell}\ mg_s^{-1}$)	b (h^{-1})	Reference
Phenol (0–115)	PNP (0–14)	*Ralstonia eutropha*	0.304	36.11	0.00404	9.54	86.02	2.36	0.0448	0.334	1.15	0.0209	This study
Phenol (0–600)	PNP (0–20)	Immobilized *Ralstonia eutropha*	0.020	236.1	0.00018	10.35	1,207.79	27.42	0.00905	–	–	–	This study
Phenol (0–600)	PNP (0–50)	Immobilized *Ralstonia eutropha*	0.032	442.9	0.0056	181.2	–	–	–	–	–	–	[20]
Phenol (0–100)	Ethanethiol (0–40)	*Ralstonia eutropha*	0.38	14.16	0.01	1.56	–	–	–	0.2	1.21	0.07	[34]
Phenol (0–10, 100)	Trichloroethylene (0.2–20)	*Pseudomonas putida*	0.68	1.8	0.015	5.6	–	–	–	0.025	0.57	0.03	[33]
Phenol[a] (0–800)	4-chlorophenol[a] (0–200)	*Pseudomonas putida*	0.819	2.19	0.633	5.55	0.193	6.49	3.88	–	–	–	[32]
Methane (0.05–6)	Trichloroethylene (0.5–9)	Methanotrophic mixed culture	0.1571	6.85	0.0063	1.94	0.119	10.8	4.01	0.06	0.54	0.0229	[15]

[a]Ternary substrate system (phenol, 4-CP, and SG).

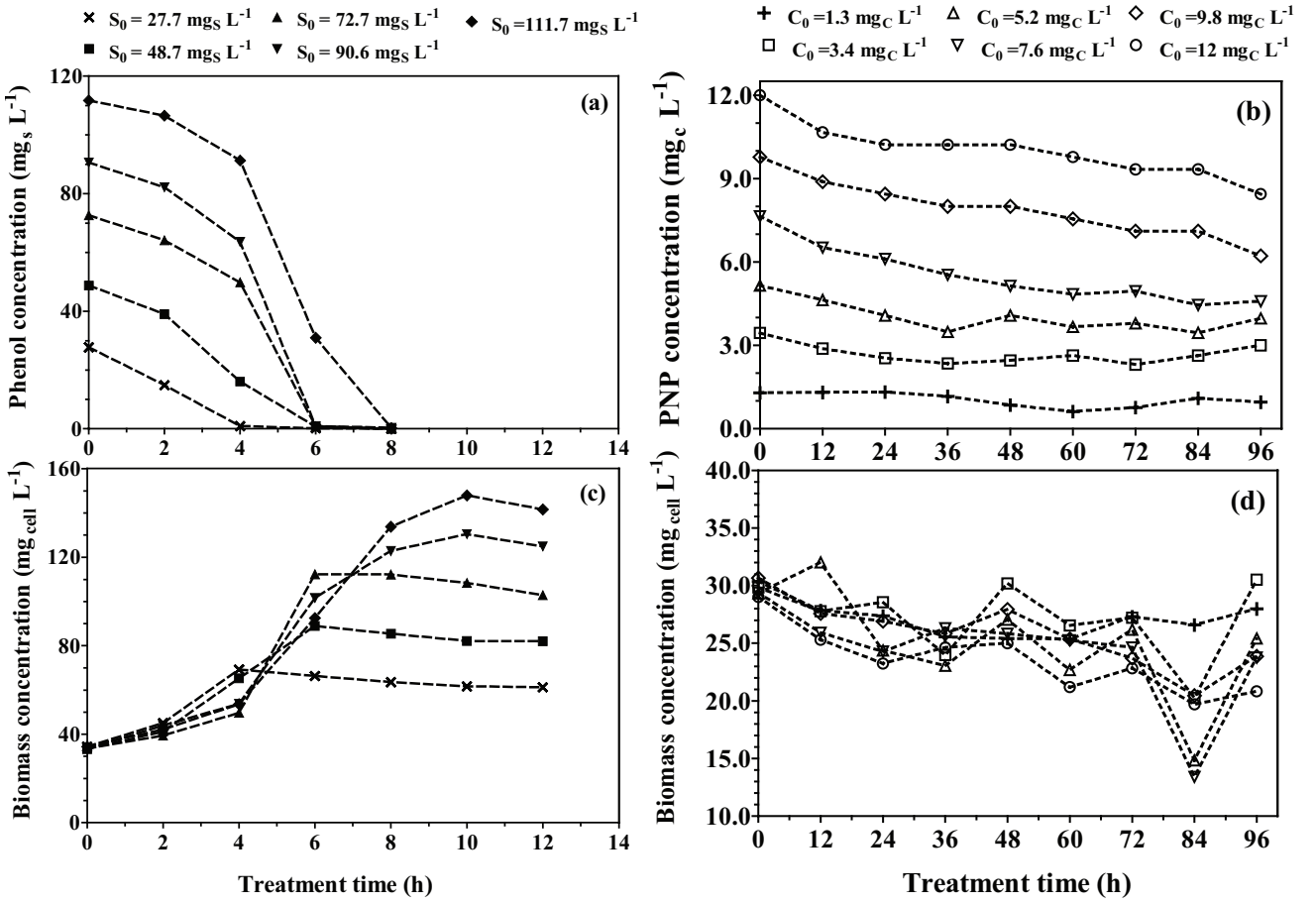

Fig. 2. Degradation of phenol (a) and PNP (b) each at different initial concentrations in flask studies using free cells of *R. eutropha*. The time changes for the formed biomass concentration are also shown ((c) and (d)).

cometabolization, then, different kinetic parameters should be determined experimentally, and weight of their presence on the model has to be evaluated quantitatively. According to the specifications given in Table 1, experiments with serial numbers of C and D were conducted, where changes of phenol, PNP, and biomass contents of the test samples taken during the treatment time were determined. Eleven test flasks were prepared by adding a constant and high level of one substrate along with 5–6 different initial concentrations of the other substrate to a medium containing microbial culture and MSS. Curves of the time dependences for the measured changes are presented in Fig. 3.

The time dependences of changes of phenol and biomass at the constant PNP concentration (Figs. 3(a) and (c)) were compared with those in which phenol was used as the single substrate (Figs. 2(a) and (c)). A comparison between lag phase period of 6 and 2 h, as revealed in Figs. 2(a) and 3(a), indicated threefold increase of the lag time when phenol, at the highest initial concentration (≈115 mg_s L^{-1}), was degraded by the test bacterium in the presence of a fixed amount of PNP. Thus, treatment time of 48 h was corresponded to almost complete degradation of phenol although it took only 8 h for complete biooxidation of phenol, when phenol was the only involved substrate and PNP was absent. Considerable increase of the lag time for the former could be interpreted as the lower possibilities of the microbial cell in producing more enzymes required for degradation of PNP as the cells biomass decrease from ≈148 mg_{cell} L^{-1} (Fig. 2(c)) to ≈93 mg_{cell} L^{-1} (Fig. 3(c)). This 37% decrease in biomass content appeared to be crucial in cometabolization process. Thus, the cells should recover themselves (producing more biomass) and recommence their activities toward degradation of PNP. Phenol feeding may be considered as an effective strategy in performing coenzyme-dependent oxidative reactions [13]. There is a positive influence of the biomass concentration and enzymatic abilities of the cells to degrade the cometabolic substrate as it is shown in Fig. 3(b), where the higher phenol and biomass concentrations resulted in higher degradation rate of PNP.

The time dependences of changes of PNP and biomass at the constant phenol concentration (Figs. 3(d) and (f)) were compared with those in which PNP was used as the sole source of carbon and energy (Figs. 2(b) and (d)). Almost no fluctuation in biomass formation was observed in the presence of phenol (see Figs. 2(d) and 3(f)). The obtained results for trend of PNP degradation in the absence of phenol was shown in Fig. 2(b), where decrease of PNP was almost constant, and the level was very low and negligible. Figs. 3(d) and (f) are indicative of enhancing effect of phenol on the formation of biomass and PNP degradation.

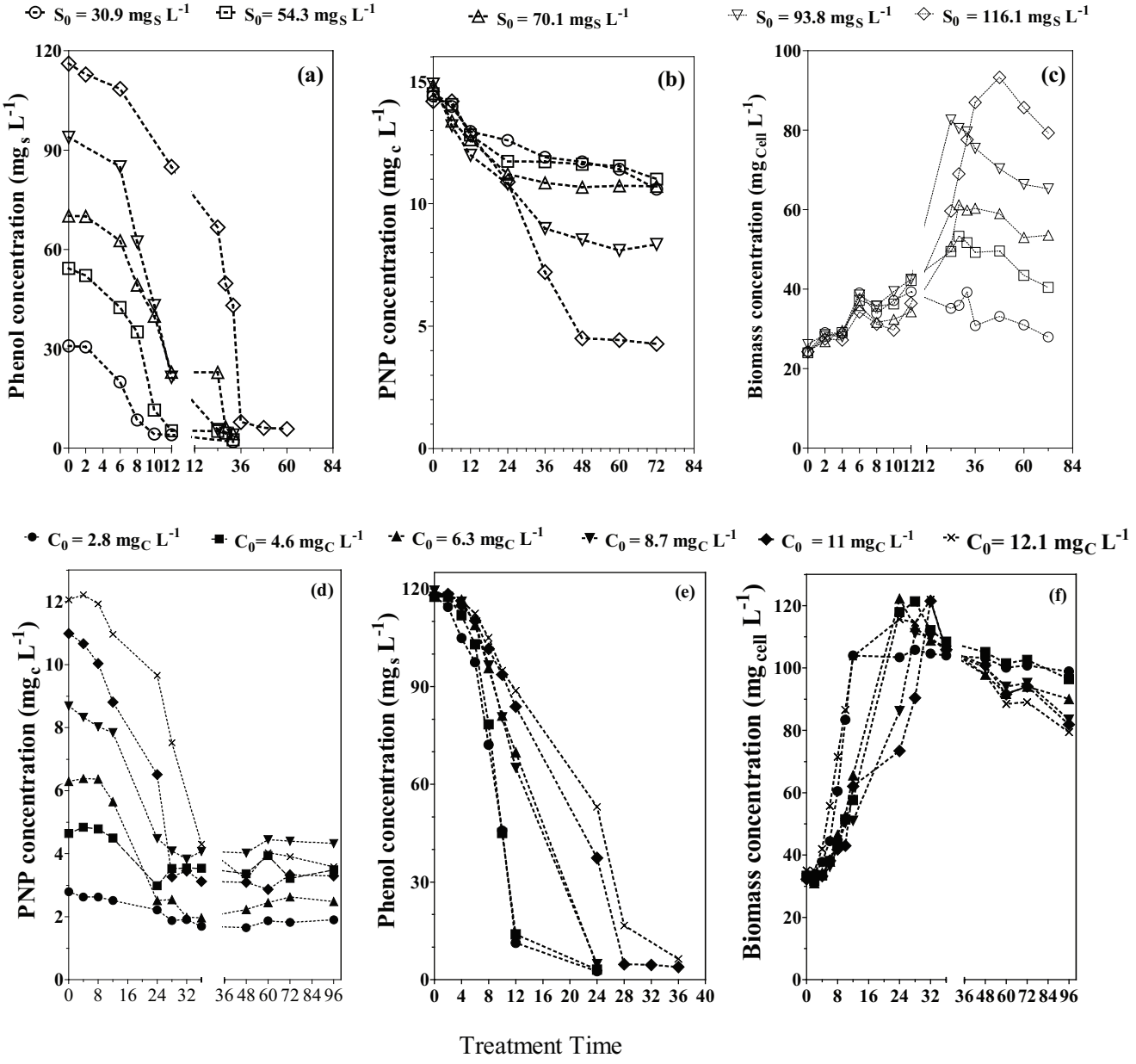

Fig. 3. Degradation of phenol (a) and PNP (b) in the presence of PNP at 14.6 mg L^{-1} initial concentration. The data of the formed biomass concentrations (c) are also shown. Cell responses to utilization of different initial concentrations of PNP in the presence of phenol at 118 mg L^{-1} initial concentration were also presented in terms of PNP, phenol, and the formed biomass concentrations ((d),(e), and (f) plots, respectively).

Cell abilities to transform the cometabolic substrate is directly related to the use of growth substrate: involvement of growth substrate in synthesis of coenzymes, induction of catabolic enzymes, and production of new enzymes (i.e., inactivation of the enzyme in response to cometabolic substrate usage) [17,32]. While, PNP negatively affected cell consumption of phenol: 36 h as the total time of phenol degradation in the presence of the highest level of PNP (≈12 mg_c L^{-1}) as compared with 8 h as the time of phenol utilization in the absence of PNP (36 h vs. 8 h). A noticeable decline in growth rate with increasing concentration of the cometabolic substrate and decreasing concentration of the growth substrate, as shown in Figs. 3(c) and (f), was previously reported in the literature [33,34].

The results of specific degradation rate for either phenol (with or without of PNP) or PNP (with or without phenol) against different initial concentrations of the relevant substrate shown in Fig. 4 are in favor of the findings described above (inhibitory and enhancing effect in substrate interactions). All the q_s values in the presence of PNP were lower when compared with the values obtained in the absence of cometabolic substrate (Fig. 4(a)). The results shown in

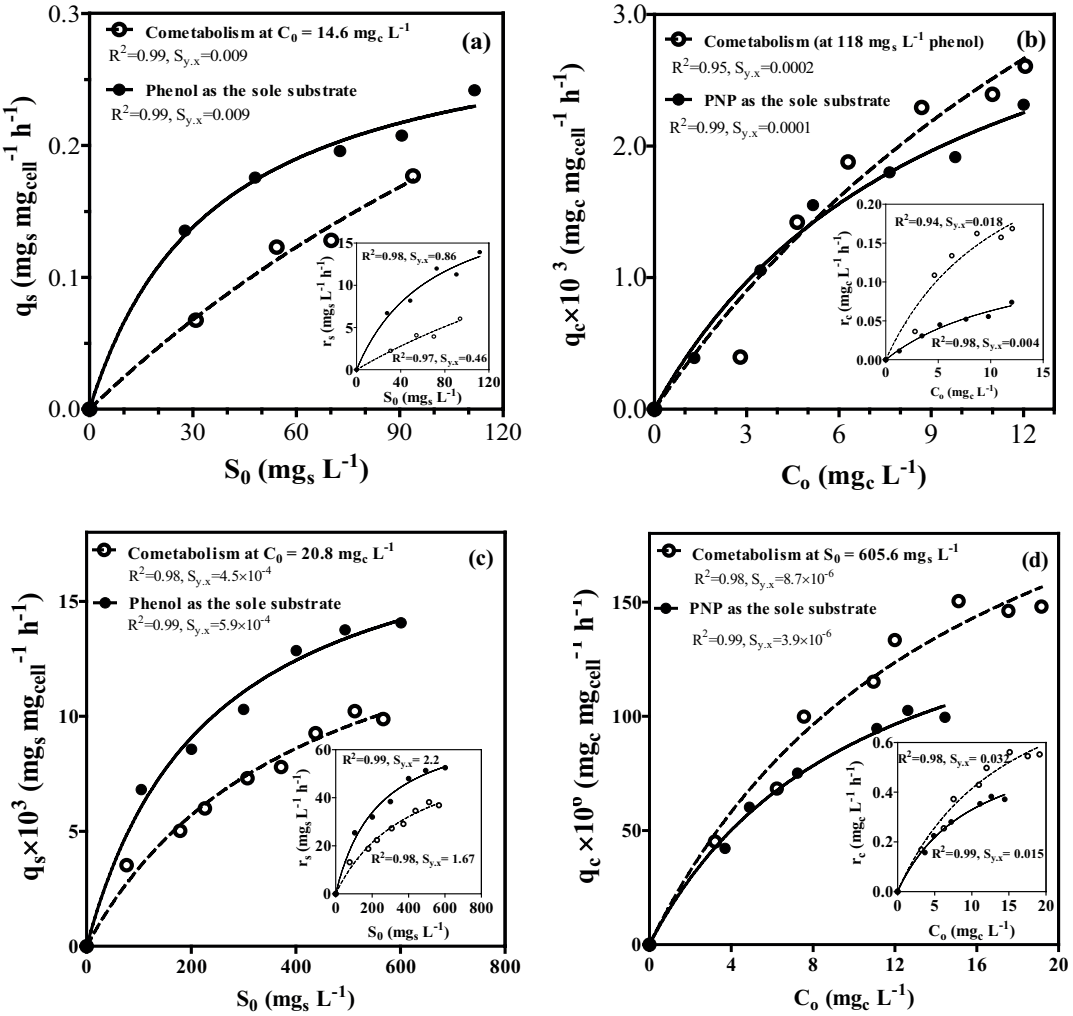

Fig. 4. Shake flask experiment specific degradation rates of the substrate either phenol (q_s) or PNP (q_c) (with or without the other substrate) as a function of the initial concentration ((a) and (b) plots for phenol and PNP, respectively). Results of the TBR experiments showing specific degradation rates of the substrate either phenol (q_s) or PNP (q_c) (with or without the other substrate) as a function of the initial concentration are also shown ((c) and (d) plots for phenol and PNP, respectively). Data for volumetric degradation rates in each case are presented as inset(s).

Fig. 4(b) are indicative of the enhancing role of phenol on PNP utilization by the cells. Based on nonlinear regression technique used here, the experimental data were matched to the values predicted by model. To determine kinetic parameters of simultaneous degradation of phenol and PNP, first, the least squares method was used to estimate values of $(K_s)_{obs}$, $(K_c)_{obs}$, and $(k_c)_{obs}$. Then, according to the data obtained in single substrate experiments (see Table 1 for A and B treatments and Eqs. (10)–(12)), the values of K_{is}, K_{ic}, and T_y were calculated. Additional note on findings of these experiments was to determine transformation yield as the amount of cometabolic substrate consumed per amount of growth substrate used by the cells. Comparison between K_{ic} and K_{is} (determined through experiments with serial numbers of C and D in Table 1) revealed that value of K_{ic} was considerably lower than the K_{is} value (Table 2). According to the competitive enzyme inhibition concept, the lower the value of K_i, the greater is the degree of inhibition. Thus, PNP inhibitory action on phenol utilization rate by *R. eutropha* was greater than that of the phenol action on PNP utilization rate by the test bacterium. The K_{ic} and K_{is} findings reported here are not in agreement with the cometabolization results reported by Chang and Criddle [15] on trichloroethylene (TCE) degradation by a methanotrophic mixed culture and also on utilization of 4-chlorophenol (4-CP) by *P. putida* using phenol as the growth substrate in the presence of sodium glutamate (SG) [32] (Table 2). In the latter study on ternary substrate system (phenol, 4-CP, and SG), SG was inhibitory to phenol, but SG did not express inhibitory function to 4-CP. Besides, SG was able to decrease the toxicity of 4-CP to phenol [32]. When one considers the ratio of amount of 4-CP degraded to amount of phenol degraded, the comparisons of T_y values would be interesting. The ratio is higher in the presence of SG than

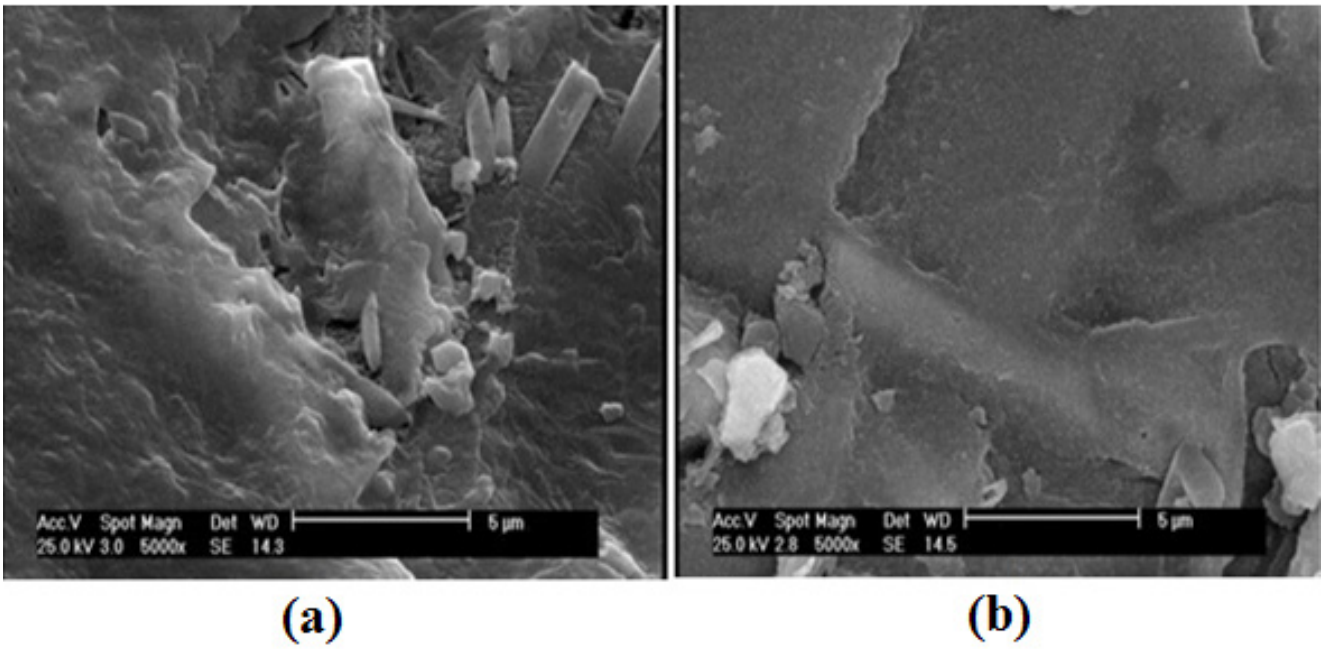

Fig. 5. SEM micrographs of the Kissiris-immobilized *R. eutropha* (a) and the bare Kissiris pieces (b) at magnification of 5,000×.

that in the absence of this substrate. The condition is different in the present study: no third substrate was included. In fact, addition of SG as an amino acid derivative seems to change the nitrogen control signals in microbial cells, and treatment of the growth culture should be considered either under nitrogen excess or nitrogen limitation conditions [35]. High value of T_y reported for simultaneous degradation of TCE and methane appears to be related to the mixed culture used as methanotrophic bacteria, which were derived from an aquifer material [15]. It is noteworthy to compare the use of complex MSS in those two experiments with the simple formulation used in the present study.

3.4. TBR studies

Microbial cells attachment to bed of packed material and biooxidation reactions are the major sequence of events in trickling biological filter extensively used for the treatment of real wastewaters, where long duration of the liquid feeding along with system's aeration create considerable differences in concentrations of mixed substrates and in densities of mixed microorganisms [36]. These complexities, although, are the cause of some real difficulties that periodically appear in efficient handling of the real system, but the depth of these problems in the present study with the use of single pure culture adapted to phenol oxidation would not be considerable. Thus, it is reasonable to assign the cause of obtaining low q_c and q_s values in the TBR to the excessive biomass immobilized throughout the Kissiris pieces, restricting availability and diffusion of the nutrients including air, within the biofilm and the bed from top to bottom. The role of the involvement of the formed biomass in the aforementioned limitations is confirmed by comparing the results in terms of relationship expressed between volumetric- and specific-degradation rate (r and q, respectively). It is noteworthy to compare the value of DCW in TBR as 3,731 mg_{cell} against ≈100 mg_{cell} L^{-1} in the flask studies (Fig. 4). Additional note is on the results of simultaneous degradation of phenol and PNP by Kissiris-immobilized cells (TBR studies), which were similar to those obtained in shake flasks experiments in terms of substrate interactions (enhancing effect of phenol on PNP utilization and inhibitory influence of PNP on phenol degradation) (see Fig. 4).

The values of the kinetic parameters in shake flask and TBR studies are summarized in Table 2 and are compared with the values determined in some other studies in the scope of cometabolism.

Dense growth of *R. eutropha* cells on Kissiris pieces in TBR is clearly observed through SEM micrographs presented in Fig. 5 (the images were obtained at the end of the TBR experiments).

Fig. 6 shows the plots of experimental vs. model predicted values for the biokinetic coefficients. Measurements of the formed biomass during the flask study considering high initial concentrations of phenol showed marked fluctuations, and the points in the resulting plot deviated greatly from diagonal. Morphological changes occurring in response to the utilization of growth substrate (with or without cometabolic substrate) have not been considered in the suggested model (Figs. 6(a) and (c)). However, in TBR study, the biomass measurement was made at the end of the experiments, and expectedly, no fluctuation was experienced in the data collection. Accordingly, the deviations in the relevant parity plots are insignificant (Figs. 6(d) and (e)). It is worth mentioning that low deviations observed in Fig. 6(b) would be interpreted in terms of K_{is}, which was calculated as higher value than K_{ic}, and the contribution of both K_{is} and K_{ic} values to half saturation constants (K_s and K_c) in Eqs. (1) and (2) should be considered in this regard.

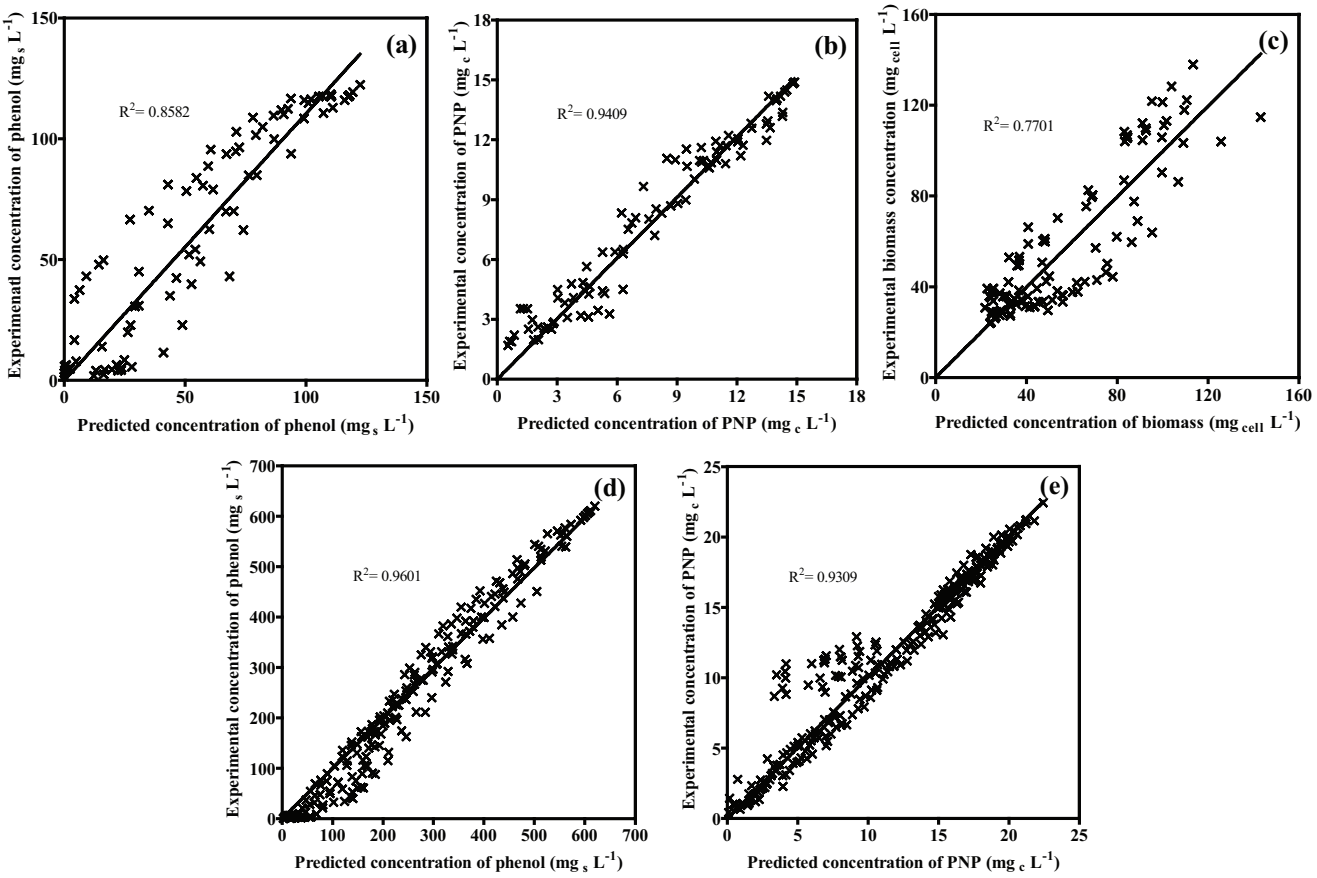

Fig. 6. Comparison of the experimentally obtained values vs. the model predictions for the two sets of experiments: shake flask ((a), (b), and (c)) and the TBR ((d) and (e)).

4. Conclusions

The behavior of *R. eutropha* was examined kinetically in simultaneous utilization of phenol and PNP. The TBR and flask studies were both conducted. A mathematical model was used in describing the events with consideration of saturation kinetics (k_s, K_s, k_c, and K_c) and with biokinetic parameters about product toxicity (T_c), competitive inhibition (K_{ic} and K_{is}), cell growth and decay (Y_m and b), and transformation yield (T_y). Model-predicted values for biokinetic parameters were satisfactorily reflected in the experimental data.

With respect to the initial concentration of phenol, when this compound was used alone, the test bacterium would efficiently degrade this substrate after passing a certain lag phase. But, when PNP was the only substrate present in the culture medium, low amount of this cometabolic substrate was degraded within the treatment time, and no cell growth was observed despite significant fluctuations seen for the biomass formation process. The necessity for the induction of certain enzymatic pathways in the presence of PNP without noticeable utilization of this substrate could lead to biomass fluctuations. The presence of phenol in the culture medium positively affected PNP degradation, and more biomass was produced (flask studies). This behavior was not observed in the TBR studies, and degradation of PNP was noticeable, although not complete, within the treatment time. Actually, the inhibitory effect of PNP on degradation rate of phenol by *R. eutropha* cells was greater than inhibitory function of phenol on PNP degradation rate, and these behaviors were observed for free cells as well as immobilized cells in the TBR ($K_{ic} < K_{is}$). Excessive biomass growth on Kissiris pieces in the TBR reduced available free spaces for passing air and liquid through the bed and restricted accessibility of the cells to nutrients such as oxygen. The probability of inactive biomass presence could increase, and therefore, the values for specific degradation rates for both of the substrates in the TBR were lower than those attained for free cells.

Despite extensive studies on the cometabolic degradation of halogenated aliphatic hydrocarbons reported in literature and although there exists substantial information about the mechanism of PNP utilization by gram positive and gram negative bacteria, only few studies on cometabolic degradation of PNP using phenol as the growth substrate have been reported. This is of great importance when it comes to the kinetics modeling, and difficulties observed in experimental validation of the assumptions used in modeling process [16,37]. In the present study, the validity of using biokinetic coefficients obtained through modeling process was evaluated by employing TBR [38].

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Symbols

b	—	First-order endogenous decay constant, h^{-1}
C	—	Concentration of cometabolic substrate, $mg_c\ L^{-1}$
C_0	—	Initial concentration of cometabolic substrate, $mg_c\ L^{-1}$
C_∞	—	Ultimate concentration of cometabolic substrate at $t = \infty$, $mg_c\ L^{-1}$
k_c	—	Maximum specific rate of utilization of cometabolic substrate, $mg_c\ mg_{cell}^{-1}\ h^{-1}$
$(k_c)_{obs}$	—	Maximum specific rate of utilization of cometabolic substrate in the presence of growth substrate, $mg_c\ mg_{cell}^{-1}\ h^{-1}$
k_s	—	Maximum specific rate of utilization of growth substrate, $mg_s\ mg_{cell}^{-1}\ h^{-1}$
K_{ic}	—	Cometabolic substrate's inhibition coefficient indicating the effect of cometabolic substrate concentration on growth substrate utilization rate, $mg_c\ L^{-1}$
K_{is}	—	Growth substrate's inhibition coefficient indicating the effect of growth substrate concentration on cometabolic substrate utilization rate, $mg_s\ L^{-1}$
K_c	—	Half-saturation coefficient of cometabolic substrate, $mg_c\ L^{-1}$
$(K_c)_{obs}$	—	Half-saturation coefficient of cometabolic substrate in the presence of growth substrate, $mg_c\ L^{-1}$
K_s	—	Half-saturation coefficient of growth substrate, $mg_s\ L^{-1}$
$(K_s)_{obs}$	—	Half-saturation coefficient of growth substrate in the presence of cometabolic substrate, $mg_s\ L^{-1}$
q_c	—	Specific rate of utilization of cometabolic substrate, $mg_c\ mg_{cell}^{-1}\ h^{-1}$
q_s	—	Specific rate of utilization of growth substrate, $mg_s\ mg_{cell}^{-1}\ h^{-1}$
r_c	—	Volumetric rate of utilization of cometabolic substrate, $mg_c\ h^{-1}$
r_s	—	Volumetric rate of utilization of growth substrate, $mg_s\ h^{-1}$
S	—	Concentration of growth substrate, $mg_s\ L^{-1}$
t	—	time, h
$(T_c)_{obs}$	—	$-q_c/\mu$, observed transformation capacity, $mg_c\ mg_{cell}^{-1}$
T_c	—	Theoretical transformation capacity in the absence of endogenous decay, $mg_c\ mg_{cell}^{-1}$
T_y	—	Theoretical transformation yield, $mg_c\ mg_s^{-1}$
X	—	Active organism concentration, $mg_{cell}\ L^{-1}$
X_0	—	Initial concentration of active organism, $mg_{cell}\ L^{-1}$
Y_m	—	Maximum yield or true growth yield, $mg_{cell}\ mg_s^{-1}$
Y	—	Observed growth yield, $mg_{cell}\ mg_s^{-1}$

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