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Modeling of *p*-nitrophenol biodegradation by *Ralstonia eutropha* via application of the substrate inhibition concept

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ABSTRACT

In this study, the capability of *Ralstonia eutropha* H16 to degrade *p*-nitrophenol with or without a supplementary substrate (glucose or yeast extract) was investigated. Using PNP as the sole energy and carbon source, the biodegradation behavior of the bacterium was modeled by applying a modified form of the Monod equation that considers substrate inhibition, as suggested in the literature ($\mu = (\mu_m S/k_s + S)(1 - (S/S_m)^n)$). PNP at a 6 mg/L initial level was degraded within 20 h under the defined incubation conditions (shaking at the reciprocal mode, pH 7 and temperature of 30 °C) however the biodegradation was enhanced when yeast extract included in the test medium (50% reduction in the time for complete degradation). When glucose was used instead of yeast extract in the test medium *R. eutropha* growth was not supported by this carbohydrate and PNP was degraded in about 14 h indicating degradation time reduced by 1/3. Comparison of *R. eutropha* growth pattern showed that biomass formation was insignificant when the bacterium grew in the test medium containing only PNP or PNP plus glucose. But by use of yeast extract considerable biomass formation was observed (OD_{546} = 0.35 versus 0.1). The presence of organic pollutants in natural ecosystems at low levels frequently occurs in form of mixture with other compounds. The findings of the present work were discussed in terms of secondary substrate utilization for *R. eutropha* at low PNP level.

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1. Introduction

Commercial production of various compounds such as pesticides, herbicides, explosives, dyes and plasticizers depends on use of *p*-nitrophenol (PNP) [1], which has a severe impact on environmental pollution (air, soil and ground water). PNP has been classified as a priority pollutant by the US Environmental Protection Agency (EPA), and its acceptable concentration in natural waters has been set at levels below 10 ng/L [2].

The capability of certain groups of bacteria (*Psedumonas*, *Arthobacter*, *Burkholderia*, *Trichosporon*, *Rhodococcus*) to degrade nitroaromatic compounds has been documented in the literature [3–7]. In spite of complexity of these types of phenomena as related to microbe's growth, often a single enzymatic reaction having a simple expression is used to describe the overall rate of growth. However simple model such as Monod kinetics for growth may fail in some cases for instance when inhibitory effects are due to excess of a particular substrate [8]. The relationship between substrate and μ during biological adaptation of the microorganism to an organic pollutant have been quantified by use of several mathematical models which are generally adaptations of equations derived from theories on the inhibition of a single enzyme (i.e., action of a single inhibitor) [9]. Generalization of Monod kinetics for analysis of microbe growth data considering substrate inhibition has been found to be a suitable approach for treating the data; however, drawbacks exist among the suggested kinetics models. For instance, Haldane kinetics are described by the following equation: $\mu = (\mu_m S/(k_s + S)(1 + (S/k_i))$, where the model implies indefinite growth for the cells, and importantly, it implies that, in reality, a definite substrate concentration exerts a limit above which microbe growth will cease [9]. Based on these and other kinetic models such as linear, exponential and Tissier, Levenspiel proposed a nonlinear equation as follows: $\mu = (\mu_m S/k_s + S)(1 - (S/S_m)^n)$, where μ is the microbial growth rate constant, μ_m is the maximal constant, k_s is the empirical Monod constant and S_m is the maximum substrate concentration above which growth is completely inhibited. The relationship between μ and substrate concentration (S) is thus described by a constant termed '*n*' whose magnitude indicates the type of the relationship: for $n > 1$, a nonlinear plot of μ/μ_m ratio versus S/S_m shows 'upward concavity,' while the plot for $n < 1$ shows 'downward concavity' [9]. A sharp increase in the accumulation of a substrate results in a turning point of substrate accumulation that

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extrapolates almost nearly to the maximum growth rate (μ_m), and the inhibitory effect of the accumulated substrate is seen thereafter [10].

Moreover, the presence of a readily metabolizable compound as a mixture with the organic pollutant may enhance the biodegradation of the test substrate (secondary substrate utilization) [11]. This situation occurs frequently in natural ecosystems which are carbon-limited environments where varieties of organic substances are present at low concentration. This condition therefore is advantageous to organisms whose genetic responses (pollutant degradative ability) are meaningful both from ecological context as well as application in the waste treatment operation within reasonable time. For instance *Pseudomonas* sp. ability in degrading PNP in presence and absence of glucose has been tested [11]. Pseudomonas sp. was unable to mineralize PNP at 10 g/mL in the presence of glucose at 20 μg/mL however, PNP at concentrations of 50 ng/mL or higher levels was biodegraded and this enhanced degradation was found to be the result of the simultaneous use of glucose and PNP and increased rate of the growth of *Pseudomonas* sp. on glucose [11].

The objective of the present work was to study the biodegradative ability of *R. eutropha* towards PNP when this energy-deficient compound was used as the sole energy and carbon source with or without presence of a supplementary substrate such as glucose or yeast extract in the test medium. Moreover, by using the generalized form of the Monod equation as described by Luong [9], biodegradation was modeled with consideration of substrate (PNP) inhibition kinetics.

2. Materials and methods

2.1. Microorganism and cultivation medium

R. eutropha was supplied by Japan Collection of Microorganisms (JCM) (accession number of strain: JCM 20644 in 2007). The growth medium (liquid culture 'LC' medium) was prepared as described elsewhere [12].

2.2. Chemicals

PNP was provided by Kanto Chemical Company (Japan). All chemicals used in this study were of analytical grade with the highest purities.

2.3. Analytical methods

HPLC analysis was used to determine PNP concentration [Shimadzu HPLC system equipped with an Inertsil ODS-3 V reverse phase column and a UV detector at 280 nm; acetonitrile and $NaClO_4$ (pH adjusted to 2.5) were the system solvents for this measurement]. Analysis was done on the cell-free supernatants obtained after centrifugation at 12000 rpm for 20 min. Cell growth measurement was performed spectrophotometrically at 546 nm.

2.4. Preparation of the inoculum for the adaptation study

For *R. eutropha* adaptation, the liquid culture medium (LC) containing PNP at some specified initial concentration was used. Whenever necessary, glucose, peptone and yeast extract were added to the LC medium (sterilization of the medium was done in an autoclave at 121 °C for 15 min). Each of these three components was removed in successive experiments to provide PNP-adapted *R. eutropha* (Table 1). Addition of 0.5 g/L yeast extract to the LC medium was found to be necessary after repeated transfer of *R. eutropha* inoculum (9 times). With PNP at an initial concentration of 13 mg/L, the recorded time for complete degradation of PNP was 14 h (i.e., no PNP was detected through HPLC analysis). For the biodegradation study, 5 mL of the latter grown *R. eutropha* culture was transferred to freshly prepared LC medium (50 mL) containing PNP at a pre-determined concentration. Yeast extract at 0.25 g/L was added to the LC medium (pH 7, incubation conditions: shaking at the reciprocal mode, temperature of 30 °C for 20 h). The inoculated culture grew and was centrifuged (2700 rpm, 20 min), and the sediment was used to prepare a *R. eutropha* cell suspension.

3. Results and discussion

3.1. Biological adaptation

In most of studies on PNP removal from the environments by microbes which are primarily responsible for this event, there are lag phases up to even 6 weeks and thereafter the PNP degraders perform faster. No lag phases were observed upon subsequent the microbe's exposure to PNP [6]. In this study biological adaptation processes were performed stepwise on the basis of using PNP as the main substrate and inclusion of the test supplementary substrate as needed, to examine PNP degradative capability of *R. eutropha*. Effects of these secondary substrates on the growth of the bacterium were evaluated through successive inoculum transfer operations and the focusing point was on developing a growth medium containing PNP with the lowest amount of each of these test substrate while the growth would be determined as a function of a single variable of PNP. As it is seen in Table 1 proper growth of *R. eutropha* along with PNP degradation was observed in the absence of glucose and peptone and at decreased levels of yeast extract (i.e., 80% reduction in the initial concentration of yeast extract). The adaptation protocol thus provided a growth medium without any noticeable source of carbon, while nitrogen was supplied mainly in the form of yeast extract and no peptone was present in this

Table 1
Results obtained from the bacterial adaptive study (see text for details).

Initial PNP concentration (mg/L)	Treatment specification (gr/L)			Time for complete degradation of PNP (h)
	glucose	peptone	yeast extract	
15	3	2	2	16
41	2	1	1	44
63	2	1	1	90
52	2	1	1	42
37	1	1	1	48
39	0.5	1	1	72
24	0.25	1	1	48
15	0	1	1	48
41	0	1	1	36
13	0	0	0.5	14
13	0	0	0.25	20

medium composition (Table 1). In fact changes in the expression of various genes are in progress for the microbe's adaptation to a highly specialized environment, i.e., the presence of an organic pollutant (PNP). In general adaptation is involved in synthesis of new types of protein [13].

3.2. Kinetics of PNP biodegradation

Progress curve of PNP degradation and time course of *R. eutropha* were shown in Fig. 1. Complete degradation of PNP occurred in less than 10 h when yeast extract as the only supplementary substrate was included in the LC medium. The metabolic strategies of organisms are influenced not only by the concentration of pollutant but also by the character of variation of its concentration. The growth pattern was monitored using the same medium compositions and a prompt increase in absorbance is also seen (Fig. 1). Flagellated bacteria possess a temporal gradient sensing system, which is a type of memory device provides the cell ability to compare present and past concentrations of the chemical of interest over a short interval of time [4]. Several studies have concentrated on chemotaxis behavior of *R. eutropha* a well-known flagellated bacterium. This process of sensation and response of the motile bacteria to low levels of organic compounds was not quantitatively evaluated in the present work. Every usable source of energy for the bacterium is not necessarily an attractant [14]. In fact *R. eutropha* showed little growth upon cultivation on a medium containing PNP with or without glucose and in the absence of yeast extract and PNP removal efficiency decreased by 33% when the growth medium contained only PNP (Fig. 1A and B). *R. eutropha* is chemotactic to PNP with chemotaxis index (CI) of 3, this response is evaluated for different nitroaromatic compounds at their optimum concentration of 200 μM with CI's ranged from 3 to 16 fold [15]. PNP concentration used in the present study was less than 200 μM and further works are needed to describe dependency of chemotaxis behavior of *R. eutropha* to PNP levels with or without any supplementary substrate.

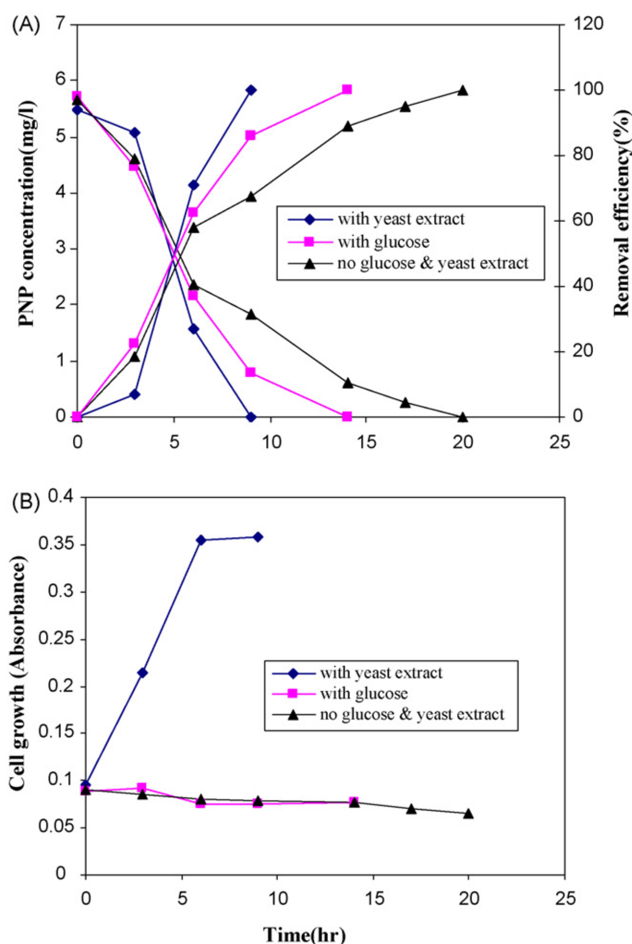

Fig. 1. Progress curve of PNP biodegradation (A) and time course of *R. eutropha* growth (B) (initial PNP concentration: 6 mg/L).

To describe the inhibitory effect of PNP in quantitative terms (to obtain a description from the kinetic aspect), it is important to assess the inhibition along with the growth of the test organism. This approach which was introduced in an ethanol fermentation study (the inhibitory effect of ethanol on the performance of *Saccharomyces cerevisiae*) had considerable impact on related research subjects thereafter. With this concept in mind, in the present work, the level of PNP that could not be degraded by *R. eutropha* within a reasonable incubation time was defined as the PNP limiting substrate concentration. Based on the results obtained from the adaptation process, 24 h or less was considered as a reasonable incubation time for observing the degradative ability of *R. eutropha* towards PNP. Efficient degraders of PNP in nature (soil, sediment, groundwater) are found to have lag phases ranging from 2 to 24 days, which is indicative of either the time required to select PNP degraders among other present organisms or the time required for adaptation of PNP-degrading bacteria [16–18]. As mentioned above, Levenspiel proposed a generalized nonlinear equation to describe product inhibition in the ethanol fermentation process [19]. Luong suggested that the product inhibition term could be replaced by a substrate inhibition term similar to one that has actually been used in enzymatic reactions [9]. In the following equation, $\mu = (\mu_m S/k_s + S)(1-(S/S_m)^n)$, when the exponent '*n*' is equal to zero, a general growth equation is obtained (Monod equation). In the present work, the time course of PNP biodegradation was recorded when the initial level of PNP was set at about 6 mg/L, and by considering a general stoichiometric equation:

$$\text{substrate (PNP)} \xrightarrow{\text{microorganisms}} \text{products} \quad (1)$$

and following the biodegradation pattern shown in Fig. 1, an autocatalytic reaction kinetic was assumed to prevail [19]:

$$-\frac{dC_p}{dt} = kC_pC_c \quad (2)$$

where C_p and C_c are the concentration terms for PNP and the microorganism, respectively, and k is the second-order rate coefficient. A Monod-type equation was applied next (the exponent '*n*' in the Luong model was assumed to be zero, and the term with the exponent was dropped, reducing the suggested Luong model to the Monod equation):

$$\frac{dC_c}{dt} = \frac{\mu_{\max} C_c C_p}{K_c + C_p} \quad (3)$$

where K_c is the Monod constant and $\mu_{\max}$ is the maximum specific growth rate. By dividing Eq. (2) by Eq. (3) and integrating the resulting term, the following expression is obtained:

$$\frac{C_c}{C_{c_0}} = 1 + \frac{\mu_{\max}}{kC_{c_0}} \ln \frac{K_c + C_{p_0}}{K_c + C_p} \quad (4)$$

Table 2
Kinetic parameters.

Kinetic parameter	Value
K_c (dimensionless)	2.37 ± 0.33
k (h^{-1})	(1.136 ± 0.125) × 10^{-2})
$\mu_{\max}$ (h^{-1})	0.16118 ± 0.03

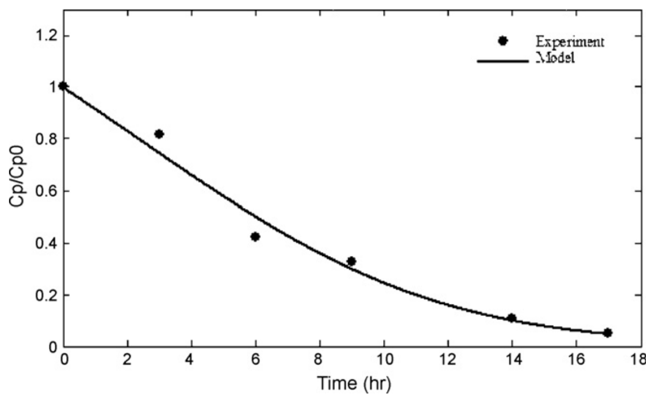

Fig. 2. Comparison between the experimental data and model calculations based on Eq. (5) for the degradation of PNP by *R. eutropha.*

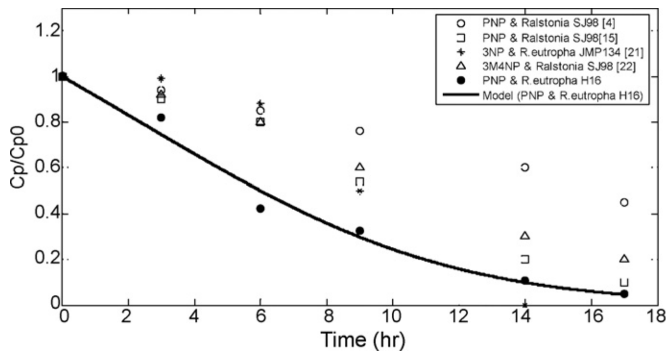

Fig. 3. Comparison made between the results of the present work and the data reported in the literature.

where C_{c_0} and C_{p_0} are the initial concentrations of bacteria and PNP, respectively. By replacing C_C from Eq. (4) in Eq. (3) followed by some arithmetic manipulations, the following equation can be obtained to describe the rate of PNP conversion as a function of only PNP concentration, initial concentration of cell and PNP:

$$\frac{dC_p}{dt} = \mu_{\max} C_p \ \ln\left(\frac{K_c + C_p}{K_c + C_{p_0}}\right) - kC_{c_0} C_p \quad (5)$$

In the above equation three kinetic parameters, $\mu_{\max}$, k, K_c should be determined using the experimental variation of PNP against time. The initial PNP concentration of the experiments was taken about 6 mg/L ($C_{p_0} = 5.64 \pm 0.79$), the initial cell concentration was kept around 74 mg/L ($C_{c_0} = 74.12 \pm 8.25$) and variation of PNP was measured versus the time at a constant temperature of 30 °C and the solution pH of 7. The kinetic parameters were evaluated by a nonlinear least square error technique [20] using the experimental results. The optimized kinetic parameters and their standard errors are shown in Table 2, and the coefficient of determination (R^2) of this model is calculated as 98.3%. Fig. 2 shows a comparison between data obtained experimentally at a temperature of 30 °C and pH 7 and the values predicted by Eq. (5). According to the coefficient of determination, the applicability of the kinetic model to PNP biodegradation was reasonably good.

Fig. 3 shows a comparison between the results of the present work and the data reported in the literature [4,15,21,22]. The exhibited literature data show lower rate of substrate consumption in which the reason may arise from using a particular strain of *Ralstonia* and different molecular structure of the test pollutant.

4. Conclusions

The capability of adapted *R. eutropha* for PNP biodegradation was studied using a simple protocol, showing the necessity of performing biological adaptation to provide appropriate growth conditions for the bacterium in treating and organic pollutant. The presence of yeast extract in the test medium was found to be necessary for complete PNP degradation by the adapted bacterium in a reasonably short period of time. For instance PNP at a 6 mg/L was degraded within 20 h (no residual PNP was detected) while including yeast extract in the test medium reduced the time of complete degradation by half and considerable growth was observed. A kinetic model for PNP biodegradation was suggested based to a model presented in literature.

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