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Kinetic studies on mineralization of *p*-nitrophenol by *Pseudomonas putida* at low and high concentration

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Pseudomonas putida isolated from ecological habitat, has been evaluated for its potential to mineralize high (2.143 mM) concentration of *p*-nitrophenol (PNP). The bacterium utilized PNP as the sole source of carbon, nitrogen and energy. Its half saturation constant (K_s values) of PNP degradation was 0.22 mM, as determined by Michaelis- Menten curve. Further, its maximum rate of PNP degradation as per Lineweaver-Burk plot was 4.5 μmol $min^{-1}g^{-1}$ dry biomass L^{-1}. Its kinetics of growth in minimal medium containing low and high PNP concentrations was Monod type of curve. Various metabolic parameters like specific growth rate (μ), specific PNP uptake rate and specific degradation rate at initial concentrations of 0.143, 0.2143, 0.357, 0.714, 1.43, 2.143 and 3.75 mM PNP were determined.

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Nitrophenolic compounds are used as chemical feedstock for synthesis of solvents, explosives and azo dyes, fungicides, insecticides, herbicides and pharmaceuticals[1]. An indiscriminate use of nitrophenolics in the past due to its wide range of applications has naturally resulted in inexorable environmental pollution. Secondly, nitro group of nitrophenolics renders oxidative attack by microbes difficult. Besides this, upon entry into biosphere, nitrophenolics are reduced to more reactive and carcinogenic derivatives. Intestinal microflora catalyses the conversion of nitro groups to nitroso- and hydroxylamino-groups. Therefore, epidemiological data has considered nitrophenolics as carcinogens[2]. Hence, nitrophenols are recognized as priority pollutants and given High Risk-3 rating by US-EPA[3]. Of these, *p*-nitrophenol (PNP) has been recognized as a model compound for studying various aspects of nitroaromatic degradations. The half-life of PNP in soil and subsoil is up to 10-40 days under aerobic conditions[4]. Several studies[5-7] have accorded focus mainly on metabolic pathways, enzymes and degradation profile at low concentration (20 mgL^{-1}). However, no effort has been made yet on selective performance and kinetic behaviour of microbes at higher concentration of PNP. These studies are essential to (i) generate biodegradation data for devising effective pollution strategy, (ii) evaluate residual persistence of pollutant, (iii) assess effects of its exposure on living things and (iv) assess its potential risk to ecosystem. Such findings will have both, practical and ecological significance. Towards these objectives, present study has attempted to find out (i) the kinetics of biodegradation by *P. putida* at higher PNP concentration, (ii) mineralization parameters and potential and (iii) kinematic behaviour of a potential trait.

Experimental Procedure

Bacterial strain

Bacterial strain used was an isolate from effluent-sediment collected from a local pesticide industry in the vicinity of Jalgaon. It was identified using an array of biochemical tests, API system and 16s rRNA analysis as *P. putida*[8]. The strain was maintained on mineral salt glucose (MSG) medium containing PNP (20 mg L^{-1}) at 4°C.

Chemicals

PNP was obtained from Sigma Chemicals, USA. All other chemicals used were of highest purity grade.

Culture medium and conditions

Mineral salt (MS) medium (*p*H 7.0 ± 0.5) contained: K_2HPO_4, 0.75; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.09 and $FeSO_4$, 0.06 gL^{-1}. It was supplemented with filter-sterilized (0.45 μm, septrane membrane filter) PNP at various concentrations

(0.143, 0.214, 0.357, 0.714, 1.43, 2.14 and 3.75 mM). MS medium supplemented with yeast extract (0.5 gL^{-1}) was incubated aerobically on a rotary shaker (120 rpm, 30°C) to obtain biomass. The cells were harvested, washed twice and used for assay.

Measurement of mineralization

Mineralization of PNP in pure culture was measured by following the release of CO_2 from PNP[9]. Accordingly, (i) 20 mL of MS medium containing 2.214 mM of filter sterilized PNP was added in pre-sterilized glass bottles (each of 60 mL capacity in triplicate), (ii) the medium was inoculated with washed cells of *P. putida* to a final density of 0.5 O.D. (0.2 g dry weight L^{-1}) (1×10^7 cells/mL) at A_{600}, (iii) the bottles sealed with rubber cork and aluminum seal were kept on a rotary shaker (120 rpm, at 30°C), (iv) uninoculated control bottle was similarly treated, (v) samples (0.5 mL) were withdrawn for CO_2 analysis from the head space of the bottle at an interval of 10 h using a sterile gas syringe, (vi) liberated CO_2 was analyzed on porapack Q column by GC analyzer (NUCON-5765) using TCD detector, at oven temperature 60°C and injector/detector temperature at 70 and 100°C, respectively, with hydrogen gas as a carrier and (vii) mineralization of PNP was qualitatively determined.

Kinetic studies

For kinetic studies of PNP degradation by *P. putida*, its whole cells were used as an enzyme system and studies carried out as per Bhushan *et al*[10].

Mineralization period was optimized by monitoring progress of PNP degradation by *P. putida* at various time intervals. For this purpose, minimal medium supplemented with 0.5 mM PNP, 1.0 g dry biomass L^{-1} at 30°C (150 rpm) was employed. Samples were withdrawn from the medium at 5 min interval for residual PNP determination.

To study the effect of biomass concentration, pre-grown cell biomass, harvested by centrifugation, washed twice, was added in varying quantities (0.54-6.0 g dry weight L^{-1}) to minimal medium supplemented with PNP (0.5 mM) for a constant (10 min) reaction time and the corresponding dry weights of biomass were determined.

Similarly, effect of varying concentrations of PNP (0.1-1.0 mM**)** was studied for constant (10 min) reaction time and using 1.0 g dry weight biomass L^{-1}. The values of half saturation constant (K_s) and rate of PNP degradation were determined from the Michaelis-Menten and L.B plot, respectively. The rate of PNP degradation was calculated as µM of PNP degraded min^{-1} g^{-1} dry weight of biomass L^{-1}.

To study kinetic behaviour of *P. putida* at yet higher PNP concentration, cells were initially acclimatized for growth in minimal medium containing varying concentrations of PNP (0.143-3.75 mM). Biomass accumulation and PNP degradation under aerobic conditions were monitored at various time intervals. The data was analyzed in terms of specific growth rate (h^{-1}), growth yield (Yx/s), specific PNP uptake rate (q_s) and degradation rate (K_s).

Analysis

PNP was determined as per Leung *et al*.[11] and nitrite as per Montgomery and Dymock[12]. Cell growth was monitored at regular intervals at A_{600} and corresponding dry weights were determined.

Results and Discussion

Several studies have reported biodegradation of PNP and their respective metabolic pathways[6,11,13-15], but very few have focused on the kinetics of PNP degradation[10,16,17]. However, kinetics of biodegradation is essential not only for evaluation of the persistence of recalcitrant(s) in biosphere, its subsequent acute/chronic exposure to biotic components in ecological *niche* but also required for the assessment of potential risks associated with exposure of sensitive species to the recalcitrant.

Mineralization of PNP

The evolution of CO_2 in the sealed assay bottles was analyzed using GC analyzer with TCD column. Upon addition of filter sterilized PNP (2.14 mM) to the medium, no CO_2 was detected initially for 10 h. However, a peak of CO_2 emerged after 20 h, which increased after 30 h (Fig. 1) and persisted even after 40 h vis-à-vis standard CO_2 (0.5 mL). Corresponding values of residual PNP at 10, 20 and 30 h were 2.129, 1.35 and 0.30 mM, respectively. Measurement of released CO_2 confirmed mineralization of PNP by *P. putida*. These results unequivocally established that *P. putida* utilized PNP as a source of carbon, nitrogen and energy. While mineralization of PNP at low concentration (10 ng-0.40 mM) has been reported[11,18], present results of CO_2 analysis indicated higher concentration of PNP (2.14 mM) mineralization by *P. putida*.

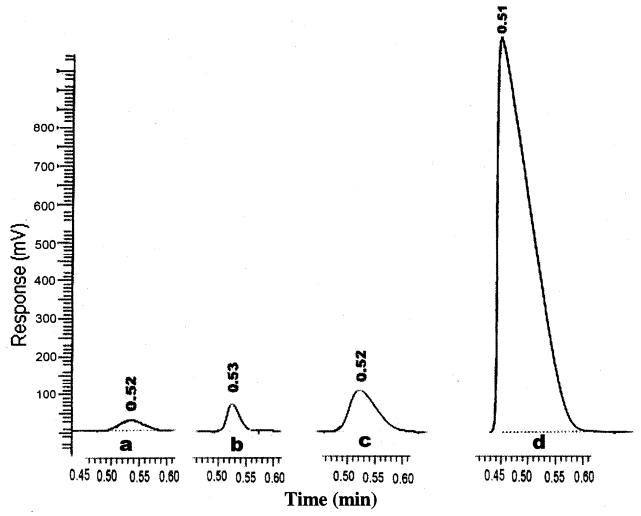

Fig. 1—Mineralization of high concentration of PNP (2.143 mM) on the basis of emergence of CO_2 peak detected by GC analyzer at a (10 h, 84935 µV.s), b (20 h, 218860 µV.s), c (30 h, 324091µV.s) and d (standard CO_2, 10325786 µV.s).

Kinetics of PNP degradation

The growing *P. putida* cells acclimatized with PNP were used for studying the kinetics of degradation. As evident from Fig. 2, PNP degradation was initially slow in the first 15 min, thereafter it was faster and finally reached stationary phase after 25 min with almost no residual PNP. The pattern of disappearance of PNP by *P. putida* was, however, different from earlier reports[10,16]. The kinetics of degradation of PNP at 0.5 mM concentration followed Monod pattern with growth kinetics as evident from the pattern of PNP disappearance at this concentration. The shape of the curve reflecting Monod with growth kinetics was in accordance with the kinetics described by Alexander[19]. Similar kinetic studies have described the metabolism of benzoate by *Pseudomonas* sp. at benzoate levels near K_s and mineralization of *p*-nitrophenol in lake water[16,20].

Further studies on the effect of biomass concentration from 0.54 g dry weight to 6.0 g dry weight L^{-1} showed regular increase in PNP degradation in a minimal medium containing 0.5 mM PNP at a constant reaction time (10 min) and thereafter, remained constant (Fig. 3). Further increase in biomass concentration fails to increase the degradation of PNP. The PNP degradation pattern by *P. putida* in the present study was similar to that reported by Bhushan *et al*[10].

In order to determine half saturation constant for growth (K_s), pre-grown 1.0 g dry biomass of *P. putida* L^{-1} were incubated with various concentrations of

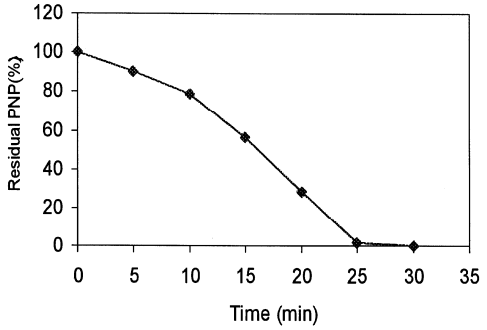

Fig. 2—Pattern of PNP degradation in minimal medium containing 0.5 mM PNP by *P. putida* as a function of time.

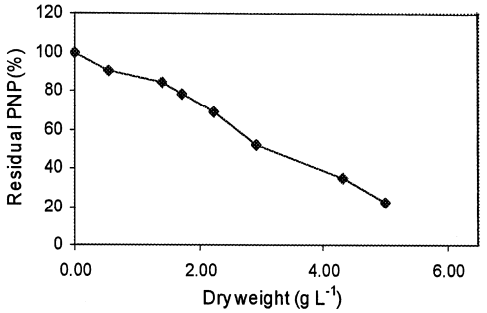

Fig. 3—Profile of PNP degradation by *P. putida* at various biomass concentrations in minimal medium supplemented with 0.5 mM PNP concentration keeping reaction time (10 min) constant.

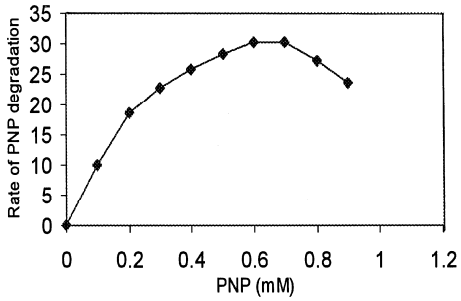

Fig. 4—Michalis-Menten curve for PNP degradation by *P. putida* as a function of PNP concentration

PNP (0.1 to 1.0 mM) in minimal medium. At these concentrations, pattern of PNP degradation drawn as PNP concentration (mM) versus µM of PNP degradation min^{-1} g^{-1} of dry weight L^{-1} were hyperbolic in shape. Hence, the apparent K_s value of PNP degradation by *P. putida* was 0.22 mM (Fig. 4), while the maximum rate of PNP degradation as determined from Lineweaver-Burk's plot drawn as reciprocal of PNP concentration in mM versus reciprocal of µM of PNP degraded was 4.5 µM of PNP degraded min^{-1} g^{-1} dry biomass L^{-1}. The apparent K_s values and rate of PNP degradation

Table 1—Growth and PNP degradation kinetics of *P. putida* in batch mode using minimal medium supplemented with varying PNP concentration

PNP (mM)	Specific growth rate μ (h^{-1})	Growth yield $Y_{(x/s)}$	Specific PNP uptake rate (μm g^{-1} h^{-1}) (q_s)	Specific PNP degradation rate (μm g^{-1} h^{-1}) (q_p)
0.143	0.1701	4.05	0.6889	0.0425
0.214	0.1438	2.75	0.3954	0.0523
0.357	0.1064	1.80	0.1912	0.0589
0.714	0.0532	1.095	0.0582	0.0486
1.428	0.0234	0.673	0.0157	0.0347
2.143	0.0174	0.54	0.0094	0.0314
3.571	0.0087	0.34	0.0003	0.0257

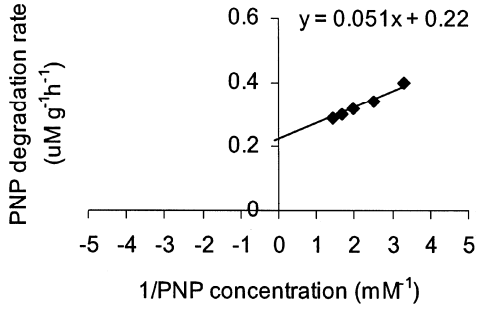

Fig. 5—Lineweaver-Burk plot of PNP degradation as a function of concentration for evaluating rate constant of PNP degradation by *P. putida.*

observed in *P. putida* was similar to the earlier report[10].

PNP degradation experiments were also performed in order to determine the influence of moderately high level of PNP with low level of biomass on the kinetics. The growth and PNP degradation kinetics of *P. putida* was evaluated in batch mode by inoculating pre-acclimated 0.2 g dry weight of *P. putida* L^{-1} in minimal medium supplemented with varying concentrations of PNP (0.143 to 3.57 mM). The specific substrate uptake rate (μM of PNP taken up g^{-1} h^{-1}) and specific rate of degradation (μm of PNP degraded g^{-1} h^{-1}) values decreased with an increasing concentration of PNP (Fig. 5). Similarly, the growth rate does not rise markedly with an increasing concentration of PNP. Ultimately, at high PNP concentration (2.14 and 3.57 mM), the growth rate did not increase with further rise in concentration. These values are given in Table 1. The pattern of kinetics agrees to the observations reviewed by Alexander[19]. From the data presented in Table 1, it is evident that the values vary enormously, permitting no generalization. Secondly, in the absence of similar reports, comparison is not possible.

Conclusion

The significance of the information obtained from such experiments may aid in the studies pertaining to the modeling of contaminated environment as well as in the interpretation of kinetic data from natural ecosystem for designing an industrial effluent treatment plant. In absence of similar reports, not only with respect to PNP but also for other nitroaromatics, comparison is not possible. The study demonstrates the effects of both low and high concentration of substrate (PNP) on the kinetics of PNP degradation by *P. putida*. However, knowledge gained from such studies may aid in finding means by which to enhance the degradation of nitroaromatics at waste disposal sites.

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