Biodegradation of the chelator 2,6-pyridine dicarboxylic acid (PDA) used for soil metal extraction

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Abstract

In this research the biodegradability of 2,6-pyridine dicarboxylic acid (PDA) was evaluated. This compound has been used as a chelating agent for extraction of metal ions from contaminated soils. Initial experiments indicated that PDA was not biodegraded by unacclimated mixed cultures to any degree. However, acclimated mixed cultures degraded more than 80% of this compound within 12 h under different conditions without showing any sign of inhibition even at high concentrations up to 6 mM (1000 mg/l). The results of PDA biodegradation at all concentrations tested in aqueous solution were very similar to those observed in presence of soil slurry, except that a slight lag of microbial growth occurred at all PDA concentrations in the latter case. No toxicity was evident to the microorganisms during the biodegradation of lead complexed PDA after an initial lag. The PDA complexed with lead was easily available for metabolism by the acclimated mixed culture.

Keywords: Biodegradation; 2,6-Pyridine dicarboxylic acid (PDA); Heavy metals; Chelating Compounds

Nomenclature

\( \mu \) specific growth rate (T\(^{-1}\))

\( \mu_{\text{max}} \) maximum specific growth rate (T\(^{-1}\))

\( S \) concentration of substrate in solution at given time (M\( L^{-3} \))

\( S_0 \) initial substrate concentration (M\( L^{-3} \))

\( K_s \) half-velocity constant (i.e. substrate concentration at which the specific growth rate is one-half the maximum specific growth rate, \( \mu_{\text{max}} \)) (M\( L^{-3} \))

\( X \) biomass concentration at given time (M\( L^{-3} \))

\( Y \) true yield coefficient (M\( L^{-3} M^{-1} \))

\( k \) maximum specific substrate utilization rate (M\( L^{-3} T^{-1} \))

\( b \) endogenous decay coefficient (T\(^{-1}\))

\( \mu' \) net specific growth rate (T\(^{-1}\))

1. Introduction

Contamination of soil with heavy metals has become a serious environmental problem for which “permanent” remedial solutions have been pursued for many years. Heavy metals are toxic to human as well as to other organisms. Their detrimental effects to the environment and organisms persist for a substantial period of time because unlike organic compounds they do not decay or degrade naturally in the environment. Because heavy metal cations are strongly adsorbed onto the negatively charged soil particles, chelating extraction has been perceived to be an effective method for the remediation of soils contaminated with heavy metals. In this method the contaminated soil is treated in slurry reactors in which metal ions are separated from the soil matrix by complexation with a particular chelating agent. The metal has a stronger affinity for the chelating agent than for the soil. The chelating agent later can be recovered just by manipulating the pH level of the solution and the metal is precipitated as an insoluble hydroxide [1]. The chelating compounds used for heavy metal complexation such as 2,6-pyridine dicarboxylic acid (PDA, also known as 2,6-dipicolinate) are organic compounds that could be subjected to biodegradation under field conditions. The biodegradation of these compounds during metal extraction from soil would be unwanted. When it is recycled during consecutive extraction operations, the chelating agent remains in solution for a prolonged duration. As most soil extractions are carried out at room temperature and a pH range of 6 to 9, these conditions are favorable for biodegradation of the chelate...
[2]. During the chelating extraction process, naturally occurring microorganisms in the soil may have or develop an enzymatic system capable of metabolizing these chelating compounds, thereby reducing the amount present in the solution phase. The degree of biodegradability of a particular chelate, therefore, becomes an important factor in their selection for soil extraction and in estimation of their reuse frequency. The main objective of this research was to evaluate the aerobic biodegradability of PDA using mixed cultures in absence or presence of a lead salt in aqueous and in soil slurry system.

2. Literature review

Pyridine molecule is readily biodegraded by natural microorganisms, but the degradation of pyridine derivatives depends upon the nature and position of the substituents [3]. It has been reported that pyridine and pyridinecarboxylic acids are more biodegradable under aerobic conditions than chloro- and aminopyridines [3]. The former compounds have been known to disappear in soil suspension in 7 days whereas the latter group required more than 30 days for their removal. Arima and Kobayashi [4] did an extensive study to determine biodegradability and metabolic pathway of PDA by selected microorganisms. They reported that many strains of soil microorganisms were capable of degrading PDA. They found that 0.1% PDA solution biodegraded completely within 18 h. They also showed that the lag period for the reaction was about 9 h. Increase of PDA concentration to 0.5% in nutrient broth caused a suppression of bacterial growth with maximum growth occurring after a lag period of about 36 h. With 1.0% PDA concentration the maximum growth occurred after a lag of about 60 h. The byproducts of the PDA oxidation were found to be CO₂, NH₃, and H₂O. An Achromobacter sp. was reported to convert PDA to 3-hydroxy derivative and then to 2-oxoglutarate after ring fission [5]. PDA is a widely occurring natural compound found in the spores of gram positive bacteria and is released from them without oxidation [4]. Taylor and King [6] reported that phthalate-degrading marine bacteria were capable of partially transforming the PDA structural analogs, including PDA. Degradation of PDA resulted in increased absorbance in the 300 to 350 nm region, suggesting that the metabolites might be susceptible to photodegradation. Metabolism of PDA may occur via 3- or 4-hydroxydipicolinate and the 3-hydroxypicolinate is further oxidized to α-ketoglutaric acid [5]. Very little research has been reported about PDA biodegradation with mixed cultures thus far. Nonetheless, it has been established that PDA can be rapidly biodegraded by pure microbial cultures and that it is susceptible to photodegradation.

3. Materials and methods

- Chemicals: PDA, constituents of growth media, and basal salts were obtained from Aldrich Chemical Company, Milwaukee, WI.
- Growth Media, Basal Salt Media, and Microorganisms: The enrichment as well as acclimation of microorganisms to PDA was carried out in the presence of peptone-tryptone-yeast-extract-glucose (PTYG) and basal salt media.
- The PTYG growth media consisted of the following compounds added to 11 of distilled water: peptone, 0.25 g; tryptone, 0.25 g; yeast extract, 0.5 g; glucose, 0.5 g; MgSO₄·7H₂O, 0.6 g; and CaCl₂·2H₂O, 0.6 g.
- The basal salt media consisted of the following compounds added to 11 of distilled water: NH₄NO₃, 0.4 g; K₂HPO₄, 0.1 g; KH₂PO₄, 0.05 g; MgSO₄·7H₂O, 0.005 g; MnCl₂·4H₂O, 0.02 g; CaCl₂·2H₂O, 0.2 g; and FeSO₄, 0.005 g.

During microbial growth experiments, the amount of basal salts used in the reactor was determined based on the ratio of chemical oxygen demand (COD): nitrogen: phosphorus (COD:N:P) 100:8:1.

The seed microorganisms were obtained from the municipal wastewater treatment plant, in Columbia, MO. The mixed liquor was withdrawn from the recycle line of the secondary activated sludge treatment unit of the wastewater treatment plant. The seed culture was cultivated in fill and draw batch reactors using PTYG and basal salt media. Cells wasted from this reactor were used for all unacclimated experiments and also for acclimation experiments.

3.1. PDA Analyses

PDA concentrations in aqueous samples were determined by directly measuring UV absorptions at 260 nm in a spectrophotometer as described by Arima and Kobayashi [5]. The concentration of PDA was determined from standard calibration curve assuming that other absorbing byproducts were absent in samples.

3.2. COD Analysis

The test tube colorimetric procedure of the Hach Chemical Co. [7] was used to estimate chemical oxygen demand (COD). The filtered aliquots of 2 ml each were added to premixed reagents in test tubes, which were tightly closed and heated for 2 h at 150°C. The COD values were evaluated using a colorimetric procedure that measures the absorbance of the samples at 620 nm (for a COD range of 0–1500 mg/l) in a spectrophotometer.
3.3. Ammonia analyses

Ammonia-N was measured using Nessler Method as described in Water Analysis Handbook [7]. To 25 ml of a sample and a blank sample containing distilled water, three drops of mineral stabilizer and three drops of polyvinyl alcohol dispersing agent were added. The samples were then well mixed. One ml of Nessler reagent was then added to each sample followed by adequate mixing. After allowing some time for the reaction to complete, the ammonia-N was measured by reading the absorbance of the samples at 425 nm against the blank using a spectrophotometer.

3.4. Acclimation of microorganisms for PDA biodegradation

Acclimation of the cultures were carried out by exposing the seed microorganisms grown on PTYG media to PDA by gradually increasing its concentration and decreasing the concentration of the growth media (PTYG) over time. The acclimation process was continued for two weeks with ultimately a constant feeding of PDA up to 6 mM (1000 mg/l) as a sole source of carbon without any supplemental growth media. The biodegradation of PDA was assessed by evaluating the growth of microorganisms [as measured by mixed liquor suspended solids (MLSS) concentration] and the elimination of PDA (as measured by PDA concentration and COD) from the solution over time.

3.5. Biodegradation of uncomplexed and complexed PDA in aqueous solution

Uncomplexed PDA solution was made by adding a certain concentration of PDA, basal salts, and acclimated seed in the test reactor. For complexed PDA solutions, a specified amount of a lead nitrate solution, as determined by the metal–chelator ratio, was added to a known concentration of PDA and shaken for 24 h at pH 3.5–5 to attain complete complexation. The concentrations of PDA tested were: 1.5, 3.0, and 6.0 mM (250, 500 and 1000 mg/l). The metal to chelator ratio (Pb: PDA) used was 1:2. To each duplicate sample solution at chosen PDA concentration appropriate amount of metal solution was added and its pH was adjusted to 6.5 to 7.0. Each sample was then inoculated with the same amount of microbial biomass (MLSS) from the acclimation units and appropriate amount of basal salts were added. All experiments were carried out at room temperature (20–25°C) and in the dark to avoid any possible photodegradation. The 125 ml-flasks containing 100 ml of sample solution (with chelate/metal, microorganisms and basal salts) were placed on a shaker and shaken at 200 rpm which provided a good mixing. Ten ml of aliquots were withdrawn from the flask after 0, 3, 6, 9, 12, and 24 h of incubation and filtered through a 0.45 µm membrane filter paper. The MLSS, PDA concentration, ammonia-N, and COD values were measured for each sample. A separate abiotic test was also carried out by adding 5000 mg/l of HgCl2 to the test solution.

3.6. Biodegradation of uncomplexed and complexed chelators in 5% soil slurry

A 5% soil slurry was prepared by adding 5 g of soil to 100 ml of basal media. For uncomplexed PDA soil solutions, the test solutions were prepared in the same manner as described for the aqueous solutions. However, in case of complexed PDA soil slurry, 5 g of soil was added first to a flask containing 50 ml of a known concentration of lead nitrate solution. The solution was then allowed to equilibrate for 24 h on a shaker. A known amount of PDA solution was added to the soil slurry to make up the desired metal to chelator ratio and the pH was brought down to 3.5, the soil solution was mixed on the shaker for another 24 h to ensure a complete complexation. The pH of the solution was then raised to 7.0. Basal salts and an appropriate amount of acclimated microbial seed were added to determine the biodegradability of PDA in the same manner as described above. A separate abiotic test was also carried out by adding 5000 mg/l of HgCl2 to the test solution.

3.7. Biokinetics modeling

The experimental results of PDA biodegradation were modeled by using Monod biokinetic approach. Monod equation can be written as:

\[ \mu = \mu_{\text{max}} S / (K_s + S) \]

Rate of bacterial growth is described as:

\[ \frac{dX}{dt} = k Y X S / (K_s + S) \]

Rate of substrate utilization is described as:

\[ \frac{dS}{dt} = -k X S / (K_s + S) \]

Actual specific growth rate was calculated as follows:

\[ \mu' = \mu - b = \mu_{\text{max}} S / (K_s + S) - b \]

These two differential equations were solved simultaneously using a numerical second order Runge–Kutta method.

The second order Runge–Kutta method can be described as follows:

\[ \frac{dX}{dt} = f(X, S) \]
\[ \frac{dS}{dt} = (X, S) \]
\[ K1 = \Delta t f(X_n, S_n) \]
\[ L1 = \Delta t g(X_n, S_n) \]
\[ K2 = \Delta t f(X_n + K1, S_n + L1) \]
\[ L2 = \Delta t g(X_n + K1, S_n + L1) \]
The change in biomass and substrate over time are determined as follows:

\[ X_{n+1} = X_n + (K_1 + K_2)/2 \]
\[ S_{n+1} = S_n + (L_1 + L_2)/2 \]

The optimization of curve fitting was achieved by nonlinear regression. The error, \( E \), between predicted and measured values of \( S \) was calculated as:

\[ E = S_{measured} - S_{predicted} \]

Then, the sum of squared errors, \( \Sigma e^2 \), was minimized by varying values of \( Y \) and \( \mu_{max} \). The value of \( K_s \), however, was determined graphically from batch experimental growth data by linearizing the Monod equation:

\[ S_o/\mu = S_o/\mu_{max} + K_i/\mu_{max} \]

where \( S_o \) is initial concentration of substrate \[8\].

The endogenous decay parameter, \( b \), was determined from the slope of a linearized curve during the endogenous growth phase assuming the decay to be first order, i.e. \( \ln(X/X_o) = -bt \).

The \( K_s \) and \( b \) determined from the batch growth experiments were assumed to be constant and applied to other PDA biodegradation experiments.

4. Results

4.1. Biodegradation of PDA by unacclimated microorganisms

Fig. 1 shows the results of uncomplexed PDA biodegradation in presence of unacclimated microorganisms. In this experiment PDA at about 3.0 mM (500 mg/l) concentration was the sole carbon source for the organisms. It can be seen that there was no perceptible biodegradation of the PDA by the culture or growth of biomass in 36h. The slight increases in the COD values with time in the blank and the PDA flask was possibly due to cell lysis and release of cellular organic matter into the media.

4.2. Biodegradation of uncomplexed PDA by acclimated mixed culture in aqueous solution

Fig. 2 shows the experimental results of biodegradation of PDA by acclimated mixed culture microorganisms. The experimental data points are shown in the figure as well as the predicted PDA removal by the model. The loss of PDA with time was evident at all concentrations studied (250, 500 and 1000 mg/l). The % removal of PDA varied from 96 to 98% in 24h. As expected the abiotic experiment with mercuric chloride showed no removal of the compound and no growth of biomass (MLSS) in the system. The PDA loss occurred with an increase of the MLSS concentration (data not included in the Fig. 2) in all the systems tested except in the abiotic system, indicating possible conversion of the substrate to microbial cells. The experimental data fit the Monod biokinetic model very well. The biokinetic parameters calculated for the PDA biodegradation were as follows: \( Y \approx 0.53; \mu_{max} = 0.071 \text{ h}^{-1}; k = 0.134 \text{ h}^{-1}; K_i = 1200 \text{ mg/l}; b = 0.005 \text{ day}^{-1} \).

4.3. Biodegradation of uncomplexed PDA by acclimated mixed culture in soil slurry

The PDA biodegradation in soil slurry by acclimated mixed cultures is shown in Fig. 3. More than 80% of the substrate (PDA) at all concentrations tested was removed within 12h of incubation. The biodegradation pattern for all concentrations tested were, however, characterized by a slight lag that occurred during the first 3h of incubation. The initial lag may be associated with PDA adsorption onto the soil particles, which was then less available to the microorganisms in the environment.
the suspended phase. However, following the lag, the biodegradation of the substrate occurred rapidly with similar kinetics as was observed in the aqueous system (Fig. 2).

The lack of substrate removal (and biomass growth) in the abiotic experiment and concomitant substrate removal (and biomass growth) in all the biotic soil slurry experiments clearly show that PDA was being biodegraded. The soluble COD values determined at the beginning and the end of incubation with 3 mM of PDA (500 mg/l) were 365 and 48 mg/l, respectively. The small amount of COD remaining in the solution at the end indicated that the PDA was being metabolized almost completely producing little residual organic matter.

The biokinetic parameters determined for the biodegradation of PDA in soil slurry system were as follows: $Y_0 = 575$, $\mu_{\text{max}} = 0.070 \ \text{h}^{-1}$, $k_0 = 122 \ \text{h}^{-1}$, $K_s = 1200 \ \text{mg/l}$, $b = 0.005 \ \text{h}^{-1}$.

4.4. Biodegradation of Pb–PDA complex (1:2) by mixed culture in aqueous solution

Fig. 4 shows that the biodegradation of Pb–PDA complex (1:2) in aqueous solution at PDA concentrations of 1.5, 3.0, and 6.0 mM (250, 500 and 1000 mg/l). More than 90% of the substrate (PDA) was removed during the first 12 h of incubation. The COD reductions for these systems varied from 63 to 80%. The initial and final COD values for the 1000 mg/l PDA system were 675 and 135 mg/l, respectively, which indicated that some residual metabolic products remained in solution after 24 h. At no PDA concentrations tested was there a noticeable lag observed during the biodegradation process. The presence of lead in the solution did not have any significant impact on the overall degradation pattern. The substrate PDA was easily available to microbial attack despite the strong metal complexation. It is possible that the lead was precipitated out during the biodegradation process as has been reported by others for other metal chelate systems [9,10]. The reduction in COD values over the course of the experiment indicated that PDA was indeed metabolized aerobically with some accumulation of degradation products by the acclimated cultures.

The predicted results of substrate biodegradation appeared to fit the experimental data fairly well. The modeling of the experimental data yielded the following biokinetic parameters: $Y = 0.575$, $\mu_{\text{max}} = 0.068 \ \text{h}^{-1}$, $k = 0.129 \ \text{h}^{-1}$, $K_s = 1200 \ \text{mg/l}$, $b = 0.005 \ \text{h}^{-1}$.

4.5. Biodegradation of Pb–PDA complex (1:2) by acclimated mixed culture in soil slurry

As shown in Fig. 5, the overall degradation rate of Pb–PDA complex in soil slurry appeared to be slightly slower than that observed in the aqueous solution. The metabolic processes of acclimated cells at PDA concentrations of 3.0 and 6.0 mM (500 and 1000 mg/l) were slightly retarded under these conditions as compared to that observed in aqueous solution. A slight, but distinctive lag occurred at the beginning stage of the biodegradation. Nevertheless, more than 90% of substrate was removed from the system in 24 h. After the 3 h lag, there was no sign of inhibition of the microbial activity due to the presence of lead and soil slurry. The strong complexing capacity of PDA toward lead did not cause any complication for PDA cleavage by the acclimated mixed cultures. As the biodegradation of Pb–PDA complex proceeded, some portion of lead was possibly precipitated and some remained in the solution as reported by Tiedje [11].

The initial and final COD values measured during the incubation with 6.0 mM (1000 mg/l) PDA were 678 and 123 mg/l, respectively. The final COD value appeared to be high, which indicates that there was some residual organic matter accumulated in the solution after the substrate was essentially depleted.

The modeling performed on the experimental growth data obtained during the biodegradation of Pb-PDA complex in soil slurry showed that the Monod model...
predictions were quite satisfactory. The modeling of the experimental growth data yielded the following biokinetic parameters: \( Y = 0.545, \quad \mu_{\text{max}} = 0.065 \, \text{h}^{-1}, \quad k = 0.119 \, \text{h}^{-1}, \quad K_i = 1200 \, \text{mg/l}, \quad b = 0.005 \, \text{h}^{-1}. \)

4.6. Respirometric results of PDA biodegradation

To further confirm the aerobic biodegradation of the PDA substrate by acclimated mixed culture, a respirometer study was performed. The test was conducted in aqueous solution as per the procedures given in BODTrak manual [12].

Fig. 6 represents the results of a respirometer experiment to determine the biodegradation of PDA at concentrations of 1.5 and 3.0 mM (250 and 500 mg/l). The biodegradation of PDA at these concentrations experienced a substantial lag which continued for about 6 h, in contrast to virtually no lag observed during the batch experiments in aqueous solution at any concentrations of PDA. However, after the lag period the biodegradation rates were very similar to the respective concentrations and environmental conditions as observed in the batch aqueous experiments. More than 80% of the substrate was mineralized within 15–18 h, which was comparable to that observed in the batch reactor systems.

4.7. Discussions

The experimental results show that PDA is easily biodegraded by acclimated mixed cultures. However, despite its natural origin, unacclimated microorganisms were not able to metabolize PDA as a sole source of carbon. After acclimation the microorganisms degraded this substrate readily in its complexed as well as uncomplexed form within 12 h without displaying any sign of significant inhibition even at high concentrations of PDA (up to 1000 mg/l).

The following observations confirm that the observed reduction of PDA concentrations in the test flasks were due to biodegradation:

1. oxygen uptake by the microorganisms occurred in the system with simultaneous loss of the substrate;
2. an increase in the biomass occurred in the system with time;
3. a reduction in COD value occurred over time indicating loss of carbon in the system; (PDA was the sole carbon source);
4. a complete avoidance of light eliminated any possibility of photodegradation of the substrate; and
5. lack of sorption of PDA on soils or biosolids or volatilization as evidenced by the absence of aqueous phase PDA loss when incubated abiotically.

The calculated value of \( b \), the endogenous decay coefficient, of 0.005 h\(^{-1}\) (0.12 day\(^{-1}\)) was quite reasonable as the reported values for this constant for synthetic wastes in the literature vary between 0.09 to 0.18/day [8]. The calculated value for \( K_c \), the half-velocity constant, of 1200 mg/l was quite high. In general, the values of \( K_c \) for many synthetic organic compounds have been reported to be under 100 mg/l [13]. However, the value of \( K_c \) depends on the compound being degraded and the microorganisms in the system. Even for the same substrate (phenol), Rozich, et al. [14] reported a large variation in the calculated values of \( K_c (5 - 272 \, \text{mg/l}) \) in batch and flow through chemostat experiments. Using respirometric studies for biodegradation of oil contaminated soil extract, Li et al. [15] found values of \( K_c \) in their systems as high as 824 mg/l. Thus, the high value of \( K_c \) observed in the experiments reported herein may be due to factors that could not be easily quantified.

The biodegradation rate for PDA at 6 mM in aqueous solution was \( 4.60 \times 10^{-3} \, \text{mM PDA/mg SS/day} \) which was considerably higher than that observed with 3 mM PDA (2.3 \times 10^{-3} \, \text{mM PDA/mg SS/day}).

The biodegradation results obtained for PDA at various concentrations in soil slurry were very similar to that obtained in aqueous solution. The calculated overall degradation rates given in Table 1 show that the rate at 6.0 mM in soil slurry appears to be slightly slower than that observed in aqueous solution. Furthermore, there was no noticeable lag observed when degraded by acclimated microorganisms in aqueous solution, whereas the presence of soil caused a lag of about 3 h. The lag may be attributed to adsorption of PDA on the soil particles making it less available for microbial attack. Organic substances present in soil, such as humic acids may have also competed with PDA to complex with trace metals necessary for microbial growth, therefore making them unavailable to microorganisms for their catabolic activities.

The results obtained for the biodegradation of complexed PDA at metal–chelator ratio of 1:2 indicated that the degradation rates at different PDA concentrations did not change significantly from that for uncomplexed...
PDA. This suggests that the presence of lead did not cause any metabolic retardation. Although very stable, the metal–chelator complexes are easily broken down by microbially mediated reaction making the complexed chelator available for microbial metabolism [11]. It is also possible that the released lead ions were precipitated from the solution which would eliminate its toxic property. Other researchers have observed during biodegradation studies of metal–organic chelates that metals can get precipitated [9,10].

5. Conclusions

- PDA at all concentrations tested was not degraded to any degree by unacclimated microorganisms.
- PDA up to 6.0 mM (1000 mg/l) was metabolized by acclimated mixed culture equally with or without the presence of soil. The acclimated microorganisms were able to metabolize greater than 80% PDA in 12 h at all concentrations tested without any sign of inhibition. There was no lag observed during the biodegradation of PDA in aqueous solution.
- The biodegradation rate of PDA in 5% soil slurry at all concentrations tested were very similar to those observed in the aqueous system. However, a slight lag occurred at all concentrations of PDA in soil slurry biodegradation experiments.
- The biodegradation rate of metal complexed PDA did not differ much from that observed for uncomplexed PDA. Thus, the presence of lead did not cause any toxicity to the microorganisms present.
- The results of respirometric studies of PDA at 1.5 and 3.0 mM (250 and 500 mg/l) in aqueous solution were somewhat similar to those observed in the batch reactors at these respective concentrations. However, the biodegradation kinetics in the respirometer showed a longer lag period compared to the batch studies at all concentrations tested.

- Some chelators such as PDA are biodegradable, therefore their use for soil metal extraction and reuse could be limited.

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