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Effects of organic compounds on the degradation of *p*-nitrophenol in lake and industrial wastewater by inoculated bacteria

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Abstract

Many microorganisms fail to degrade pollutants when introduced in different natural environments. This is a problem in selecting inocula for bioremediation of polluted sites. Thus, a study was conducted to determine the success of four inoculants to degrade *p*-nitrophenol (PNP) in lake and industrial wastewater and the effects of organic compounds on the degradation of high and low concentrations of PNP in these environments. *Corynebacterium* strain Z4 when inoculated into the lake and wastewater samples containing 20 μg/ml of PNP degraded 90% of PNP in one day. Addition of 100 μg/ml of glucose as a second substrate did not enhance the degradation of PNP and the bacterium utilized the two substrates simultaneously. Glucose used at the same concentration (100 μg/ml), inhibited degradation of 20 μg of PNP in wastewater by *Pseudomonas* strain MS. However, glucose increased the extent of degradation of PNP by *Pseudomonas* strain GR. Phenol also enhanced the degradation of PNP in wastewater by *Pseudomonas* strain GR, but had no effect on the degradation of PNP by *Corynebacterium* strain Z4.

Addition of 100 μg/ml of glucose as a second substrate into the lake water samples containing low concentration of PNP (26 ng/ml) enhanced the degradation of PNP and the growth of *Corynebacterium* strain Z4. In the presence of glucose, it grew from 2×10^4 to 4×10^4 cells/ml in 3 days and degraded 70% of PNP as compared to samples without glucose in which the bacterium declined in cell number from 2×10^4 to 8×10^3 cells/ml and degraded only 30% PNP. The results suggest that in inoculation to enhance biodegradation, depending on the inoculant, second organic substrate many play an important role in controlling the rate and extent of biodegradation of organic compounds.

Abbreviations: PNP – *p*-nitrophenol

Introduction

One of the major problems currently confronting environmental science professionals is the biodegradation of toxic organic chemicals in wastewater treatment facilities, ground waters and hazardous waste sites. In these situations, organic pollutants frequently occur in mixture with other synthetic as well as natural organic compounds. Therefore, it is necessary to understand how the biodegradation of the polluting compounds is affected by the presence of alternate substrates. Recent work (Hess et al. 1990; LaPat-Polasko et al. 1984; Schmidt & Alexander 1985; Schmidt et al. 1987) has shown that the degradation of low concentrations of organic compounds can be stimulated by the addition of readily degradable organic substrates. Several pure culture studies have demonstrated the effectiveness of supplemental substrates for enhancing the rate of biodegradation of toxic chemicals (LaPat-Polasko et al. 1984; Schmidt et al. 1987; Scow et al. 1989). Schmidt et al. (1987) showed that glucose could increase the growth rate of PNP mineralization. Lapat-Polasko et al. (1984) demonstrated that methylene chloride degradation by *Pseudomonas* sp. can be enhanced if acetate

is supplied to the organism as a supplementary substrate. In batch cultures of mixed populations, glucose inhibited the degradation of 2,4-dichlorphenoxy acetic acid even though the two substrates were used simultaneously (Papanastasiou et al. 1982; Rozich et al. 1986). Likewise, glucose inhibited the degradation of phenol by a heterogenous population that had previously been acclimated to phenol, but the use of an inoculum acclimated to phenol and glucose reduced inhibition (Rozich et al. 1986). Such findings have practical significance. It would be beneficial if these results could be applied to the operation of wastewater treatment systems to stimulate the break-down of synthetic compounds.

Because the use of naturally occurring or genetically engineered microorganisms represent a potentially promising means of destroying polluting chemicals in wastewater treatment systems, natural waters, or soil, a study was initiated to investigate the effects of organic substrates on the degradation of PNP in lake and industrial wastewater by the inoculated bacteria.

Materials and methods

Samples of wastewater from the Mayaguez, P.R. industrial zone were obtained and used within 1 h after collection. Lake water was obtained from Beebe and Cayuga Lakes, Ithaca, N.Y.

Corynebacterium was isolated from Cayuga lake, Ithaca, N.Y. Other bacteria were isolated from soil and fresh water samples collected from a petrochemical complex site in Guayanilla, Puerto Rico. To isolate the bacteria capable of degrading PNP, samples of soil, wastewater and fresh water were added to a mineral medium (0.1 g of NH_4NO_3, 0.8 g of K_2HPO_4, 0.2 g of KH_2PO_4, 0.1 g of $CaCl_2 \cdot 7H_2O$, 0.1 g of $MgSO_4$, and 10 mg of $FeCl_3$ per liter of distilled water) amended with 50 μg PNP/ml. The enrichments were incubated for 7 days at 29° C. When the yellow color of PNP had disappeared, enrichment culture was transferred to a fresh medium. After three such serial transfers, a sample of liquid was plated on a medium containing 0.3 percent powdered Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) and 1.5% agar. Individual colonies were transferred to the mineral medium amended with 10 μg PNP/ml to test degradation activity.

To determine whether the organisms would lose their ability to degrade PNP when grown in the absence of this compound, the isolated strains were grown in 0.3% Trypticase soy broth for three serial cultures. The cultures were then plated on agar containing mineral medium and 10 μg PNP/ml, and 15 to 20 colonies were randomly picked. Each colony degraded PNP when tested in the salts solution amended with 50 μg PNP/ml.

Corynebacterium sp. strain Z4, which is a mutant of a strain originally isolated from Cayuga lake water, is resistant to 10 mg of kasugamycin, 100 mg of streptomycin, and 25 mg of spectinomycin per liter (Zaidi et al. 1988). The population size of this bacerium in lake water samples was determined by the drop plate method (Hoben & Somasegaran 1982) on 0.3% Trypticase soy agar containing the three antibiotics at the indicated concentrations in 250 ml flasks. Triplicate counts were made of each dilution. The plates were incubated at 29° C. The enrichments were incubated for 7 days at 29° C, and dilutions were then plated on the same medium containing agar. *Pseudomonas* sp. strains GR, MS and UBG were isolated from the Guayanilla River, a waste stream flowing through the Mayaguez industrial zone, and from soil of a former manufacturing site, respectively. The *Psseudomonads* were identified by the Biolog GN Micro plate method (Biolog, Inc. Hayward, CA) using MicroLog 1 software for the identification of non-clinical organisms.

To measure mineralization, duplicate 50-ml samples of inorganic salts solution, lake water or industrial waste water were placed in 250-ml Erlenmeyer flasks. Washed cells (100 μl) from 24 hours old cultures grown in salts solution amended with 10 μg PNP/ml were added to give initial densities between 10^3 and 10^4 cells/ml. All bottles received similar amounts of C^{14}-labeled compounds (1,000 to 2,000 dpm/ml), but the final substrate concentration was varied by adding different quantities of unlabeled compounds. Duplicate flasks for each treatment were incubated in the dark at 29° C. At regular intervals, 1.0-ml samples were transferred to the scintillation vials, and the liquids were acidified with 200 microliter of 1.0 M sulfuric acid. Air was bubbled vigorously through the liquid for 5 min to drive off CO_2, and then 3.5 ml of Liquiscint scintillation cocktail (National Diagnostic, Inc., Somerville, N.J.) was added to the acidified samples. The radioactivity was counted with a liquid scintillation counter (model LS 7500; Beckman Instruments, Inc., Irvine, Cal.). This procedure has been shown to measure mineralization of PNP, since the loss of ^{14}C from labeled PNP added to the liquid is parallel to the formation of $^{14}CO_2$ (Zaidi & Alexander 1988, 89). To determine

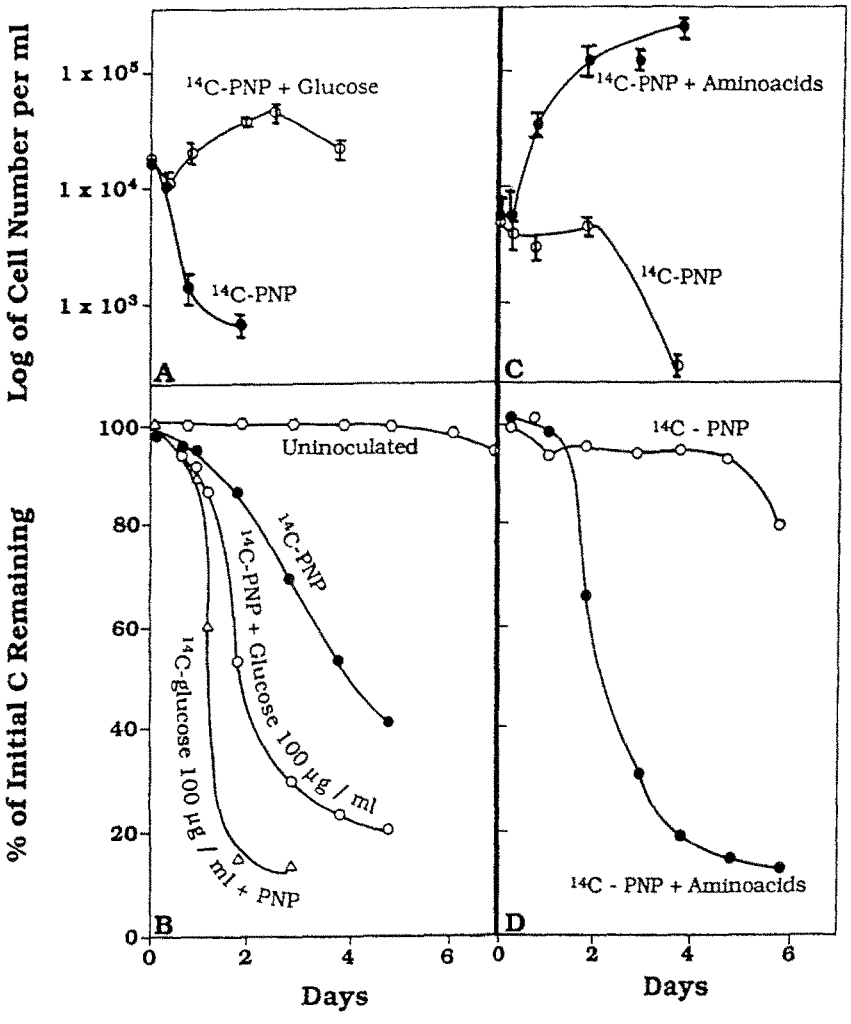

Fig. 1. Effect of glucose and amino acids on growth (A and C), and mineralization of 26 ng of PNP per ml (B and D) by *Corynebacterium* sp. strain Z4 in lake water.

the biodegradation at high substrate concentration, the loss of UV absorbancy at 300 nm was measured with a Beckman spectrophotometer, model 25.

p-Nitro[U-^{14}-C]phenol (1.1 GBq/mmol; 98% radiopurity) was obtained from California Bionuclear Corp., Sun Valley, Ca, and [U-^{14}C]glucose (12,900 MBq/mmol) was purchased from New England Nuclear Corp., Boston, Mass.

Results

Characterization of bacteria

The bacteria isolated from the petrochemical complex in Puerto Rico were all gram negative, motile, oxidase positive, catalase positive and oxidized glucose. Biolog test indicated that all belong to genus *Pseudomonas* with correlation coefficient from 0.547 to 0.869.

Lake water

Corynebacterium sp. strain Z4 rapidly mineralized 20 µg/ml of PNP in lake water, and 90% of PNP disappeared in less than a day (data not shown). No degradation of PNP at that concentration was observed in 7 days in uninoculated lake water. Addition of 100 microgram of glucose did not enhance the degradation of PNP. Similar results of degradation of 20 µg of PNP per ml were obtained when this bacteria was added to ground and pristine water (data not shown).

When the bacterium was added to lake water containing 26 ng of PNP per ml, the cell numbers declined from 2×10^4 to 8×10^2 cells per ml in 2 days (Fig. 1A). The degradation was slow, and only 50% of PNP was mineralized in 5 days (Fig. 1B). Addition of 100 μg/ml of glucose enhanced the degradation of 26 ng/ml of C^{14}-PNP, and approximately 80% was mineralized in 5 days. The cell density initially increased from 2×10^4 to 5×10^4 in 3 days and then declined. Because of a slight increase in numbers of *Corynebacterium* sp. strain Z4 in the glucose amended water, the organism used very little of the sugar. Figure 1B reflects that under these conditions, glucose was mineralized before PNP, when labeled glucose and unlabeled PNP were added to the water samples. PNP was not mineralized in uninoculated lake water samples for 6 days.

At the initial density of 5×10^3 cells/ml, *Corynebacterium* sp. strain Z4 mineralized only 10% of PNP at 26 ng/ml in 4 days, and the cell number decreased to 3×10^2/ml (Fig. 1C). However, it readily mineralized PNP when amino acids (casamino acids vitamin free) at 400 μg/ml were added (Fig. 1D), and its density increased from 5×10^3 to 2×10^5 cells/ml (Fig. 1C). However, the addition of 400 μg/ml of amino acids did not enhance the degradation of 10 μg of PNP/ml in lake water by *Corynebacterium* sp. strain Z4 (data not shown).

The addition of 100 μg/ml of sodium acetate into the lake water samples slightly enhanced the degradation of 26 ng/ml of PNP by *Corynebacterium* sp. strain Z4 (Fig. 2). The bacterium mineralized 90% of 100 μg/ml of PNP, in 3 days, but addition of 100 μg/ml of sodium acetate completely inhibited degradation of 100 μg/ml of PNP by this strain.

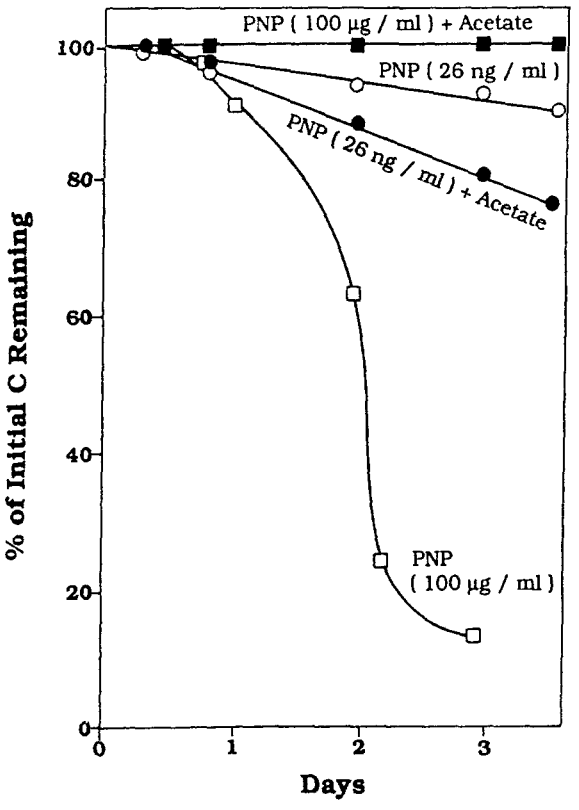

Fig. 2. Mineralization of 100 μg and 26 ng of PNP per ml by *Corynebacterium* sp. strain Z4 in lake water in the presence or absence of 100 μg of sodium acetate per ml.

Industrial wastewater stream

A study was conducted to determine whether *Corynebacterium* sp. strain Z4 and *Pseudomonas* sp. strains GR, MS and UBS can enhance the degradation of PNP in an industrial wastewater stream, and to establish the effect of a second organic compound on the degradation of PNP. Mineralization was not observed in uninoculated wastewater in 7 days, but inoculation of *Corynebacterium* sp. strain resulted in mineralization of more than 90% of 20 μg/ml of PNP in less than a day (Fig. 3). Phenol at 20 μg/ml concentration level had no effect on this activity. However, *Pseudomonas* sp. strain GR added to the wastewater spiked with phenol mineralized PNP in 2 days, whereas a longer acclimation period was evident in wastewater without phenol.

The addition of 100 μg/ml of glucose had no effect on the degradation in wastewater of 20 μg/ml of PNP by *Corynebacterium* sp. strain Z4 and 90% was mineralized in one day with or without glucose (Fig. 4). Biodegradation by *Pseudomonas* sp. strain GR was initially not affected by the addition of glucose, but a major influence was evident after 2 days. After 3 days, 55% of PNP was mineralized in samples without glucose, but 98% of PNP was mineralized in samples amended with sugar. The effect of glucose was especially pronounced when *Pseudomonas* sp. strain MS was used as inoculant. *Pseudomonas* sp. strain MS mineralized 98% of PNP in 2 days in wastewater samples not amended with glucose compared to 5% when the samples contained the second carbon source.

The addition of glucose into the wastewater had little or no influence on PNP mineralization in samples inoculated with *Pseudomonas* sp. strain UBG (data not shown). On the other hand, addition of 100 μg/ml of

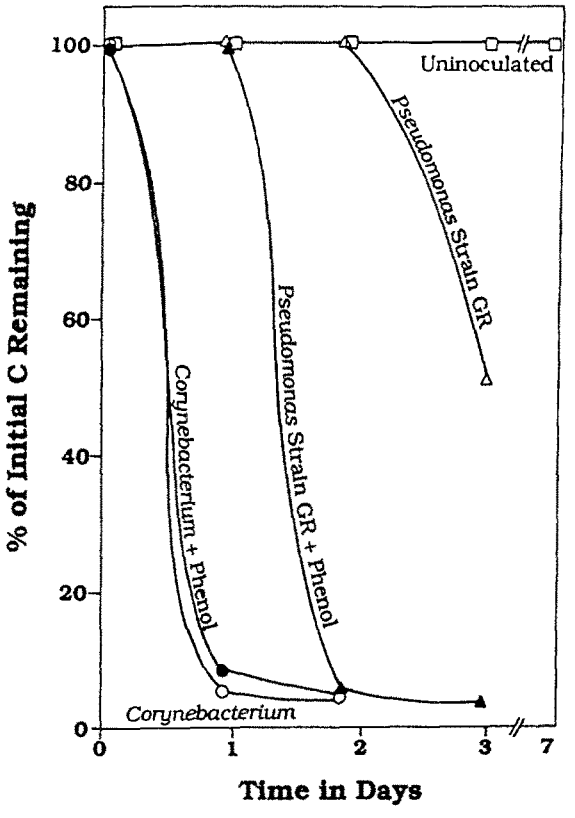

Fig. 3. Mineralization of 20 μg of PNP per ml by *Corynebacterium* sp. Z4 or *Pseudomonas* sp. GR in wastewater amended with or without 20 μg of phenol per ml.

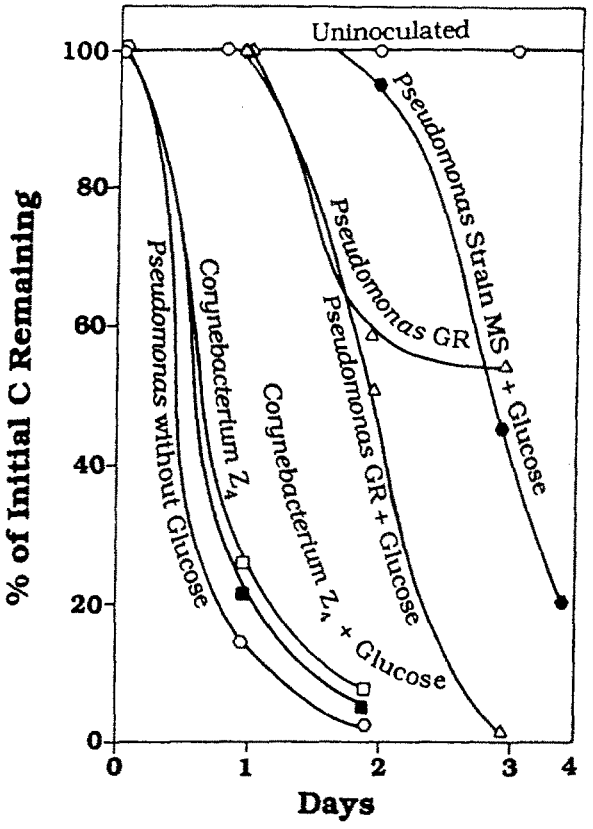

Fig. 4. Mineralization of 20 μg of PNP per ml by three bacteria in wastewater amended with and without glucose at 100 μg/ml.

sodium acetate in wastewater enhanced the degradation of 20 μg/ml of PNP by *Pseudomonas* sp. strain UBG (Fig. 5). More than 95% of PNP was mineralized by this strain in one day in wastewater amended with acetate, whereas similar extent of mineralization required 3 days with no acetate additions. Some degradation of PNP was evident in addition of acetate into uninoculated wastewater after 3 days, but acetate retarded or inhibited the degradation of PNP by the indigenous wastewater microorganisms.

Discussion

A microorganism can grow by using an organic pollutant as a carbon source in pure culture but may fail to degrade the compound when inoculated into the natural environment. The added bacterium may fail to bring about the degradation because it may use other organic substrate present in the natural environment. The concentration of the target compound may be too low to support the growth of the inoculated species, and other reasons that have been proposed (Goldstein et al. 1985). This study was designed to test the success of four inoculants to degrade high and low concentrations of PNP in lake and industrial wastewater and the effect of a second organic substrate on the degradation of PNP by the inoculated bacteria in these environments.

Heterogeneous bacterial populations are known to metabolize mixture of carbon compounds simultaneously (LaPat-Polasko et al. 1984; Rozich et al. 1986; Schmidt et al. 1985). Our data indicate that *Corynebacterium* strain Z4 when inoculated into the lake water containing high concentration of PNP (20 μg/ml) degraded PNP rapidly, while addition of glucose (100 μg/ml) did not enhance the degradation of PNP. Utilization of PNP and glucose appears to be simultaneous when PNP is present at high concentration levels. However, utilization of glucose is not simultaneous when PNP concentration is low. Presence of high concentration of glucose enhanced the degradation of low concentration of PNP (26 ng/ml). The glucose was

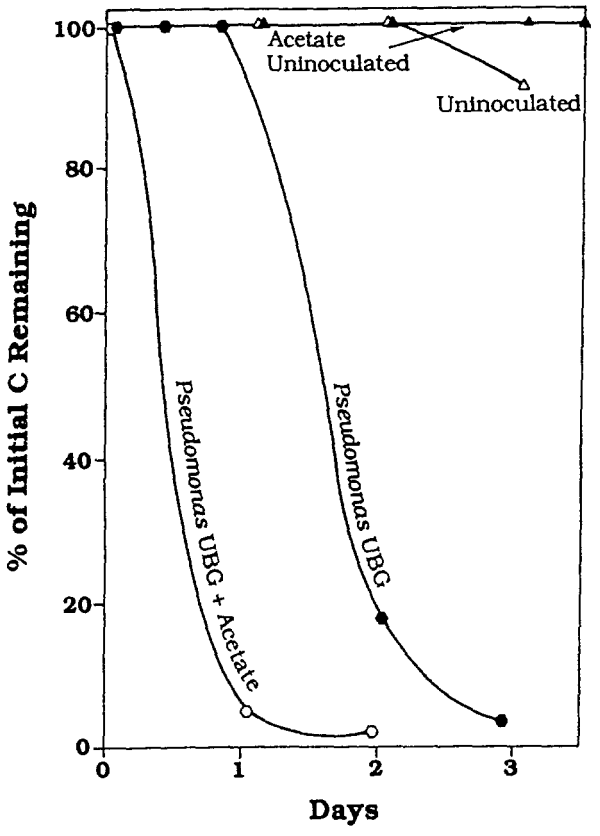

Fig. 5. Mineralization of 100 μg of PNP by *Pseudomonas* sp. UBG in wastewater with or without 100 μg of sodium acetate per ml.

mineralized first, only when glucose had almost disappeared did the mineralization of PNP start. However, since 100 μg/ml of glucose supports the growth of 10^8 cells per ml, it is clear that *Corynebacterium* sp. strain Z4 used very little of the glucose. This may be due to inability of *Corynebacterium* sp. strain Z4 to compete with indigenous lake water microbial population for glucose. Similarly, presence of high concentration of amino acids (400 μg/ml) also enhanced the degradation of low concentration of PNP.

In lake water, degradation of PNP at high concentration levels by *Corynebacterium* sp. strain Z4 is completely inhibited in the presence of sodium acetate (100 μg/ml). This effect, however, is reversed when PNP in lake water is present at low concentration. The presence of high concentration of sodium acetate in industrial wastewater enhanced the degradation of high concentration of PNP (100 μg/ml) by *Pseudomonas* sp. strain UBG. However, the effect of glucose on the mineralization of PNP by *Pseudomonas* sp. strain GR and MS was quite different. The presence of glucose greatly increased the extent of mineralization of PNP by *Pseudomonas* sp. strain GR, but the degradation of PNP by *Pseudomonas* sp. strain MS was inhibited in the presence of glucose.

Conclusions

The inoculation results with four bacteria suggest that the stimulation or inhibition by the second organic compound on the biodegradation of toxic chemicals in natural environments may depend on (1) the organism; (2) concentration of the toxic chemical present in the environment; and (3) second substrate. It is possible that the reasons why *Corynebacterium* strain Z4 is successful as an inoculant at high concentration levels of PNP, is because of its inability to compete for other easily utilizable compounds present in lake and wastewater. However, further work is needed to really establish that.

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