BIODEGRADATION OF α,β-DICHLOROALKANES BY *Rhodococcus rhodochrous* AND *Pseudomonas oleovorans*

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Introduction

Polychlorinated n-alkanes (PCAs), also known as chlorinated paraffins, encompass a broad group of compounds containing varying carbon chain lengths and chlorine content. They are grouped into three main categories with regard to their carbon chain length as either short (C10-C13), medium (C14-C17) or long (>C17), and are further classified by their chlorine content, typically between 30 and 70% by mass. Because of their high chemical stability under varying conditions, PCAs are used for numerous applications, most commonly as extreme pressure, anti-wear additives used for metal machinery or as plasticizers in both plastics and paints (1).

In 1992, worldwide production of PCAs was estimated at 300,000 tonnes per year, and although estimates vary, studies have found that up to 50% of PCAs produced make it into the environment (2). Investigations into the environmental fate of these compounds have determined that they achieve long-range transport via sediments, are toxic to marine life, and bioaccumulate in biota worldwide (3-5). Due to their carcinogenicity, short chain PCAs are considered to be toxic under the Canadian Environmental Protection Act and have been placed on the US Environmental Protection Agency Toxic Release Inventory.

Unfortunately, the very properties that make PCAs appealing for industrial applications make them an environmental concern. Due to the chemical stability of the carbon-chlorine bond, abiotic degradation occurs slowly, giving PCAs estimated half-lives ranging form months to several hundred years (4). Consequently, interest in the study of biodegradative pathways is paramount in order to find possible bioremediation strategies and to gain further insight into the environmental fate of these compounds.

To date, studies into the biodegradation of PCAs have been limited. Omori *et al.* (6) isolated bacterial strains capable of cometabolically dechlorinating between 15 and 57% of different PCA mixtures using n-hexadecane as a co-substrate. Allpress and Gowland (7) found that *Rhodococcus* sp. S45-1 was able to use a PCA mixture as a sole source of carbon and energy, releasing up to 49% of the chloride in the mixture. In order to address the partial recalcitrance observed in both of these studies, the biodegradation of pure PCA congeners has been investigated. Numerous studies have reported the mineralization of α-chloroalkanes of various chain lengths (C10 to C18). Also, *Pseudomonas* sp. 273 was shown to use 1,10-dichlorodecane and other α,ω-dichloroalkanes as a sole sources of carbon and energy (8).

The present study seeks to offer more insight into the biodegradation potential of PCA mixtures by investigating microbial interactions with α,β-dichloroalkanes.

Materials and Methods

Chemicals. Alkenes (1-octene, 1-decene, 1-dodecene, 1-tridecene, 1-tetradecene, 1-hexadecene, 1,9-decadiene) as well as 1,10-dichlorodecane were procured from Sigma-Aldrich Canada Ltd. (Mississauga, Ontario, Canada). Adipic acid, n-hexadecane, dichloromethane (HPLC grade) and chloroform (HPLC grade) were purchased from Fisher Scientific Ltd. (Nepean, Ontario, Canada).

Synthesis of α,β-dichloroalkanes and 1,2,9,10-tetrachlorodecane. The reaction mixture consisted of 2 ml of alkene and 10 ml of dichloromethane in a 50 ml round-bottom flask. The mixture was stirred gently with a magnetic stirrer while chlorine gas was bubbled through at a rate of about 4 bubbles per second. The reaction extent was monitored by gas chromatography. The endpoint was signaled by the initially clear reaction mixture turning yellow, indicating the presence of dissolved chlorine gas. At this point the mixture was purged with nitrogen. The solvent was removed by roto-evaporation and the

structure of the product was verified by ^{1}H and ^{13}C NMR spectroscopy (300 MHz Varian Mercury).

Culture and culture conditions. Both *Rhodococcus rhodochrous* ATCC 13808 and *Pseudomonas oleovorans* ATCC 29347 were obtained from the American Type Culture Collection (ATCC). The organisms were grown in a mineral salts medium described previously (7) supplemented with a carbon source. For *R. rhodochrous*, this consisted of 2 g l^{-1} adipic acid with 0.1 g l^{-1} yeast extract and 0.96 g l^{-1} NaOH. The carbon source for *P. oleovorans* consisted of 1% (v/v) octane. All experiments, with the exception of the bioreactor run, were carried out in 500 ml Erlenmeyer flasks containing 100 ml of growth medium. To minimize the evaporation of substrate, the flasks were capped with rubber stoppers covered in aluminum foil. Abiotic controls were conducted in the same manner but using autoclaved biomass. For the bioreactor run, a 4 l glass cyclone reactor was used with a working volume of 1 l. A centrifugal pump was used to cycle the broth through the reactor while sterile air was bubbled through the system at a flowrate of 0.2 l min^{-1}.

Analysis of chloride release. The concentration of chloride ions in the growth medium was measured using ion chromatography. A Dionex DX-100 ion chromatograph (Dionex Canada Ltd., Oakville, Ontario, Canada) equipped with an ion pack anion exchange AS 12A guard column (4 x 200 mm) and a self-regenerating suppressor was used. Anion detection was performed by electrical conductivity. The eluent consisted of a solution of 2.7 mM Na_2CO_3 and 0.3 mM $NaHCO_3$ at a flow rate of 1.5 ml min^{-1}. Samples of the growth medium were withdrawn and centrifuged at 10,000 rpm for 5 minutes, the supernatant withdrawn and diluted 10x with an internal standard solution containing 40 mg l^{-1} KBr.

Analysis of 1,2-DCD concentration. 10 ml samples were withdrawn from the reactor growth medium and extracted with 5 ml of chloroform containing 6.00 x 10^{-3} % (v/v) n-pentadecane as an internal standard. Quantification was carried out in a Varian CP-3800 gas chromatograph equipped with a Supelco SPB-5 column (30 m x 0.32 mm ID, 0.25 µm film thickness) and a flame ionization detector. The carrier gas was UHP grade helium (Megs Specialty Gases, Montreal, Quebec, Canada) at a column flow of 4 ml min^{-1} and a split ratio of 10:1. The temperature regime was as follows: initial 60°C, hold for 2 min, ramp to 160°C at 10°C min^{-1}, ramp to 300°C min^{-1}, hold for 2 min. The injector temperature was 250°C and the detector temperature 320°C.

Results and Discussion

After conducting a preliminary screening, it was determined that both *Rhodococcus rhodochrous* ATCC 13808 and *Pseudomonas oleovorans* ATTC 29347 were able to biodegrade 1,2-dichlorodecane (1,2-DCD) while in the presence of a co-substrate. *R. rhodochrous* was able to use both adipic acid and n-hexadecane (C16) as a co-substrate. Figure 1 illustrates the chloride released from 1,2-DCD by two flasks containing 100 ml of adipic acid-grown *R. rhodochrous* in mineral media. After 42 days of incubation and an apparent plateau in the chloride release, each flask was spiked with 0.01% (v/v) of either C16, a growth substrate, or heptamethylnonane (HMN), an inert oil exhibiting similar physical properties to those of C16. The resulting increase in the rate of chloride release upon addition of C16 indicates the necessity of a growth co-substrate for the dechlorination of 1,2-DCD by *R. rhodochrous*.

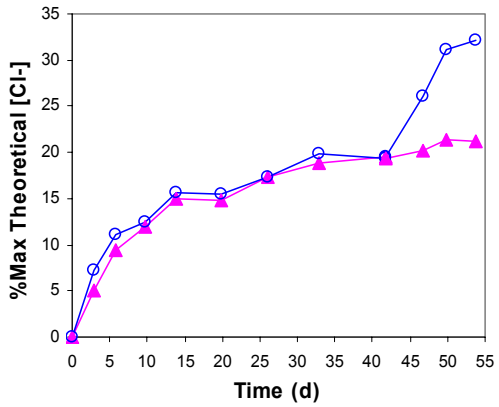

Figure 1. Chloride release from 1,2-DCD monitored in batch fermentations of *R. rhodochrous* with 2 g/L adipic acid as a carbon source. After 42 days one flask was spiked to obtain a concentration of 0.1% (v/v) C16 (triangles) and one with an equal amount of HMN (circles).

To investigate the recalcitrance of the α,β-chlorination pattern, adipic acid-grown *R.rhodochrous* cells were harvested in late exponential phase and subjected to 1 mM of either 1,2-DCD, 1,10-dichlorodecane, or 1,2,9,10-tetrachlorodecane (TCD). The results are summarized in Figure 2.

The recalcitrance of TCD compared to 1,2-DCD hints at the possibility that the degradative pathway begins with enzymatic action at the non-chlorinated end of the 1,2-DCD. As indicated by the relative ease with which 1,10-DCD is dechlorinated, the presence of a terminally

chlorinated carbon is not in itself a reason for recalcitrance. Thus, it appears that it is the presence of vicinal chlorinated carbons that prevents degradation of TCD and slows the rate of dechlorination of 1,2-DCD when compared to 1,10-DCD.

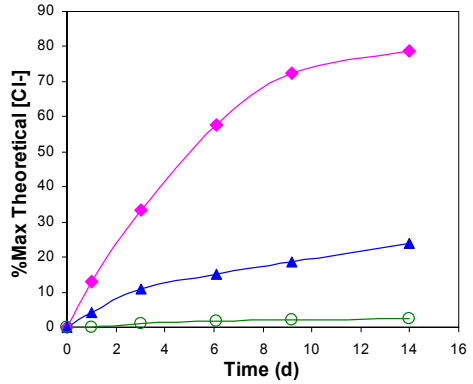

Figure 2. Chloride release from batch fermentations of adipic acid-grown *R. rhodochrous* spiked with 1 mM of either 1,10-dichlorodecane (diamonds), 1,2-DCD (triangles), or TCD (circles) during late exponential phase. Each data point represents the mean of duplicate flasks.

Dechlorination of 1,2-DCD by *P. oleovorans* was achieved in the presence of both succinic acid and octane as co-substrates (data not shown). To monitor the disappearance of 1,2-DCD during metabolism by the organism, a well-mixed cyclone bioreactor was employed. *P. oleovorans* was grown on octane to late exponential phase, at which point the growth medium was spiked with 1,2-DCD to a concentration of 1 mM. Figure 3 shows the resulting disappearance of 1,2-DCD and the accompanying chloride release.

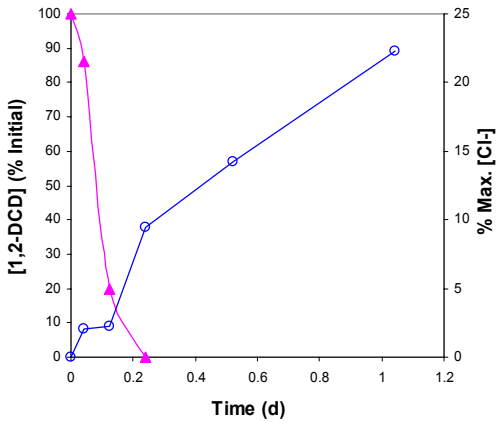

Figure 3. Disappearance of 1,2-DCD (triangles) and resulting chloride release (circles) measured in *P. oleovorans* cells grown to late exponential phase on 1% (v/v) octane and spiked with 1 mM 1,2-DCD. Fermentation conducted in a cyclone bioreactor.

The rapid disappearance of 1,2-DCD in the bioreactor is followed by chloride release. However, the latter is delayed, and continues even when no 1,2-DCD is detected in the growth medium. These observations indicate that, as in the case with *R. rhodochrous*, metabolism of 1,2-DCD appears to be initiated on the non-chlorinated end of the molecule. Previous studies have also offered evidence of similar pathways in the bacterial degradation of chlorinated alkanes. Upon their examination of 1-chlorohexadecane degradation by *R. rhodochrous* NCIMB 13064, Curragh *et al.* (9) postulated that metabolism was initiated on the non-chlorinated end of the molecule by an oxygenase enzyme. In a separate study, *Rhodococcus* sp. S45-1 degraded 1-chlorotetradecane to produce metabolites that suggested a similar metabolic pathway (7).

The ability of *P. oleovorans* to degrade C6-C12 n-alkanes has been extensively studied (10). The pathway begins with oxidation of the terminal methyl group to the corresponding terminal acyl-CoA derivative, which is then able to enter the β-oxidation cycle. Various strains of the *Rhodococcus* genus have been shown to possess enzymes, particularly alkane monooxygenases, capable of degrading n-alkanes of various chain lengths (11). It thus seems likely that these same enzymes are responsible for the initial step in metabolism of 1,2-DCD.

In order to assess the ability of each organism to dechlorinate α,β-dichloroalkanes of varying chain length, both *R. rhodochrous* and *P. oleovorans* were exposed to 1,2-dichlorooctane, 1,2-DCD, 1,2-dichlorododecane, 1,2-dichlorotridecane, 1,2-dichlorotetradecane, and 1,2-dichlorohexadecane. *R. rhodochrous* was grown to late exponential phase having adipic acid as a carbon and energy source and triplicate flasks were spiked with one of the α,β-dichloroalkanes to a concentration of 1 mM. After a 48 hour incubation period in a rotary shaker, the chloride concentration was measured. Abiotic controls consisting of autoclaved biomass were also monitored and the resulting abiotic chloride release was subtracted from that of the biotic flasks. The same procedure was repeated for *P. oleovorans* cells grown on octane. The results are summarized in Figure 4.

The resulting data exhibits dramatically different trends for each organism. The greatest rate of chloride release for *R. rhodochrous* occurs with 1,2-dichlorotridecane and 1,2-dichlorotetradecane. As the carbon chain length increases or decreases, the rate drops. Very little dechlorinating activity is observed with 1,2-dichlorooctane. This latter compound, however, is the most easily dechlorinated by *P. oleovorans*

when compared to the longer-chained α,β-dichloroalkanes. Minimal dechlorinating activity by *P. oleovorans* is observed with 1,2-dichlorohexadecane.

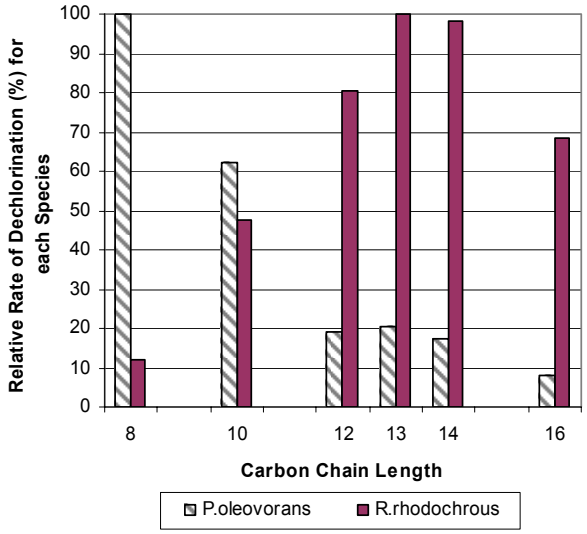

Figure 4. Relative rates of chloride release during fermentations of *R. rhodochrous* and *P. oleovorans* grown to late growth phase on adipic acid and octane, respectively, and spiked with 1mM of α,β-dichloroalkane. Rates for *R. rhodochrous* are taken relative to 1,2-dichlorotridecane, and those for *P. oleovorans* relative to 1,2-dichlorooctane.

The trends observed in the *R. rhodochrous* fermentations suggest that the organism has an optimum carbon chain length for dechlorination of α,β-dichloroalkanes. A number of studies have found that species within the *Rhodococcus* genus are able to degrade n-alkanes within a specific range of chain lengths. Rapp and Gabriel-Jürgens (12) determined that *Rhodococcus* sp. strain MS11 degraded n-alkanes with chain lengths of C10-C17 as readily as or better than those of shorter chain length, even though the latter compounds had greater water solubility. Sorkhoh *et al.* (13) found isolates *R. rhodochrous* KUCC 8801 and KUCC 8802 could grow on C10-C20 n-alkanes but not on those of shorter chain length. The recalcitrance of n-alkanes with chain lengths less than 10 may depend in part on the toxicity of these compounds (12). Furthermore, the production of biosurfactants to enhance the uptake of these compounds may play a factor. *Rhodococcus* sp. strain MS11 produced biosurfactants in the presence of C10 to C17 n-alkanes (12). *P. oleovorans* has been shown to possess an oxygenase enzyme capable of oxidizing C6-C12 n-alkanes (10). Although these studies were conducted on non-chlorinated n-alkanes, the trends are similar to those observed in with α,β-dichloroalkanes, suggesting that similar enzymes are involved in the degradation of both classes of compounds.

Conclusions

The present work presents two organisms, *R. rhodochrous* ATCC 13808 and *P. oleovorans* ATCC 29347, capable of degrading α,β-dichloroalkanes. Although the metabolic pathway for both organisms appears to begin with oxidation of the non-chlorinated terminus of the molecule, the preferred range of carbon chain length varies for each organism. In considering the environmental fate of PCAs, it is thus necessary not only to consider the chlorination pattern found on the n-alkane, but also the chain length of the compounds involved.

The inability of 1,2,9,10-TCD to be degraded demonstrates the recalcitrance imposed by vicinal chlorinated carbon atoms. In the case of α,β-dichloralkanes, the non-chlorinated end of the molecule allows a pathway to be initiated which eventually leads to dechlorination. Further study into identification and characterization of the enzymes involved in the dehalogenation step of the pathway is required.

Acknowledgements

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Bibliography

1. US-Environmental-Protection-Agency, *List of Toxic Chemicals within the Polychlorinated Alkanes Category and Guidance for Reporting*. 1999, US Environmental Protection Agency, 1-14.
2. Environment-Canada, *Priority Substances List Assessment Report: Chlorinated Paraffins*. 1993. p. 1-26.
3. Madeley, J.R.B., D. N. Robin, *Chlorinated Paraffins and the Environment.* American Chemical Society, 1980. **14**(10): p. 1215-1221.
4. Vogel, T.M., C.S. Criddle, and P.L. McCarty, *Transformation of halogenated aliphatic compounds.* Environmental Science Technology, 1987. **21**(8): p. 722 - 736.
5. Tomy, G.T., et al., *Levels of C10-C13 Polychloro-n-Alkanes in Marine Mammals from the Arctic and the St. Lawrence River Estuary.* Environmental Science & Technology, 2000. **34**(9): p. 1615-1619.
6. Omori, T., T. Kimura, and T. Kodama, *Bacterial Cometabolic Degradation of Chlorinated Paraffins.* Applied Microbiology and Biotechnology, 1987. **25**: p. 553-557.

7. Allpress, J.D., and P. C. Gowland, *Biodegradation of Chlorinated paraffins and long-chain chloroalkanes by Rodococcus sp. S45-1.* International Biodeterioration & Biodegradation, 1999. **43**: p. 173-179.
8. Wischnak, C. and R. Muller, *Degradation of Chlorinated Compounds.* Biotechnology, 1999. 11b Environmental Processes II - Soil Decontamination: p. 243-271.
9. Curragh, H., et al., *Haloalkane degradation and assimilation by Rhodococcus rhodochrous NCIMB 13064.* Microbiology, 1994. **140**: p. 1433-1442.
10. van Beilen, J.B., Wubbolts, M.G., and B. Witholt, *Genetics of alkane oxidation by Pseudomonas oleovorans.* Biodegradation, 1994. **5**(3-4): p. 161-174.
11. Whyte, L.G., Smits, T.H.M., Labbe, D., Witholt, B., Greer, C.W., and J.B. van Beilen, *Gene cloning and characterization of multiple alkane hydroxylase systems in Rhodococcus strains Q15 and NRRL B-16531.* Applied and Environmental Microbiology, 2002. **68**(12): p. 5933-5942.
12. Rapp, P. and L.H.E. Gabriel-Jürgens, *Degradation of alkanes and highly chlorinated benzenes, and production of biosurfactants, by a psychrophilic Rhodococcus sp. and genetic characterization of its chlorobenzene dioxygenase.* Microbiology, 2003. **149**: p. 2879-2890.
13. Sorkhoh, N.A., et al., *Crude oil and hydrocarbon-degrading strains of Rhodococcus rhodochrous isolated from soil and marine environments in Kuwait.* Environmental Pollution, 1990. **65**: p. 1-17