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ORIGINAL PAPER

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Isopropanol and acetone induces vinyl chloride degradation in *Rhodococcus rhodochrous*

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Abstract In situ bioremediation of vinyl chloride (VC)-contaminated waste sites requires a microorganism capable of degrading VC. While propane will induce an oxygenase to accomplish this goal, its use as a primary substrate in bioremediation is complicated by its flammability and low water solubility. This study demonstrates that two degradation products of propane, isoproponal and acetone, can induce the enzymes in *Rhodococcus rhodochrous* that degrade VC. Additionally, a reasonable number of cells for bioremediation can be grown on conventional solid bacteriological media (nutrient agar, tryptic soy agar, plate count agar) in an average microbiological laboratory and then induced to produce the necessary enzymes by incubation of a resting cell suspension with isopropanol or acetone. Since acetone is more volatile than isopropanol and has other undesirable characteristics, isopropanol is the inducer of choice. It offers a non-toxic, water-soluble, relatively inexpensive alternative to propane for in situ bioremediation of waste sites contaminated with VC.

Keywords Biodegradation · Isopropanol · Acetone · Vinyl chloride

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Introduction

Vinyl chloride (VC) is a human carcinogen [14] and is listed by the United States Environmental Protection Agency (USEPA) as a priority pollutant [7]. It is a colorless, flammable gas at ambient temperature and only slightly soluble in water. VC in the environment originates from the manufacture of polyvinyl chloride and the anaerobic microbial breakdown of dichloroethylene, trichloroethylene, and tetrachloroethylene [13]. Under anaerobic conditions, reductive dechlorination occurs, but at a relatively slow rate [8]. Hence, VC is known for its long-term persistence in soil [18]. However, VC is reported to be cometabolized with such primary substrates as ethene [9], ethane [9], methane [2], propane [12], propylene [5], isoprene [6], and 3-chloropropanol [2]. Basically, all of these primary substrates (often referred to as the inducting agent) produce a non-selective oxygenase that is able to degrade VC. There have also been reports of aerobic biodegradation of VC within environmental samples [4], but it is unclear whether the VC was serving as a primary substrate or was degraded cometabolically. While organisms capable of growth on VC as a sole carbon and energy source have been reported, VC-degrading ability appears to be unstable [10]. An aerobic isolate capable of growth on VC that retained its ability to use VC for at least 24 days has been described [17] but, in the absence of oxygen for 2–5 days, the culture did not regain the ability to grow on VC [17].

In dealing with VC-contaminated waste sites, one of the protocols for bioremediation involves seeding the area with propane-utilizing microorganisms and stimulating their growth in situ by sparging the area with propane and oxygen [3]. The propane elicits the production of an oxygenase that attacks the VC molecule but, obviously, growth of the microorganism in situ is dependent on both a nitrogen and a phosphorous source in addition to the primary substrate.

As cited above, a number of compounds have been reported to be primary substrates, but the gases pose operational problems in the field. These include the need for specialized gas transfer unit processes, high volumes of water having to be introduced into the biologically active zone due to the low solubility of propane in water, and the handling of an explosive gas that could build up under man-made surface features such as building foundations. Further, since the nitrogen and phosphorous sources are water-soluble, it is highly desirable to have a non-toxic, water-soluble, primary substrate that requires only a mixing step which could be done at the same process point as nutrient-amending. In this connection, it is noteworthy to point out that resting cells of a *Mycobacterium* sp. grown on isopropanol metabolized propane, suggesting that metabolism of isopropanol also elicits production of an oxygenase [15]. Furthermore, since isopropanol is metabolized via acetone, it is probable that acetone can also elicit the production of an oxygenase [15, 16, 19]. In this light, both isopropanol and acetone are highly soluble in water, are not considered a hazardous waste by the USEPA, and are highly transportable within soil matrices due to their high aqueous solubilities. As stated earlier, these characteristics make them excellent candidates for use as inducting agents for stimulating in situ bioremediation of VC within contaminated aquifers.

Another challenge during the application of in situ bioremediation to contaminated aquifers is the provision of appropriate levels of microorganisms capable of producing targeted oxygenases via metabolism of the inducting agents at sites that do not naturally contain these organisms. Preparing a sufficient number of microorganisms to seed such an area poses some technological problems. For example, in most laboratory settings, the production of the number of cells needed is not economically feasible, since the conventional method of cultivating large numbers of cells using large fermentors is expensive and outside of the capability of most laboratories. Therefore, the question arises as to whether the cells can be grown on conventional solid bacteriological media and then induced to produce enzymes to degrade VC by exposing the cells to an inducing substrate. Growth on a solid medium using propane requires a closed system and can be time-consuming. Furthermore, large numbers of cells cannot be grown easily on solid media when either isopropanol or acetone is the substrate, because of the volatility of the compounds. Hence, closed systems are required.

Therefore, the objectives of this investigation were to determine whether: (1) isopropanol- and acetone-grown cells of *Rhodococcus rhodochrous* can degrade VC, and (2) resting cell suspensions of *R. rhodochrous* grown on conventional bacteriological media can be induced to degrade VC.

Materials and methods

Microorganism and culture conditions

The microorganism employed was *R. rhodochrous* (ATCC 21198) obtained from the American Type Culture Collection (Manassas, Va.). The organism was cultivated in a mineral salts medium (MSM) containing 1.0 g of NH_4Cl, 0.38 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.05 g of $FeCl_3 \cdot 6H_2O$ per liter of distilled water. The pH was adjusted to 7.0 with 10% KOH (w/v). The MSM was dispensed in 50-ml amounts into 6-oz (ca. 180-ml) prescription bottles and autoclaved at 121 °C for 15 min. Mineral salts agar (MSA) was prepared by adding 17 g of Bacto agar (granulated; Difco Laboratories, Detroit, Mich.) per liter of MSM. Bottle slants contained 30 ml of MSA in 6-oz prescription bottles with the agar solidified on the flat side of the bottle. When propane was used as the carbon source, the bottle was stoppered with a serum stopper and the atmosphere was replaced with a gas mixture containing 65% propane, 30% oxygen, and 5% carbon dioxide. When isopropanol or acetone was used as the carbon source, 0.2 ml of pure isopropanol or acetone diluted in 2 ml of water was added to the side of a capped bottle slant opposite to the agar slant.

Nutrient agar (NA), tryptic soy agar (TSA), and plate count agar (PCA) were obtained from Difco Laboratories.

Stock cultures of *R. rhodochrous* were maintained in 6-oz bottles containing MSM with an atmosphere of propane, oxygen, and carbon dioxide. After incubation for 5 days at 30 °C, the cultures were streaked on NA, to insure purity, and then stored at 4 °C.

Preparation of resting cell suspensions

A *R. rhodochrous* cell suspension (100 µl) from the stock culture was inoculated onto the agar surface of each of 20 MSA bottle slants. Propane, isopropanol, or acetone was employed as the carbon source. The cultures were incubated for 5 days at 30 °C.

For cultures grown on conventional bacteriological media, 100 µl of *R. rhodochrous* stock culture were spread onto the agar surface of each of 20 Petri plates containing NA, TSA, or PCA and incubated for 48 h at 30 °C. After incubation, cells were harvested by washing them from the agar surface with physiological saline [0.85% NaCl (w/v)] and centrifuging at 2,500 *g* for 20 min. The supernatant was decanted, the cellular pellet was washed two times in 0.15 M sodium phosphate buffer, and the pellet was resuspended in the buffer.

The protein content of cell suspensions was determined using the method described by Lowry et al. [11].

VC-degrading ability of *R. rhodochrous* cells grown on propane, isopropanol, or acetone

A resting cell suspension (2 ml) of *R. rhodochrous* grown on propane, isopropanol, or acetone was transferred to a 6.7-ml vial capped with a 13-mm Mininert valve. The atmosphere of each vial was supplemented with 9.4 µmol of oxygen and 8.9 µmol of VC. Endogenous controls were employed with no added carbon source and were subtracted from all values reported. Abiotic controls containing VC but no cells were also employed. The samples were incubated on a rotary shaker at 96 rpm at 30 °C and the atmospheres of the vials were analyzed for consumption of oxygen and VC and production of carbon dioxide after 1 h and 2 h of incubation. A 50 µl sample of the headspace was removed through the Mininert valve and analyzed using a Fisher Gas Partitioner model 1200 (dual column, dual detector GC). Column 1 was a 610×0.3 cm aluminum column packed with 37.5% DC 200/500 mesh chromosorb P-AW. Column 2 was a 180×0.5 cm aluminum column packed with 60/80 mesh molecular sieve, 13X. Helium was

Table 1 The ability of propane-grown *Rhodococcus rhodochrous* cells to use isopropanol, acetone, and vinyl chloride. The carbon dioxide produced (0.05 μmol) and oxygen (0.21 μmol) consumed by the endogenous controls were subtracted from the values shown (mean ± SD). *NA* Not applicable

Substrate	Carbon dioxide produced (μmol CO_2 mg^{-1} protein h^{-1})	Oxygen consumed (μmol O_2 mg^{-1} protein h^{-1})	Vinyl chloride consumed (μmol vinyl chloride mg^{-1} protein h^{-1})
Isopropanol	1.70 ± 0.0	1.6 ± 0.27	NA
Acetone	0.12 ± 0.03	0.62 ± 0.10	NA
Vinyl chloride	0.03 ± 0.0	0.02 ± 0.0	0.40 ± 0.0

used as the carrier gas at a flow rate of 35 ml min^{-1}. Quantitation of components was accomplished by comparing the area under the curve for a given gas with a standard curve prepared using a pure sample of that gas. The volumes of VC and oxygen consumed and carbon dioxide produced were calculated and all tests were conducted in triplicate.

In the experiment conducted to confirm that oxygen was required for VC degradation by propane-grown cells, only 4.5 μmol of VC were added per vial. Anaerobic conditions in some vials were achieved by bubbling nitrogen gas into the cell suspensions for 4 h prior to capping the vials in an anaerobic chamber. The VC content in the atmosphere of the vials was determined as described above.

VC-degrading ability of *R. rhodochrous* cells grown on NA, TSA, or PCA after treatment with either isopropanol or acetone

Resting cell suspension (10 ml) from cells grown on NA, TSA, or PCA was incubated with either isopropanol or acetone in a 2-oz (ca. 60-ml) prescription bottle containing 30 ml of phosphate buffer and 25 μmol or 50 μmol of isopropanol or acetone for 18 h at 30 °C under shake conditions. After incubation, the cells were collected by centrifugation, washed twice in 50 ml of phosphate buffer, and suspended in 50 ml of the buffer. Aliquots (2 ml) of this cell suspension were transferred to 6.7-ml vials capped with 13-mm Mininert valves. The atmosphere of each vial was supplemented with 9.4 μmol of oxygen and 8.9 μmol of VC. The samples were incubated under shake conditions at 30 °C and the atmosphere of the vials was analyzed for VC consumption over 2 h at 30-min intervals, using 50-μl samples of the headspace removed through the Mininert valve, and analyzed using the GC as described above.

Results and discussion

Resting cell studies

Resting cell suspensions of propane-grown *R. rhodochrous* were able to metabolize VC as well as isopropanol and acetone, as shown in Table 1. It is widely reported that VC is degraded by an oxygenase [5, 12]; and it is known that the initial attack on propane by *R. rhodochrous* is via an oxygenase [1]. Therefore, it seemed desirable to determine whether VC degradation by propane-grown *R. rhodochrous* required oxygen for the initial attack. To do this, propane-grown cells suspensions were offered VC in the presence of, and in the absence of, molecular oxygen. No VC was degraded in the absence of oxygen even after 3 h, while aerobically 0.41 μmol VC mg^{-1} cell protein were degraded in 1 h and 1.09 μmol VC mg^{-1} cell protein were degraded in 3 h. Additionally, it was found that *R. rhodochrous* grew on both isopropanol and acetone, although the growth obtained was less than that obtained using propane (Table 2). Cells grown on isopropanol used acetone as expected and used 75% as much propane (0.65 μmol propane mg^{-1} protein h^{-1}) as propane-grown cells (0.87 μmol propane mg $protein^{-1}$ h^{-1}). Furthermore, acetone-grown cells also used as much propane as did the propane-grown cells (0.90 μmol propane mg^{-1} protein h^{-1}, 0.87 μmol propane mg^{-1} protein h^{-1}, respectively). Thus, the fact that isopropanol-grown cells and acetone-grown cells use propane suggests that an oxygenase is involved in their metabolism, as reported by others [19]. Therefore, the question arises as to whether isopropanol or metabolites of isopropanol induce cells to degrade VC. Subsequent experiments showed that resting cell suspensions of isopropanol-grown cells and acetone-grown cells could indeed degrade VC (Table 3). Since isopropanol is converted to acetone via a dehydrogenase, not an oxygenase, the oxygenase must attack either the acetone directly or a degradation product of acetone, most probably acetol. Acetone is reported to be converted to acetol by an oxygenase [17, 19]. Finally, the rate of VC degradation per milligram of cell protein was approximately the same for both propane- and isopropanol-induced cells, but significantly less for acetone-induced cells (approximately 50% less than the two others, as shown in Table 3). This could be due to the toxicity of the acetone.

For in situ bioremediation of VC-contaminated waste sites, isopropanol would be the primary substrate of choice. Compared with propane, it is far easier to handle and less hazardous. Compared with acetone, it supported degradation of more VC per mass unit added than acetone. Additionally, at ambient temperature and

Table 2 Protein content of *R. rhodochrous* cells grown on propane, isopropanol, and acetone for 5 days. Values given represent total growth from 20 bottle slants

Growth substrate	Protein produced in 5 days (mg)
Propane	3,910.1 ± 456.8
Isopropanol	743.4 ± 160.1
Acetone	789.7 ± 152.0

Table 3 Vinyl chloride utilization by *R. rhodochrous* cells grown on propane, isopropanol, and acetone

Growth substrate	Vinyl chloride consumed (μmol vinyl chloride mg^{-1} protein h^{-1})
Propane	0.40 ± 0.0
Isopropanol	0.35 ± 0.02
Acetone	0.16 ± 0.02

pressure, propane is a gas and acetone is more volatile than isopropanol and must be kept away from a variety of materials, like plastic eyeglasses, Rayon materials, and ignition sources. It is also interesting to consider that, when oxygen is supplied to the site in the form of H_2O_2, the primary substrate (inducting agent), nitrogen source, phosphorus source, and oxygen can be supplied in the same solution.

Induction of VC-degrading ability by *R. rhodochrous* cells grown on NA, TSA, or PCA

As noted previously, growth on isopropanol and acetone was considerably less than that obtained by growth on propane or conventional bacteriological media (i.e. NA, TSA, or PCA). Also, as easily observed visually, growth on conventional media is much greater in 2 days than it is when the organism is grown on propane, isopropanol, or acetone as the carbon source for 5 days. Unfortunately, growth on any of the three media (NA, TSA, or PCA) failed to stimulate VC-degrading ability by the cells, even after extended periods of exposure to VC. This indicated that the organisms growing on these substrates did not produce an enzyme capable of degrading VC or one that could be modified to do so. Even though cells grown on these media did not elicit the required enzyme, the question arises as to whether these cells in a resting state could be induced to produce a VC-degrading enzyme by exposure to isopropanol or acetone.

Cells grown on NA and TSA and then exposed to 25 μmol of isopropanol for 18 h were able to degrade 0.15 μmol and 0.18 μmol VC mg^{-1} protein, respectively, in 2 h. Cells grown on PCA showed very little ability to degrade VC when the cells were induced with 25 μmol of isopropanol, but showed excellent VC-degrading ability (0.17 μmol VC mg^{-1} protein in 2 h) when induced with 50 μmol of isopropanol. Cells grown on NA, TSA, or PCA were able to degrade 0.16 μmol, 0.19 μmol, and 0.34 μmol VC mg^{-1} protein in 2 h, respectively, after being induced with 50 μmol of acetone for 18 h.

Having found that *R. rhodochrous* can be grown on conventional solid microbiological media and then induced to produce a VC-degrading enzyme, the question arises as to how many cells can be produced in a microbiology laboratory in this fashion.

Studies showed that 500 Petri plates (95×15 mm) containing NA yields 1.2×10^{13} cells in 2 days. Diluted for field use, this was sufficient to prepare 11,350 l of inoculum containing 1.1×10^{6} cells ml^{-1}. While the number of cells needed for a bioremediation project will vary with the operator and the site, the above amount of cells was for an operator treating an area 6.1×1.8 m with a total aquifer volume of 1,700 m^3. Thus, a reasonable quantity of microorganisms needed for a field in situ bioremediation project can be produced in most microbiology laboratories. The need to concentrate cells, as is necessary when cells are produced in a fermentor, is obviated and the ease of shipping to the contaminated site is obvious. On conventional microbiological media, a much greater growth was obtained in less time compared with that obtained by growth on inducing agents, thus saving time and money. Also, after the *R. rhodochrous* cells were harvested in physiological saline, the agar plates could be reincubated and produce another harvest of cells (data not shown). In fact, the plates could be reincubated twice and still be capable of producing a large quantity of cells, thereby further reducing the cost of agar and Petri plates.

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