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Selectivity among organic sulfur compounds in one- and two-liquid-phase cultures of *Rhodococcus* sp. strain JVH1

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Abstract The selectivity of *Rhodococcus* sp. strain JVH1 among selected sulfidic and thiophenic compounds was investigated in both single-liquid-phase (aqueous) cultures and in two-liquid-phase cultures, where the sulfur compounds were dissolved in 2,2,4,4,6,8,8-heptamethylnonane as the immiscible organic carrier phase. In the single-liquid-phase cultures, *Rhodococcus* sp. strain JVH1 showed a preference for benzyl sulfide over both 1,4-dithiane and benzothiophene. An increased lag was observed in the degradation of benzyl sulfone and benzothiophene sulfone when both compounds were present. These results were consistent with a competitive inhibition mechanism, affecting both sulfur oxidation and carbon–sulfur bond cleavage. In the two-liquid-phase cultures, the effect of partitioning between the two liquid phases dominated the desulfurization activity of the culture. This partitioning resulted in an apparent absence of selectivity, as well as decreases in lag time, extent of degradation, and time to completion of degradation. Desulfurization activity also depended on the growth phase of the cultures. Mass transfer rate limitations were not observed at the low degradation rates of 0.02 mmol day^{-1} l^{-1}. Owing to the importance of partitioning, *Rhodococcus* sp. strain JVH1 is predicted to show nonselective activity towards the sulfur species in a whole crude oil.

Keywords Competitive inhibition · Crude oil · Heptamethylnonane · Organosulfur compounds · Partitioning · Substrate selectivity

Abbreviation
HMN 2,2,4,4,6,8,8-Heptamethylnonane

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Introduction

The organic sulfur species in crude oil include both thiophenic (aromatic) and sulfidic (aliphatic) compounds. The relative amounts of these species vary greatly among different oils, e.g., in the maltene fraction of Alberta heavy crude oils the sulfide-to-thiophene ratio, based on weight percent concentration, ranges from 0.22 (Pembina) to 2.1 (Peace River) (Strausz and Lown 2003). Most work on the biodegradation of

crude oil sulfur compounds has, however, focused on thiophenic model compounds such as dibenzothiophene. Because, the aliphatic sulfides can represent a significant proportion of the sulfur compounds, and may even be more abundant than the thiophenes in some oils, it is of interest to know whether the microorganisms used for biodegradation of crude oil exhibit a preference for thiophenic or sulfidic sulfur compounds. For example, *Rhodococcus* sp. strain K1bD showed a preference for the aromatic compound dibenzothiophene over the aliphatic compound 1,4-dithiane as a sulfur source (Kirkwood et al. 2005).

Rhodococcus sp. strain JVH1 uses several compounds with aliphatic sulfide linkages as sulfur sources for growth; these include dialkyl sulfides, aryl-terminated sulfides, and alicyclic sulfides (Van Hamme et al. 2004). *Rhodococcus* sp. strain JVH1 cannot use dibenzothiophene as a sulfur source, but does use benzothiophene and methylbenzothiophenes (Kirkwood 2006). The broad substrate range of *Rhodococcus* sp. strain JVH1 for organosulfur compounds may be useful for the bioremediation of sulfur-containing petroleum contaminants.

A biodegradation process for crude oil will, of necessity, be a two-liquid-phase system, because the microbial cells require an aqueous environment, and the hydrocarbons have low water solubility and will form a separate organic liquid phase. Two transport processes are essential to the biotransformation of substrates dissolved in a second liquid phase: first, molecules must transfer from the organic phase to the cells; and second, molecules must be transported across the cell membrane to access enzymes inside the cells (Bressler and Gray 2003). Both processes can affect biotransformation rates through the rates of mass transfer and through the equilibrium partitioning of the substrate between the two phases.

Partitioning has important effects on biotransformation in two-liquid-phase systems. The partition coefficient defines the relative concentrations of a substrate in two phases at equilibrium. For hydrophobic compounds, the organic concentration may be much higher than the aqueous concentration, which can affect biotransformation in two ways. First, partitioning may enhance biodegradation of toxic substrates by reducing the aqueous concentration below an inhibitory threshold (Déziel et al. 1999). Higher total substrate loadings can therefore be used and greater conversion can be achieved. This approach has been used for the biodegradation of compounds such as phenol and benzene (reviewed by Déziel et al. 1999). Partitioning may also be detrimental to biotransformation, if the aqueous concentration falls below a minimum threshold for enzymatic activity. The extent of conversion may then be reduced due to effective sequestering of the substrate in the organic phase (Efroymson and Alexander 1995). The role of such partitioning in selective degradation of different sulfur sources has not been reported.

This work investigated the selectivity of *Rhodococcus* sp. strain JVH1 among sulfidic and thiophenic compounds (Fig. 1), both in single-liquid-phase (aqueous) cultures and in two-liquid-phase cultures, where the sulfur compounds were dissolved in 2,2,4,4,6,8,8-heptamethylnonane (HMN). The results of these experiments allowed an initial assessment of the expected activity of *Rhodococcus* sp. strain JVH1 towards the sulfur species in a whole crude oil.

Materials and methods

Chemicals

Benzothiophene (99%), benzyl sulfide (98%), benzyl sulfone (99%), 1,4-dithiane (97%), and thianthrene (99+%) were from Aldrich (Oakville, Ont., Canada). HMN (min. 95%) and

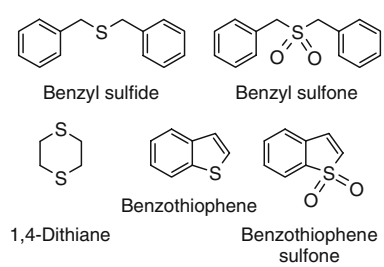

Fig. 1 Structures of organosulfur compounds used in this study

n-hexadecane (min. 99%) were from Sigma (St. Louis, MO, USA). *n*-Pentane (distilled in glass) was from Caledon (Georgetown, Ont., Canada). Dichloromethane and methanol (high-performance liquid chromatography grade) were from Fisher Scientific (Fairlawn, NJ, USA). Benzothiophene sulfone was synthesized as previously described (Bressler et al. 1999).

The commercial preparations of 1,4-dithiane and benzyl sulfide were further purified by recrystallization from methanol and methanol–water, respectively.

Growth media and amendments

Plate count agar was from BD (Franklin Lakes, NJ, USA), and was prepared using double-distilled water. Sulfate-free mineral medium with acetate was prepared in acid-washed glassware as previously described (Kirkwood et al. 2005).

Organic sulfur sources were added to sterile sulfate-free medium in a minimum volume of methanol or as filter-sterilized (0.22 μm) solutions in HMN (0.1 vol%), to give a final concentration of 0.05 mmol S l^{-1}.

Culture conditions

Rhodococcus sp. strain JVH1 was stored in glycerol at –70°C. To start an experiment, *Rhodococcus* sp. strain JVH1 was first streaked from frozen stocks onto plate count agar and grown for 3–14 days. A single colony was picked from the plate to inoculate 5 ml of sulfate-free medium with 1,4-dithiane as the sole sulfur source (0.025 mmol l^{-1}) in test tubes. After growth for 3 days, a portion of the culture was harvested by centrifugation, washed three times, and resuspended in an equal volume of potassium phosphate buffer (10 mM, pH 7). Aliquots of the final suspension were used to inoculate experimental cultures (0.4% inoculum by volume).

Cultures without HMN were grown in 5 ml of sulfate-free medium in culture tubes with PTFE-lined screw caps to prevent evaporative losses. Cultures with HMN were grown in 100 ml of sulfate-free medium in 250 ml Erlenmeyer flasks with PTFE-lined screw caps.

Tubes were incubated on a tube roller at approximately 30 rpm, and flasks were incubated on a rotary shaker at approximately 160 rpm. All cultures were incubated in the dark at 28°C.

Analytical techniques

After incubation, all cultures to be extracted were acidified with concentrated HCl to pH < 1, and either extracted immediately or stored at 4°C for up to 1 week until extraction.

For tube cultures, thianthrene was added as a surrogate standard and then the cultures were extracted by adding 2 ml of dichloromethane and mixing the two phases using a vortex mixer at high speed. After phase separation, the organic layer was transferred to a PTFE-lined screw-cap vial for analysis.

For the flask cultures with HMN, 5 ml of *n*-pentane was added to *n*-hexadecane in a small volume of dichloromethane as a surrogate standard. The flasks were swirled vigorously and the phases allowed to settle, then tap water was added below the pentane layer through a glass tube to raise the *n*-pentane layer into the neck of the flask. A sample of the organic layer was removed to a PTFE-lined screw-cap vial for analysis.

Culture extracts were analyzed for organic sulfur compounds by gas chromatography using a Hewlett-Packard 5890 series gas chromatograph equipped with an HP–1 capillary column (25 m length, 0.32 mm inner diameter, 0.17 μm film thickness) (Agilent Technologies, Wilmington, DE, USA) and a flame ionization detector, with helium as the carrier gas. The injector and detector temperatures were both 300°C; the oven temperature program started at 50°C for 2 min and then increased at 10°C/min up to 280°C.

Acetate was quantified using an adaptation of the method of Fedorak et al. (2002). Subsamples of culture medium (1 ml each) were taken at various times during incubation and frozen at –20°C. The frozen subsamples of culture medium were thawed

to room temperature, and diluted if necessary with deionized water to an acetate concentration between 25 and 250 mg l^{-1}. Ten microliters of 4 M H_3PO_4 and 10 µl of propionic acid stock (1,500 mg l^{-1}, surrogate standard) were added to 80 µl of the diluted sample for the analysis. Acetate was quantified in the prepared samples by gas chromatography using a Hewlett-Packard 5890 series gas chromatograph equipped with a DB-FFAP capillary column (30 m length, 0.53 mm inner diameter, 1 μm film thickness) (Agilent Technologies) and a flame ionization detector, with helium as the carrier gas. The injector, detector, and oven temperatures were 240, 250, and 120°C, respectively.

Results

Selectivity between thiophenic and sulfidic sulfur compounds in single-liquid-phase cultures

Rhodococcus sp. strain JVH1 was grown with binary combinations of benzothiophene, 1,4-dithiane, and benzyl sulfide as sulfur sources, and the degradation of each compound was compared to degradation as a sole sulfur source (Fig. 2). Benzothiophene and 1,4-dithiane were degraded at the same rate both as sole sulfur sources and when provided together to the culture as sulfur sources. In contrast, the biodegradation of both benzothiophene and 1,4-dithiane was delayed by the presence of benzyl sulfide in the culture medium. However, benzothiophene and 1,4-dithiane did not affect the degradation of benzyl sulfide. Degradation rates cannot be calculated precisely due to the few sample times used, especially for 1,4-dithiane, but were approximately 0.02 mmol day^{-1} l^{-1} for both benzothiophene and benzyl sulfide.

Selectivity was also tested using benzothiophene sulfone and benzyl sulfone (Fig. 3). An increased lag was observed in the degradation of the sulfones when both compounds were provided together to the culture.

Degradation and selectivity in two-liquid-phase cultures

Selectivity between benzyl sulfide and benzothiophene was further tested in two-liquid-phase cultures, to see if the same patterns were observed when the sulfur compounds were solubilized in a second liquid phase. The substrates were added in HMN as the carrier phase, giving 0.1% HMN by volume in the cultures. In the two-liquid-phase system, the degradation of benzothiophene was not affected by the presence of benzyl sulfide (Fig. 4), in contrast to the results shown in Fig. 2. Degradation ceased after 2 days, for benzyl sulfide the initial rate was not affected, but the extent of degradation decreased in the presence of benzothiophene (Fig. 4). Benzyl sulfide was fully degraded in 3 days when provided as the sole sulfur source. When benzothiophene was also present, only 75% of the benzyl sulfide was degraded, and this degradation ceased after 2 days. The degradation rates were calculated to be approximately 0.01 mmol day^{-1} l^{-1} for both compounds.

Visual inspection of the cultures suggested that growth was more rapid when benzothiophene was included as a sulfur source (alone or with benzyl sulfide) than when benzyl sulfide was the sole sulfur source. The increase in biomass could not be measured directly due to adhesion of the cells to the HMN layer. Instead, the concentration of acetate (the carbon source) in the cultures was determined at different time points as an indirect estimate of culture growth (Fig. 5). The consumption of acetate was similar in cultures containing benzothiophene (alone or with benzyl sulfide), whereas, an increased lag phase was observed when benzyl sulfide was the sole sulfur source.

Discussion

Although the degradation of benzyl sulfide by *Rhodococcus* sp. strain JVH1 was not affected by the presence of benzothiophene or 1,4-dithiane, degradation of the latter two compounds was

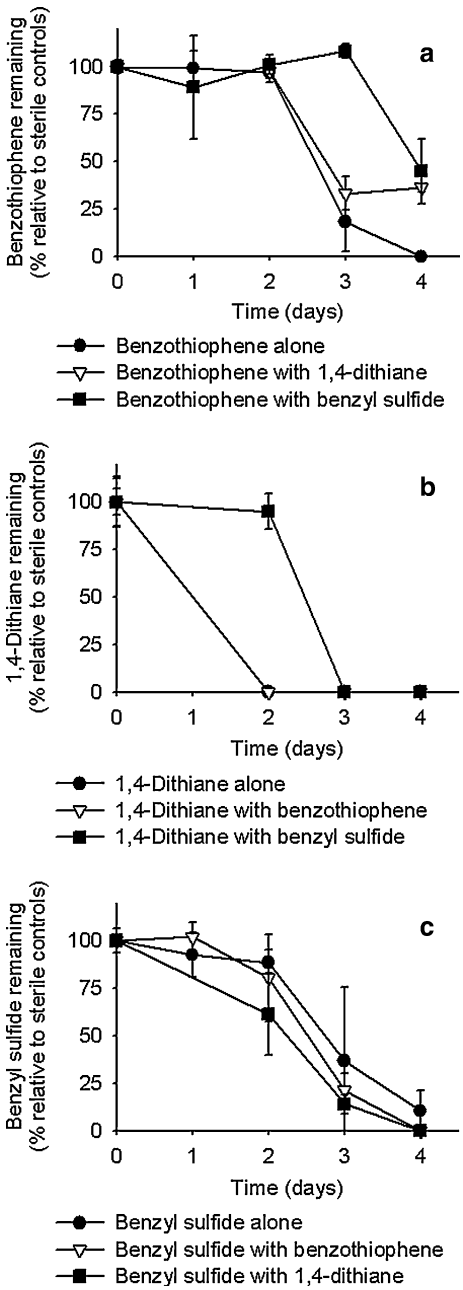

Fig. 2 Degradation of (**a**) benzothiophene, (**b**) 1,4-dithiane, and (**c**) benzyl sulfide when provided alone or in binary combinations as sole sulfur sources for *Rhodococcus* sp. strain JVH1 (average ± standard deviation, $n = 3$). Each compound was added at a concentration of 0.05 mmol S l^{-1}

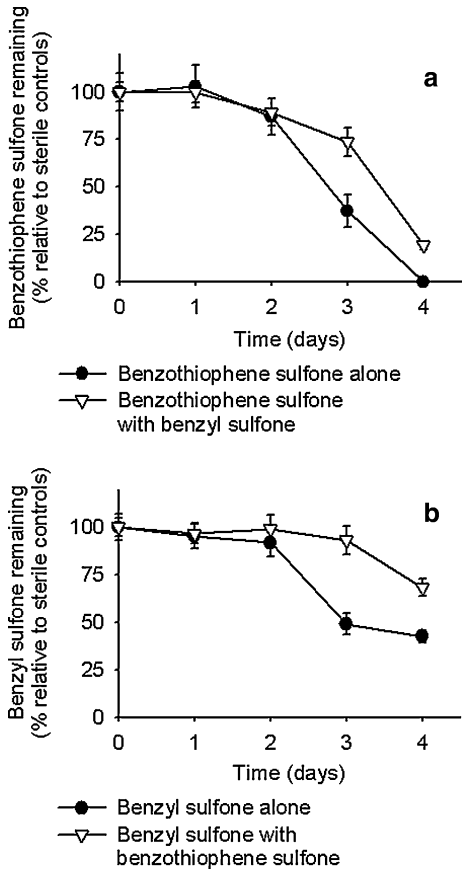

Fig. 3 Degradation of (**a**) benzothiophene sulfone and (**b**) benzyl sulfone when provided alone or together as sole sulfur sources for *Rhodococcus* sp. strain JVH1 (average ± standard deviation, $n = 3$). Each compound was added at a concentration of 0.05 mmol S l^{-1}

delayed in the presence of benzyl sulfide (Fig. 2). The desulfurization system of *Rhodococcus* sp. strain JVH1 did, therefore, exhibit some substrate preference for the aliphatic sulfur from benzyl sulfide over the aromatic sulfur in benzothiophene and the aliphatic sulfur in 1,4-dithiane. The lack of selectivity between 1,4-dithiane and benzothiophene suggests that the preferences of the organism could be due to the details of chemical structure rather than a simple distinction between aliphatic and aromatic sulfur species.

The degradation rates for both benzyl sulfone and benzothiophene sulfone were reduced when both compounds were added together to cultures of *Rhodococcus* sp. strain JVH1 (Fig. 3). These compounds are intermediates in the desulfurization pathways of benzyl sulfide and benzothiophene, respectively, and would be subject to attack by enzymes catalyzing carbon–sulfur bond cleavage reactions rather than the

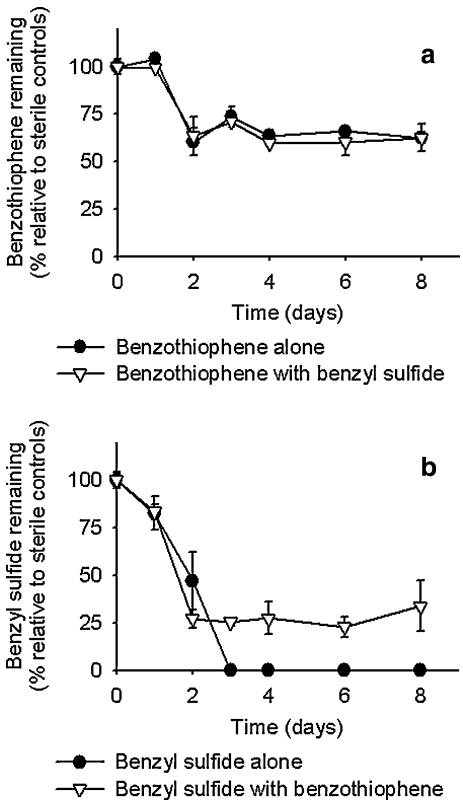

Fig. 4 Degradation of (**a**) benzothiophene and (**b**) benzyl sulfide when provided alone or together in HMN as sole sulfur sources for *Rhodococcus* sp. strain JVH1 (average ± standard deviation, $n = 3$). Each compound was added at a concentration of 0.05 mmol S l^{-1}

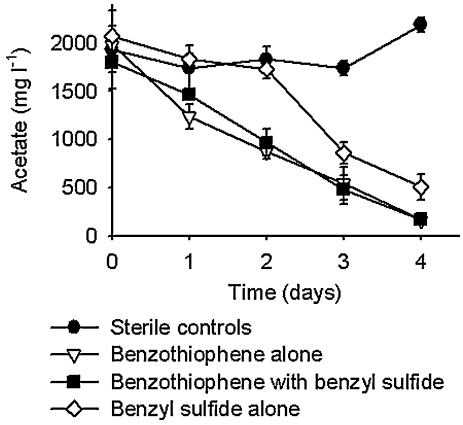

Fig. 5 Consumption of acetate in cultures of *Rhodococcus* sp. strain JVH1 grown with benzothiophene and benzyl sulfide in HMN as sole sulfur sources (average ± standard deviation, $n = 3$)

initial sulfur oxidation reactions. These results suggest competitive inhibition at both stages of the desulfurization pathway: sulfur oxidation, resulting in selectivity between benzyl sulfide and the other two reduced sulfur compounds; and carbon–sulfur bond cleavage, resulting in competition between the two sulfones.

A two-liquid-phase system is expected to have different mass transfer characteristics from the single-liquid-phase system, changing the availability of the sulfur compounds to the cells and therefore potentially the rate and extent of degradation. The addition of benzothiophene and benzyl sulfide dissolved in HMN and provided at a volume ratio of 0.1% resulted in decreases in selectivity, lag time, extent of degradation, and time to cessation of degradation compared with the single-liquid-phase aqueous system. In biodegradation studies, dissolution of an organic substrate in a second liquid phase is typically used for one of two purposes (Déziel et al. 1999): to reduce the aqueous-phase concentration and thus to promote biodegradation of a toxic compound; or to enhance the biodegradation of a compound with low aqueous solubility. In this work, however, the second liquid phase was used to simulate the use of organosulfur species in crude oil, and was therefore not necessarily expected to optimize the bioconversion of the model compounds.

The presence of a second liquid phase changes the rate of solubilization or mass transfer of the substrates into the aqueous phase, which can affect the overall degradation rate, and reduces the aqueous-phase concentration of the substrates through partitioning, which can affect the rate and extent of degradation. For example, both phenomena were seen to influence the degradation of anthracene by *Sphingomonas* sp. strain BA2 and of pyrene by *Gordonia*-like strain BP9 and *Mycobacterium gilvum* sp. strain VF1, where both the rate and extent of mineralization were decreased when the substrates were dissolved in HMN (Mutnuri et al. 2005). In both the single- and two-liquid-phase systems, degradation was completed over a period of 1 or 2 days, so mass transfer rate limitations do not appear to be a significant challenge to degradation in the two-liquid-phase system.

Partitioning between the organic and aqueous liquid phases could account for the lack of selectivity, decreased lag time, and decreased extent of degradation in the two-liquid-phase system. Benzothiophene and benzyl sulfide would be completely dissolved at 0.05 mmol l^{-1} in the aqueous phase in the single-liquid-phase system. In the two-liquid-phase system, the same nominal concentrations were used (0.05 mmol S l^{-1}), so both compounds were present below their aqueous solubility limits, but would be partitioned between the aqueous and organic phases. The octanol–water partition coefficient (log K_{ow}) for benzothiophene was reported to be 3.17 (Andersson and Schräder 1999), and for benzyl sulfide it is estimated to be 4.33 [Interactive LogKow (KowWin) Demo 2005]. The concentration of each compound would therefore be approximately 10^3–10^4 times lower in the aqueous phase than in the organic (HMN) phase in the two-liquid-phase system. Given a ratio of water:HMN of 1,000:1, this partitioning would reduce the aqueous-phase concentration by 50–90% compared to the single-liquid-phase system without HMN. The lowered aqueous concentration of benzyl sulfide may be too low to competitively inhibit the degradation of benzothiophene, removing any observable selectivity. Similarly, the lowered aqueous concentrations of benzyl sulfide and benzothiophene could relieve any inhibitory effect of the individual compounds on the growth of the culture, resulting in the reduced lag times. Finally, the lowered aqueous concentration of benzothiophene appears to be close to a minimum threshold for enzymatic activity or gene induction, accounting for the reduced extent of degradation for benzothiophene in the two-liquid-phase system.

Partitioning, however, cannot fully explain the extent of conversion of benzyl sulfide in HMN, which reached 100% when benzyl sulfide was the sole sulfur source, but was limited to 75% when benzothiophene was also present. The observed differences in culture growth with the different substrate combinations could be related to the differences in benzyl sulfide conversion. Growth of *Rhodococcus* sp. strain JVH1 was indirectly assessed by measuring the acetate concentration in the two-liquid-phase cultures. Acetate was consumed sooner when benzothiophene was present, whereas a longer lag was observed when benzyl sulfide was the sole sulfur source (Fig. 5). Also, conversion of the sulfur compounds had ceased in 2–3 days, while acetate was still being consumed by the cultures up to 4 days after inoculation. Although growth phases cannot be discerned from the acetate data, the continued consumption of acetate indicates that conversion of the sulfur compounds only occurred early in the growth of the cultures. Because growth was apparently more rapid in the cultures containing benzothiophene (Fig. 5), the active phase for conversion of sulfur compounds was shorter, resulting in incomplete conversion of benzyl sulfide (Fig. 4). This effect could also have contributed to the reduced conversion of benzothiophene in the two-liquid-phase system (Fig. 4) compared with the single-liquid-phase system (Fig. 2).

Conclusions

It was previously unknown whether the enzyme system used for desulfurization of the model aliphatic sulfide bis-(3-pentafluorophenylpropyl) sulfide by *Rhodococcus* sp. strain JVH1 (Van Hamme et al. 2004) is also used for desulfurization of other organic sulfur compounds. The experiments reported here showed that *Rhodococcus* sp. strain JVH1 exhibited selectivity, consistent with competitive inhibition, between some pairs of sulfur compounds in a single-liquid-phase system, confirming that the same enzyme system was used for more than one substrate. The observed selectivity could be due to competition for the degradative enzymes or membrane transport systems. However, selectivity was not observed in a two-liquid-phase system, most likely due to the reduced concentration of substrates in the aqueous phase, resulting from preferential partitioning of the sulfur compounds to the organic (HMN) phase. Therefore, in a system with a real crude oil, with partitioning between the organic and aqueous phases, *Rhodococcus* sp.

strain JVH1 would be predicted to exhibit broad, nonselective activity towards available sulfur groups within the substrate range of the microorganism.

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