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Kinetic coefficients for simultaneous reduction of sulfate and uranium by *Desulfovibrio desulfuricans*

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Abstract Previously it was demonstrated that bacteria are capable of transforming soluble uranyl ion, U(VI), to insoluble uraninite, U(IV); however, the rate for this transformation has not been determined. We report the kinetic coefficients for *Desulfovibrio desulfuricans* DSM 1924 grown in a continuous-flow chemostat where pyruvate was the electron donor and sulfate was the electron acceptor. The medium was supplemented with 1 mM uranyl nitrate, and the chemostat flow rate ranged from 1.12 ml/h to 4.75 ml/h with incubation at 28°C. The maximum rate of pyruvate utilization (k) was determined to be 4.7 days^{-1}, while the half-velocity constant (K_s) was 127 mg/l. The yield coefficient (Y) of cells per mole of pyruvate oxidized was calculated to be 0.021 g, while the endogenous decay coefficient (k_d) was determined to be 0.072 days^{-1}. More than 90% of U(VI) was transformed to U(VI) in the chemostat under the conditions employed.

Introduction

In areas where uranium has been milled, the ground water frequently contains soluble uranium levels that exceed levels acceptable for health (Thomson and Heggen, 1983). One of the systems proposed for the remediation of uranium-containing ground water is to transform the soluble uranyl ion, U(VI), to insoluble uraninite, U(IV), through the use of bacteria. Cultures of *Desulfovibrio desulfuricans* (Lovley and Phillips 1992), *Geobacter metallireducens* (Lovley et al. 1991), *Shewanella putrefaciens* (Lovley and Phillips 1992), and *Clostridium* sp. (Francis et al. 1994) have been demonstrated to transform U(VI). To be an effective bioremediation process, however, the bacteria must be able to grow in the presence of U(VI) and to transform UO_2^{2+}, U(VI), to UO_2, U(IV), at an appreciable rate. The purpose of this study is to evaluate the kinetics of growth and uranium reduction in a continuous culture of a sulfate-reducing bacterium.

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Materials and methods

Culture

The cultivation of *D. desulfuricans* DSM 1924 was in a growth medium containing the following in g/l: sodium pyruvate, 11.0; NH_4Cl, 2.0; Na_2SO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.5; $FeSO_4$, 0.02; and yeast extract (Difco, Detroit, Mich., USA). 1.0. The medium was adjusted to pH 7.4 with 20% KOH. For experiments using U(VI), uranyl nitrate solution was autoclaved separately and added to the sterile growth medium to give a final concentration of 1.0 mM. Immediately after autoclaving, the media were flushed with purified N_2 to displace molecular O_2. Resazurin was added to the medium at 1 mg/l. Maintaining this redox indicator in the colorless form confirmed an oxidation-reduction potential in the medium of approximately − 81 mV.

Chemostat system

The system employed for growth analysis is shown in Fig. 1. The culture vessel of the chemostat was a 125-ml Erlenmeyer flask; the volume of culture medium in it was 100 ml. Rubber stoppers with glass tubing were placed in the tops of the flasks and Nalgene tubing (1/8 inch or 3 mm i.d.) was used to make connections between the flasks. The purpose of the vacuum relief and isolation tanks, both 1-l Erlenmeyer flasks, was to prevent the formation of a vacuum in the influent tank as the medium was withdrawn by the pump. The pressure relief tank was a 1-l Erlenmeyer flask and it was used to prevent an increase of pressure in the effluent tank as it received discharge from the reactor. The influent and chemostat fluids were mixed with magnetic stirrers controlled by automatic timers. Every 30 min the timers would activate the stirrers for 2 min before the pump was started and the stirrer would continue for 2 min after the pump had ceased. The duration of the pump cycle was 5 min. The

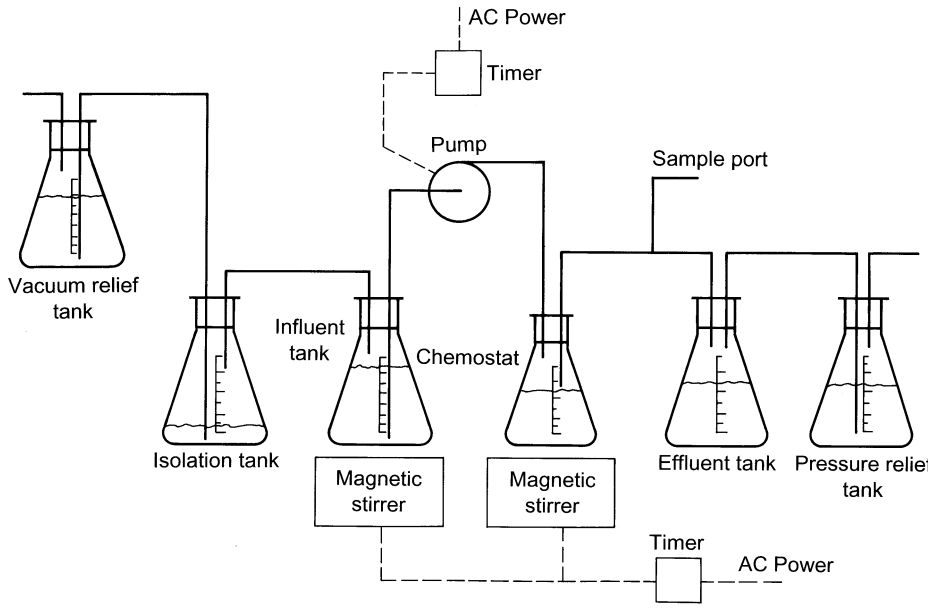

Fig. 1 Experimental design for the continuous culture system

atmosphere above each solution in the flasks was purged with purified N_2 gas for at least 10 min before the start of an experiment. Samples were collected periodically and analyzed to determine when the steady-state activity was achieved.

Analytical methods

Colorimetric procedures were used to determine pyruvate (Friedman and Haugen 1943) and uranium concentrations (Meloan et al. 1960) in the culture medium. For biomass determination, an aliquot of 20 ml was taken from the culture and the cells were collected by centrifugation at 10 min at 5,000 *g*. Cell protein was determined with the phenol reagent (Lowry et al. 1951) and conversion of cell protein to cell dry weight was made using a cell standard. Sulfate in the culture medium was measured with a Dionex 2010i ion chromatograph (Dionex, Sunnyvale, Calif., USA) following the procedure 4500–SO_4^{2-} B (American Public Health Association 1992).

Samples were taken from the sample port of the chemostat after each experimental run as well as from the growth medium. The 8-ml sample was divided into four replicates of 2 ml each to be used for the U(VI) assay. Another 4-ml sample was centrifuged and the centrifugate was used for the pyruvate assay, while the cell pellet was used to determine biomass by the protein method. The efficiency of uranium removal was calculated according to Eq. 1.

$$\eta = \left(\frac{[U]_{in} - [U]_{out}}{[U]_{in}}\right)(100\%) \quad (1)$$

Calculation of coefficients

The values for kinetic coefficients were determined according to Monod Kinetics. The maximum rate of pyruvate utilization, k, and the half-velocity constant, K_g, were determined by performing a mass balance on the pyruvate in the chemostat and employing Eq. 2 (Metcalf and Eddy 1991).

$$\frac{X\theta}{S_0 - S} = \frac{K}{k}\frac{1}{S} + \frac{1}{k} \quad (2)$$

The components in Eq. 2 refer to the biomass concentration in the chemostat, X; influent substrate concentration, S_o, substrate concentration in the chemostat, S; and hydraulic residence time (in h), θ. The inverse of the unit rate of substance disappearance ($X\theta/S_o - S$) was plotted versus $1/S$. This plot is commonly referred to as a Lineweaver-Burke graph. A straight line was fitted to the data points using linear regression. The values of k and K_s were determined from the plot since the slope of the line is equal to K_s/k and the y-intercept of the line is equal to $1/k$.

The values for the specific yield, Y, and the endogenous decay coefficient, k_d were also determined by plotting the experimental data on a linear scale using Eq. 3 (Metcalf and Eddy 1991), which was obtained from a mass balance on the biomass in the system. A straight line was fitted to the data points. The slope of this line is equal to Y and the y-intercept of the line is equal to $-k_d$.

$$\frac{1}{\theta} = Y\frac{S_o - S}{X\theta} - k_d \quad (3)$$

The yield coefficients for cell energetics were calculated according to procedures previously described (Magee et al. 1978; Vosjan 1975). The yield coefficient, Y_s, refers to the amount of bacterial mass produced in terms of dry weight (in g) for each mole of pyruvate utilized. Y_{ps} is the expression of cell protein produced (in mg) for each mole of pyruvate oxidized.

Electron microscopy

Samples were taken from the chemostat and placed directly on formvar-covered grids. After a short drying time of a few hours, the sample was examined with a Jeol 2000FX transmission electron microscope with a Noran 5500 electron dispersive spectroscopy attachment. Procedures were followed as described by Beeston (1972).

Results

The mathematical model developed for this study of *D. desulfuricans* is based on the assumption that growth in the chemostat is pyruvate limited. In previous reports, it was determined that 10 mg/l (Middleton and Lawrence 1977) and 17 mg/l (Pomeroy and Bowlus

1946) sulfate was needed in the chemostat to ensure that the growth is not limited by an electron acceptor. In our experiments the residence time had a marked effect on the amount of sulfate remaining in the chemostat. With residence times of 89.3 h and 21.1 h, the amount of sulfate remaining was 25 mg/l and 80 mg/l, respectively. While 10.8 g/l of sodium pyruvate entered the chemostat, 4.25 g/l and 0.10/l were in the effluent when the residence times were 21.1 h and 79.3 h, respectively. Clearly, the kinetics obtained in this study at > 79 h residence time would be characteristic of *D. desulfuricans* growing under conditions of limiting electron donor.

Information in Figs 2 and 3 is based on experiments where bacteria were grown in medium containing both sulfate and uranyl ions. Figure 2 illustrates the Lineweaver-Burke plot that was used to calculate k and K_s. The plot used to calculate Y and k_d is shown in Fig. 3. The r^2 values for linear fit to the data in Figs. 2 and 3 were 0.89 and 0.98, respectively. The yield coefficient for cell mass (in g) per mole pyruvate oxidized (Y_s) was calculated to be 0.021, and k_d was determined to be 0.079 days^{-1}. k was determined to be 4.7 days^{-1} and K_s was 127 mg/l. As shown in Fig. 4, a direct relationship was observed between the efficiency of uranium removal and residence time in the chemostat.

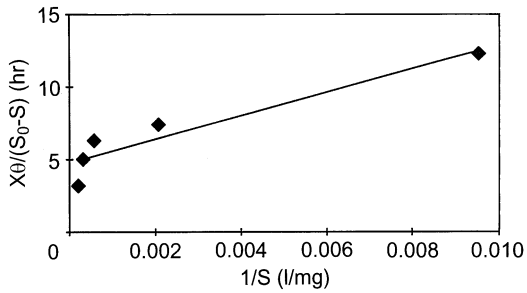

Fig. 2 Plot for the determination of the maximum rate of pyruvate utilization (k) and the half-velocity constant (K_s) for growth of *Desulfovibrio desulfuricans* in the presence of uranium. See Materials and methods for definitions of parameters

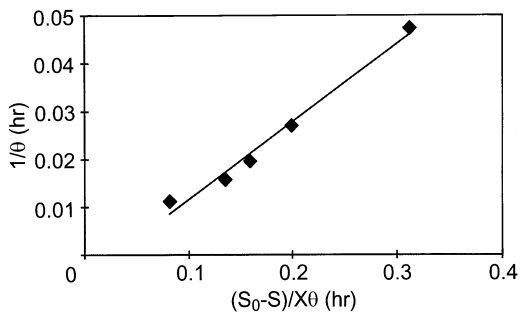

Fig. 3 Plot for the determination of the specific yield (Y) and the endogenous decay coefficient (K_d) for growth of *D. desulfuricans* in the presence of uranium. See Materials and methods for definitions of parameters

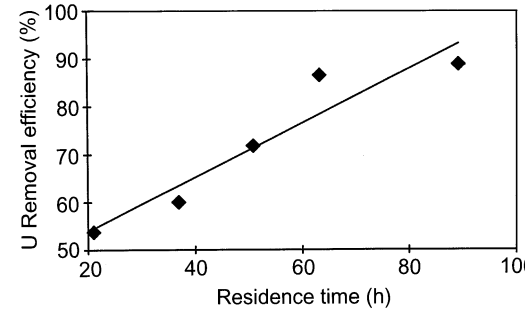

Fig. 4 The conversion of uranyl ion, U(VI), to insoluble uraninite, U(IV), as a function of residence time in the chemostat

Table 1 Yield values for *Desulfovibrio desulfuricans* growing in continuous culture with and without uranium

Parameter	Residence time (h)			
	21		79	
U(VI) present	Yes	No	Yes	No
Yield of *D. desulfuricans* (g dry wt/l cells)	0.53	0.706	0.885	1.119
Na pyruvate used (mol/l)	0.059	0.064	0.104	0.108
Protein production (g/mol of pyruvate)	15.191	18.238	14.198	17.222
Yield of *D. desulfuricans* (g dry weight/mol pyruvate)	9.122	10.949	8.518	10.333

The growth yields for *D. desulfuricans* were calculated and the resulting yield values are presented in Table 1. At two different residence times, the presence of U(VI) markedly reduced the cell yield as measured by gram protein or gram dry weight per mole of pyruvate used. This is the first report to provide quantitative information indicating the inhibition of bacterial growth by U(VI). The ratio of sulfate of U(VI) employed in these experiments was based on information by Lovley et al. (1991) and verified in our laboratory. Reduction of uranium by sulfate-reducing bacteria proceeds optimally at molar ratios of approximately 4:1.

Examination of a sample taken from the culture by electron microscopy revealed considerable amorphous material. The electron diffraction pattern was characteristic of uraninite (UO_2). A high correlation was found between the sample taken from the chemostat and uraninite in that the four rings of electron diffraction for the sample are markedly similar to the pattern for uraninite (Table 2).

Discussion

There has been considerable interest in the use of bacteria in the remediation of areas contaminated with

Table 2 Comparison of measured electron diffraction pattern to standard values for UO_2

Ring[a]	Measured Diameter (nm) of sample	Known values for UO_2 diameter (nm)[b]	Difference
111	0.325	0.315	3.2%
002	0.286	0.273	4.7%
022	0.201	0.193	4.1%
113	0.164	0.165	0.6%

[a] Listed in decreasing intensity
[b] JCPDS – International Centre for Diffraction Data, 1979

uranium such as uranium mill tailings sites or weapons firing sites. The strategies proposed are to immobilize uranium through the transformation of soluble U(VI) to insoluble U(IV) by bacteria either coupled to an extraction procedure (Phillips et al. 1995) or as an in situ bio-treatment system (Barton et al. 1995). While other reports have identified bacteria capable of producing U(IV) by reducing of U(VI) (Francis et al. 1995; Lovley and Phillips 1992; Lovley et al. 1991), this research provides kinetic and stoichiometric data to develop designs for a U(VI) reduction process.

The information on energetics of growth obtained in this continuous culture system with *D. desulfuricans* DSM 1924 contributes to our understanding of these important organisms. The value of 17.2–18.2 for protein yield per mole of pyruvate utilized by *D. desulfuricans* was considerably more than the value of 6.7 obtained with *D. desulfuricans* cultured under conditions where the electron acceptor was limiting and pyruvate was fermented (Vosjan 1975). The Y_s value for the cultures without U(VI) was similar to the value of 9.41 g/mol pyruvate (dry weight in g) utilized by *D. desulfuricans* strain canet 41 (Magee et al. 1978). This study is the first to indicate that uranium had a marked inhibitory effect on the growth of *D. desulfuricans*. While the nature of this inhibition of uranium on bacterial growth remains unexplained at this time, there was sufficient reduction of uranium to merit the application of sulfate-reducting bacteria for the biotransformation of U(VI).

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