Evaluation of the effects of various culture conditions on Cr(VI) reduction by *Shewanella oneidensis* MR-1 in a novel high-throughput mini-bioreactor

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Abstract

The growth and Cr(VI) reduction by *Shewanella oneidensis* MR-1 was examined using a mini-bioreactor system that independently monitors and controls pH, dissolved oxygen, and temperature for each of its 24, 10-mL reactors. Independent monitoring and control of each reactor in the cassette allows the exploration of a matrix of environmental conditions known to influence *S. oneidensis* chromium reduction. *S. oneidensis* MR-1 grew in minimal medium without amino acid or vitamin supplementation under aerobic conditions but required serine and glycine supplementation under anaerobic conditions. Growth was inhibited by dissolved oxygen concentrations >80%. Lactate transformation to acetate was enhanced by low concentration of dissolved oxygen during the logarithmic growth phase. Between 11 and 35°C, the growth rate obeyed the Arrhenius reaction rate-temperature relationship, with a maximum growth rate occurring at 35°C. *S. oneidensis* MR-1 was able to grow over a wide range of pH (6-9). At neutral pH and temperatures ranging from 30-35°C, *S. oneidensis* MR-1 reduced 100 μM Cr(VI) to Cr(III) within 20 minutes in the exponential growth phase, and the growth rate was not affected by the addition of chromate; it reduced chromate even faster at temperatures between 35 and 39°C. At low temperatures (<25°C), acidic (pH<6.5), or alkaline (pH>8.5) conditions, 100 μM Cr(VI) strongly inhibited growth and chromate reduction. The mini-bioreactor system enabled the rapid determination of these parameters reproducibly and easily by performing very few experiments. Besides its use for examining parameters of interest to environmental remediation, the device will also allow one to quickly assess parameters for optimal production of recombinant proteins or secondary metabolites

Keywords: pH, temperature, dissolved oxygen, chromium reduction, high throughput cultivation

Introduction

The facultative bacterium *Shewanella oneidensis* MR-1, isolated from Oneida Lake sediments (Myers and Nealson 1988), is able to use many organic carbon sources as electron donors (e.g., lactate, pyruvate, propionate, acetate, formate, and serine) and can reduce a variety of soluble or solid compounds, including iron III, manganese IV, nitrate, nitrite, thiosulfate, trimethyl-amine N-oxide, thiosulfate, fumarate, uranium, and Cr(VI) (Scott and Nealson 1994; Venkateswaran et al. 1999). Because of its metabolic versatility and its ability to reduce metals to less mobile forms, this bacterium has been considered for use in bioremediation of subsurface sites contaminated with metals and, as such, has been studied extensively over the last decade (Abboud et al. 2005; Liu et al. 2005; Middleton et al. 2003; Myers and Nealson 1988; Tiedje 2002.; Viamajala et al. 2002; Viamajala et al. 2004). Because of its environmental importance, the genome of *S. oneidensis* MR-1 was recently sequenced (Heidelberg et al. 2002).

Understanding how environmental conditions (pH, temperature, oxygen) impact its ability to reduce metals is important if *S. oneidensis* is going to be used to remediate environments contaminated with metals. However, examination and optimization of cultivation conditions for bacteria has always been a time-consuming process, in part because it is very difficult to accurately monitor and control conditions in high throughput systems, such as multi-well plates or shake flasks. At a larger scale, 1-L and 10-L bioreactors have the advantage that one can monitor and control pH, temperature, dissolved oxygen (DO), and other parameters; however, the low throughput of larger bioreactors makes it difficult to explore the many possible combinations of parameters in an efficient manner. Pharmaceutical companies and research laboratories are looking closely at small-scale bioreactors to meet the needs of higher throughput

controlled cell cultivation (Boswell 2004; Kostov et al. 2001; Kumar et al. 2004; Maharbiz 2004).

All of the cell growth and chromate reduction experiments described herein were performed using a 24-well mini-bioreactor device that was engineered to independently monitor and control pH, temperature, and dissolved oxygen in each well. As such, the mini-bioreactor device allows one to examine the impact of a wide range of parameters on cell growth and, in this case, chromium reduction. The optimal culture conditions found in these studies were similar to those previously identified as being best suited for chromium reduction by *S. oneidensis*. These findings demonstrate the utility of small-scale bioreactors for exploring many conditions in parallel, providing more data than traditional multi-well plates and at a rate faster than would be possible with larger scale bioreactors.

Materials and methods

Mini-bioreactor design. All experiments described herein were preformed using a prototype mini-bioreactor (MicroReactor Technologies Inc. and Gener8 Inc., Mountain View, California) (Figure 1). Designed to control the growth of organisms in a custom, disposable, 24-well, SBS format (The Society for Biomolecular Screening, Connecticut, USA), reactor cassette, the mini-bioreactor independently measures and controls the pH, DO, and temperature for each well of the cassette. The pH and DO are measured via a non-invasive, optically-probed sensor and controlled via the introduction of process gasses, most typically $CO_{2(g)}$, dilute $NH_{3(g)}$, nitrogen, and air. Cassettes used in the mini-bioreactor consist of a 24-well plate in a 10-mL, deep-well format. Incorporated into the plate are a set of features that enable its use as 24, independently controlled, 10-mL bioreactors. At the bottom of each well are two fluorescence-lifetime–based, chemical sensors, one to detect dissolved oxygen levels and another to detect pH

levels. Also integrated into the bottom of the plate are two thermal transfer pads, one of which is used to heat the contents of the well and the other is used to sense the well's temperature. These features surround a 0.2-μm porosity membrane through which control gases are introduced. Cassettes are γ-beam sterilized and intended for one time use. A gas permeable, peel-and-stick, sterile membrane is used to seal the top surface of the cassette. The instrument contains the optical, thermal, and gas delivery hardware, which interrogate and control the cassette during cultivation, mounted on a variable speed, orbital shaker that has a 2-mm diameter rotation. Cassettes are typically orbited at 500 rpm to create sufficient vortices to ensure gas exchange between the headspace of each individual reactor well and the medium within it. Command and control of the system is achieved through a USB interface to a laptop PC.

*1. pH and DO Sensing***.** The mini-bioreactor's optical measurement of oxygen and pH is based on the effect of dynamic luminescence quenching of a fluorophore by molecular oxygen or protons. The functional dependence of fluorescence signal on the oxygen concentration is described by the Stern-Volmer equation (Lakowicz 1983). For oxygen, this is expressed as follows:

$$\frac{I_o}{I} = 1 + K_{SV}[O_2] \quad (1)$$

I_o is the intrinsic fluorescent intensity for the particular sensor fluorophore (no oxygen quenching), while I is the measured signals in the presence of oxygen at a partial pressure entered for $[O_2]$ in equation (1). K_{SV} then describes a simple linear relationship between the quenching and the oxygen concentration.

In order to control the chemical conditions in the well, high quality micro-scale sensing is required. Traditional membrane-based pH and DO probes used in larger scale bioreactors

require sterilization before each use, are expensive, and typically require calibration before each use. An alternative technology for the micro-scale is optical lifetime-based probes (Presens-Precision Sensing GmbH**,** Josef-Engert-Str 9, D-93053 Regensburg, Germany). With this technology, small "dots" of fluorophores are deposited into each mini-bioreactor. These fluorophores have been designed so that their fluorescence only depends on the chemical environment of the well and not on extrinsic parameters such as the fluorophore density, sensor thickness, or optical alignment. Some fluorophores are designed to be sensitive to dissolved oxygen, while the others are designed to be sensitive to pH. There are several methods by which one can measure the signal from fluorescence-lifetime based sensors. Most commonly-available, pH-sensitive fluorophores have a fluorescence decay lifetime that is on a nanosecond timescale and so the direct measurement of their lifetime is costly and often plagued by errors. The mini-bioreactor uses a technique known as dual lifetime referenced (DLR) optical sensing (Presens-Precision Sensing GmbH). In this approach, two fluorophores were used. The first, referred to as the indicator, is pH sensitive and has a decay time on a nanosecond timescale. The second, referred to as a reference standard, is insensitive to pH, and has a decay time on a microsecond timescale. The two fluorophores have overlapping excitation and emission spectra. By measuring the time response signal of the sensor one can determine the relative emission ratio, and hence the pH.

The technology to probe the sensor materials is embossed into the mini-bioreactor's engine. The mini-bioreactor contains a total of 8 LED's (light emitting diode), one for each row of pH and DO sensors. The LED's have wavelengths of 470 nm for the pH sensor and 505 nm for the DO sensor and are sharply filtered to remove spectral tails that might coincide with the fluorophore's emission spectrum. To enable the measurement of lifetime, the LED's are

modulated at approximately 8 kHz for the pH sensor and 1 kHz for the DO sensor. The filtered and modulated LED light is routed to each of the 24 wells via fiber optics. The light emitted by the fluorophores is filtered to remove reflected and scattered light from the excitation source and then is captured by a total of 48 silicon photodiodes whose signals are amplified by nearby transimpedance amplifiers. To measure the lifetime of the signals the mini-bioreactor employs 3 embedded lock-in amplifiers. The amplifiers are referenced to the modulation frequency of the LED's. A lock-in amplifier is a technology that employs a pair of synchronous demodulators. One demodulator is driven in-phase with the LED's, the other is 90 degrees out of phase. Low-pass filtering and measurement of the ratio of the magnitudes of these two signals allows determination of the phase delay, and hence the lifetime, of the fluorophore.

2. *Automatic pH and DO Control.* The cassettes are mounted onto the engine via a set of 15 vacuum clamps, which ensure a uniform high force attachment. When the cassette is clamped onto the engine, a set of 24 gaskets, one for each reactor, make an air-tight seal between the engine and the cassette. This seal enables the controlled introduction of gasses into the reactors. A total of 72 solenoids independently supply any of 3 gasses to each of the 24 reactors through the 0.2-μm ePTFE membranes at the bottom of the cassettes reactors. CO_2(g) was used to drive reactor pH acidic. NH_3(g), used to drive reactor pH basic, was generated from a dilute solution of NH_3OH in dH_2O (5% v/v) in a closed vessel at 5 psi. The concentration of NH_3 in the gaseous phase for this system is approximately 0.71%. Process gasses used for DO control are pure nitrogen (to purge the reactor of oxygen) and air, enriched air, or oxygen (to increase the oxygen concentration). The amount of gas delivered by the mini-bioreactor to an individual well is controlled by the duration and frequency with which the solenoids are opened. Once a valve has been opened, the gas will continue to flow into the well at a rate that is determined by the

internal orifices of the mini-bioreactor. The mini-bioreactor used in this study can apply 0-5 gas pulses per minute, and each pulse can vary from 20 ms to 200 ms. As a result the flow rate can be controlled between 0.48 ~ 8 sccm.

The defined medium and cultivation conditions. *Shewanella oneidensis* MR-1 was purchased from ATCC (strain number 700550) and stored at -80°C. *S. oneidensis* MR-1 experiments were based on a protocol provided by Dr. Jim Fredrickson (Pacific Northwest National Laboratory, Richland, Washington State, USA). The minimal medium contained 60 mM lactate for aerobic and 7 mM for anaerobic conditions, 28 mM NH_4Cl, 1.34 mM KCl, 4.4 mM Na_2HPO_4, 1.5 mM Na_2SO_4, 1 mM $MgCl_2$, 10 mM PIPES, 100 ppm antifoam (Sigma Antifoam A-6457), and trace elements (1 L medium contained 10 mg $FeCl_2{\cdot}4H_2O$, 5 mg $MnCl_2{\cdot}4H_2O$, 3 mg $CoCl_2{\cdot}4H_2O$, 2 mg $ZnCl_2$, 0.5 mg $Na_2MoO_4{\cdot}4H_2O$, 0.2 mg H_3BO_3, 1 mg $NiSO_4{\cdot}6H_2O$, 0.02 mg $CuCl_2{\cdot}2H_2O$, 0.06 mg $Na_2SeO_3{\cdot}5H_2O$, 0.08 mg $Na_2WO_4{\cdot}2H_2O$). A solution of 1 M NaOH was used to adjust the final medium pH to 7. For anaerobic growth, 30 mM fumaric acid was used as the electronic acceptor. In the experiments to test the effect of amino acids or vitamin on growth (Table 1), an amino acid mix (25 mg/L arginine, 25 mg/L glutamate, and 50 mg/L serine) or a vitamin mix (0.02 mg/L Biotin, 0.02 mg/L folic acid, 0.1 mg/L pyridoxine HCl, 0.05 mg/L thiamine HCl, 0.05 mg/L riboflavin, 0.05 mg/L nicotinic acid, 0.05 mg/L DL pantothenic acid, 0.05 mg/L *p*-aminobenzoic acid, 0.05 mg/L lipoic acid, 2 mg/L choline chloride, and 0.01 mg/L vitamin B_{12}) was added to the minimal medium. All experiments were conducted in the 24-well, 10-mL mini-bioreactor plates. Sterile medium (by filtration) was inoculated with a 1:100 dilution of *S. oneidensis* MR-1, which was pre-cultured in Luria-Bertani (LB) medium at 30°C, 200 rpm for 16 hours. Six mL of inoculated medium was transferred to each well and the plate sealed with gas-permeable sealing tape (#AB-0718,

Abgene Inc., Rochester New York, USA). During aerobic growth, air or oxygen mixed with nitrogen was fed from the bottom of each well to obtain the desired dissolved oxygen concentration. CO_2 and nitrogen gas mix (containing >15% CO_2) was used to control pH in all wells. The shaker was set to 500 rpm to ensure optimal mass transfer. For cultivation temperatures below ambient, the mini-bioreactor was placed in a cold room (4°C), and the integrated heater at the bottom of each well was used to raise the temperature to the set point. For anaerobic cultivation, the sterile, anaerobic medium (purged with nitrogen for 20 minutes) was inoculated into the wells of the device in an anaerobic hood (Coy Laboratory Products Inc, Grass Lake, MI), and the plate was sealed with sterile tape (MICROSEAL Foil, MJ Research, Inc, Incline Village, NV) before the plate was placed in the mini-bioreactor device outside the anaerobic hood. Because the mini-bioreactor is not air tight, we continuously introduced nitrogen into the culture medium and the headspace above the mini-bioreactor plates during anaerobic cultivation. Only endpoint samples were taken to avoid the disruption of the anaerobic conditions.

Analytical measurements. For all conditions except anaerobic growth, samples of 0.5 mL were taken at each time point. The optical density at a wavelength of 600 nm (OD_{600}) was measured in a spectrophotometer (DU®640, Beckman Instruments, Palo Alto, California). Because the volume of culture in each well of the mini-bioreactor was insufficient to determine the biomass dry weight, we used a standard curve relating OD_{600} to the weight of lyophilized biomass (dried for 24 hours) to obtain the amount of biomass in each reactor.

To determine the Cr(VI) reduction rate, a 10 mM K_2CrO_4 solution was added to the medium to a final concentration of 50 or 100 μM. At several time points (10 minutes, 30 minutes, 1 hr, 2.5 hrs, and 5.5 hrs), 0.5 mL samples were quickly taken from the mini-bioreactor

and centrifuged at 10,000×g for 1 minute. Soluble Cr(VI) concentrations in the supernatant fraction were determined colorimetrically at a wavelength of 540 nm using 1,5-diphenyl-carbazide in a sulfuric acid solution (pH=2) as described by Middleton *et al*. (Middleton et al. 2003). The acetate concentration was determined using an enzyme test kit (r-Biopharm Inc., Germany). The specific growth rate (μ) was calculated using the following formula (Viamajala et al. 2004):

$$\mu = \frac{\ln \frac{(OD_{600}) \text{ at } t_2}{(OD_{600}) \text{ at } t_1}}{t_2 - t_1} \quad (2)$$

where t_2 and t_1 were two time points at the early period of exponential growth phase (OD_{600}=0.2~0.6). We avoided using data from the late exponential phase in Equation (1) because acetate (produced from lactate metabolism) could be used as carbon source and thus affect cell growth. Chromate (VI) reduction kinetics is described with a zero order kinetics expression:

$$k = \frac{C_2 - C_1}{X(t_2 - t_1)} \quad (3)$$

where C_1 and C_2 were the Cr(VI) concentrations [μM] at the two time points t_1 and t_2 [hours], respectively; k is the chromate reduction rate [μmol Cr(VI) /(g biomass hr)]; and X is the biomass dry weight (averaged at t_1 and t_2).

Results and Discussion

Control of the batch culture conditions using the mini-bioreactor. The mini-bioreactor system, developed as previously described, controls the 24 independent bioreactor vessels, each having a capacity of up to 10 mL. In contrast to experiments in shake flasks, the mini-bioreactor system monitors and controls the dissolved oxygen, pH, and temperature

simultaneously in each bioreactor vessel during cultivation. The data from a 25-hour cultivation illustrate the stability and control achieved using the system (Figure 2). The mini-bioreactor was capable of controlling temperature within a very narrow window ($\Delta T<0.2°C$).

Oxygen control was achieved through air or blend gas introduction into the wells. Cell growth was responsible for depleting the oxygen levels down to the set-point, which occurred during the early stages of culture growth (line 1 in Figure 2b). Once below the set-point, the mini-bioreactor initiated feedback control to return the dissolved oxygen levels above the set-point. The dissolved oxygen levels fluctuated (20~50%) in the middle of the exponential growth phase because of the high oxygen demands of the *S. oneidensis* culture and the enhanced gas addition by the apparatus. To achieve tighter control of the dissolved oxygen levels, an additional gas line was used to sparge pure inert gas (nitrogen or argon) into wells when dissolved oxygen exceeded the set-point. This control strategy maintained the desired oxygen level throughout the cultivation run (Figure 2b). When *S. oneidensis* reached an $OD_{600} > 1.0$, it depleted the oxygen in the medium. When necessary, enriched oxygen sources (>21%) can be used with the mini-bioreactor to overcome oxygen depletion. At 30°C under aerobic conditions, *S. oneidensis* MR-1 had a doubling time of 3.5-4 hours, similar to that reported by Middleton *et al*. (2003). Under oxygen-limited conditions (DO<20%), the doubling time was longer (5.9 hours).

The mini-bioreactor also provided pH control. The consumption of lactate increased the pH in the medium (Figure 2d). A weak acid, CO_2 (15% balanced with nitrogen), was used to control pH during growth. However, CO_2 solubility depends on temperature and pH. At high temperature (>30°C) or acidic conditions, CO_2 (15%) will eventually saturate the medium so that it cannot be used to maintain the pH of the culture in the late growth stage. Therefore, a more

concentrated CO_2 gas mix (up to 100%) had to be used in the mini-bioreactor system. The increase in the CO_2 partial pressure increased the CO_2 dissolved in the medium and enhanced the pH control capability.

Effect of medium composition and dissolved oxygen on *S. oneidensis* MR-1 growth. Examination of *S. oneidensis* MR-1 growth is important for bioremediation applications, since heavy metal reduction rates have been shown to be directly related to total biomass (Middleton et al. 2003). Although *S. oneidensis* MR-1 grows best in a rich medium, such as Luria-Bertani (LB) medium, it is capable of growing in a defined medium supplemented with only three amino acids (arginine, glutamate and serine) and vitamins (Myers and Nealson 1988). For metabolite and metabolic flux analyses, a minimal medium without amino acids or other nutrients is more desirable. Therefore, we examined medium composition on *S. oneidensis* MR-1 growth in a parallel manner using the mini-bioreactor system (Table 1). Under aerobic conditions (Figure 2), MR-1 growth did not require any vitamins or amino acids. However, MR-1 grew poorly without Mg^{2+} or trace metals (OD_{600}<0.4). *S. oneidensis* MR-1 grew to high density (OD_{600}>1.3) without Ca^{++} supplementation. When Ca^{++} was absent, antifoam was necessary to prevent serious foaming. Concentrations of Ca^{++} above 0.5 mM caused the cells to aggregate. It is possible that the aggregation protects the cells from exposure to environmental stresses including oxygen, antibiotics, chromate, and H_2O_2 (personal communication, Dr. Jim Fredrickson). It is also possible that cell aggregation is mediated by extracellular polymers, and that free calcium is essential for their structural integrity (Szomolay et al. 2005; Turakhia 1986).

When fumaric acid was used as an electron acceptor for anaerobic growth, the cells did not grow without addition of amino acids. Addition of amino acids that can be used through the serine pathway (such as glycine or serine) significantly enhanced anaerobic growth. However,

the maximum biomass concentrations were far from those obtained under aerobic conditions. Supplementation with other amino acids had little or no effect on cell growth. Indeed, the serine pathway is thought to play an important role under anaerobic conditions (Scott and Nealson 1994). The enhanced growth by supplementation with glycine and serine suggests that the key reactions in the serine pathway that convert glyoxylate to glycine (by glyoxylate transaminase) or glycine to serine (by glycine hydroxymethyltransferase) may be rate-limiting for anaerobic carbon metabolism.

Various dissolved oxygen levels were maintained by adding an oxygen-nitrogen mixture to the bottom of the growth vessel and purging the headspace with nitrogen. The OD_{600} was measured at 12 and 22 hours (Figure 3). The cells grew very poorly when the dissolved oxygen concentration was controlled over 80% of air saturation. The oxidative stress of oxygen may be due to the generation of $O_2^{\bullet}$ from oxygen (Ghosal et al. 2005). A low dissolved oxygen concentration (less than 10%) caused significant acetate production, which accumulated in the later stages of exponential growth. Thirty percent (30%) of total lactate (~17 mM) was converted to acetate and secreted into the medium under micro-aerobic conditions after 22 hrs of incubation. Once the lactate was depleted, the cells used the acetate as a carbon source for growth. With respect to oxygen's effect on Cr(VI) reduction, it has been suggested that oxygen may not directly inhibit reduction of Cr(VI) (Middleton et al. 2003). Rather the stress of the high dissolved oxygen inhibits cell growth, and thus there is less biomass for chromate reduction.

Effect of temperature and pH on growth. Although MR-1 has been reported to grow in rich medium at temperatures as high as 40°C (Venkateswaran et al. 1999), we observed no growth in the minimal medium at temperatures over 37°C. The specific growth rates in the minimal medium were calculated and plotted against reciprocal of the absolute temperature

(units of K) (Figure 4a). The specific growth rate was highest in the temperature range of 34~35°C. The specific growth rate obeyed the Arrhenius equation in the temperature range from 11 and 35°C.

To study the effect of pH on cell growth, the medium (buffered with 20 mM PIPES) was adjusted to different pH's before inoculation. The pH in the mini-bioreactor was controlled by the addition of CO_2 to the bottom of the mini-bioreactor wells during the cultivation. *S. oneidensis* MR-1 grew over a wide range of pH (5.6~9.4) and similarly under the three pH ranges (pH=6.5~6.9, 7.5~8.0 and 8.5~9.0) (Figure 5a). MR-1 was capable of growth under relatively basic pH (pH>8.5), most likely because it was originally isolated from the alkaline Lake Oneida (pH up to 9.1) (Lavallee and Pick 2002).

Cr(VI) reduction under various environmental conditions. Hexavalent chromium is a known carcinogen and can be reduced to the less soluble chromium (III) by *S. oneidensis* MR-1 under both anaerobic and aerobic conditions (Middleton et al. 2003; Myers et al. 2000; Viamajala et al. 2002; Viamajala et al. 2004). The cited reports indicate that Cr(VI) (even at concentrations as low as 15 μM) strongly inhibits cell growth and its own reduction. However, the experiments described herein demonstrate that the aerobic chromate reduction rate can be significantly improved under specific conditions. We tested MR-1's ability to reduce 50 or 100 μM Cr(VI) at three different growth stages (Figure 6). If 100 μM Cr(VI) was added to the culture before exponential growth ($OD_{600} < 0.1$), the chromate inhibited cell growth; however, the culture was able to grow and reduce 50 μM Cr(VI) within 12 hours if the chromate was added to the culture before exponential growth. Once most of the Cr(VI) was reduced, the culture grew rapidly with a relatively normal biomass yield. The cultures were capable of reducing 100 μM Cr(VI), added in mid-exponential growth, to Cr(III) in as little as 20 minutes

with little or no impact on growth. These results indicate that a high biomass concentration prior to addition of high Cr(VI) concentrations was optimal for its reduction, because Cr(VI) can be reduced within minutes and cell growth is not likely to be affected.

Culture temperature was found to be another important factor for Cr(VI) reduction. *S. oneidensis* MR-1 was grown at various temperatures into the mid-exponential growth phase and then spiked with Cr(VI) (100 μM final concentration). The specific chromate reduction rate was calculated and plotted as a function of incubation temperature (Figure 4b). The highest specific chromate reduction rate occurred at temperatures over 34°C, ranging from 35-39°C. The Cr(VI) specific reduction rate rapidly declined with temperature and was zero at temperatures below 13°C, implying that raising the temperature in contaminated sites to 35°C could substantially increase the rate of Cr(VI) reduction. We did not explore growth or chromate reduction by the cells at temperatures lower than 10°C, since both growth and chromate reduction rates were extremely slow.

pH also had a significant impact on Cr(VI) reduction. Cr(III) formed by reduction of Cr(VI) is thought to precipitate at most environmental pH's (6~9) (Ayres et al. 1994; Cherry 1982; Jardine et al. 1999; Middleton et al. 2003). At neutral to slightly alkaline pH (7~7.5), Cr(VI) (100 μM) was quickly reduced (the chromate turnover time is indicated in the caption of Figure 6). When chromate was added as a spike to a culture in exponential growth under three unfavorable pH's (Figure 5b), chromate reduction was seriously inhibited (turnover time >5 hours) (Figure 5c). Although MR-1 is adapted to live in relatively alkaline environments, MR-1 loses its ability to reduce chromate at pH>=8.5. These results indicate that Cr(VI) reductase activity may be sensitive to the pH of the culture medium, which may cause potential problems in using MR-1 for *in situ* bioremediation of very acidic and basic Cr(VI) contaminated sites.

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Figure captions

Figure 1. The mini-bioreactor. **(a)** The mini-bioreactor controls conditions in each well of the reactor cassette. Each well has monitoring and control over pH, DO and temperature. pH and DO are monitored optically through the bottom of the cassette and are controlled by addition of gas through the affixed, 0.2-um, sterile membrane in the center of each well. Individual reactor temperatures are controlled through the thermally conductive materials interaction with a heater and thermistor for each individual reactor in the cassette. **(b)** Sealed with gas permeable adhesive sealing tapes, the single use, pre-sterilized reactor cassettes are manufactured from optically clear polystyrene and are disposed of after each experiment.

Figure 2. Demonstration of control of MR-1 growth conditions in individual wells. **(a)** Controlling and monitoring temperature at 30°C and 20°C. **(b)** Dissolved oxygen control. 1, dissolved oxygen was controlled above 20% using 30% oxygen balanced with nitrogen; 2, dissolved oxygen control was off; 3, dissolved oxygen was controlled at 15% using an oxygen-nitrogen mixture (6% oxygen and 94% nitrogen) and nitrogen gas (100%). **(c)** MR-1 growth curves under aerobic (♦) and oxygen-limited (■) conditions at 30°C. **(d)** Control of pH (at 7.0) during growth on 60 mM sodium lactate using CO_2. 1, no pH control, incubation at 30°C; 2, pH control (using 15% CO_2 gas, incubation at 30°C).

Figure 3. Effect of dissolved oxygen on cell growth and acetate production in minimal medium (no Ca^{++}). Symbols represent the mean of three replicates; error bars represent the standard deviation around the mean. Cell density at 13 (♦) and 22 (■) hrs. Acetate concentrations at 13 (◊) and 22 (□) hrs. Lactate and acetate in all cultures were completely depleted after 33 hours incubation.

Figure 4. Temperature effect on MR-1 growth and Cr(VI) reduction (replicates, n=2). **(a)** Specific growth rate as a function of the reciprocal of the absolute temperature. **(b)** Cr(VI) reduction rate as the reciprocal of temperature. Symbols represent the experimental temperatures above (♦) and below (◊) 24°C. Cr(VI) (final concentration of 100 μM) was spiked into the culture (OD_{600} of 0.6~0.8 for growth temperatures below 24°C and 0.2~0.4 for temperatures above 24°C).

Figure 5. pH effect on growth and Cr(VI) reduction. The dissolved oxygen level was controlled above 15% of air saturation (n=2). **(a)** Growth without chromate: ◊ pH=6.5~6.9; □ pH=7.5~8; Δ pH=8.5~8.9. **(b)** Growth in the presence of 100 μM Cr(VI). Cr(VI) was added at 8 hours (indicated by the arrow). Symbols were the same as in (a). **(c)** Cr(VI) concentration. Symbols are the same as in (a).

Figure 6. *S. oneidensis* MR-1 cultures exposed to Cr(VI) at different growth stages. The dissolved oxygen level was controlled at 15% of air saturation, pH at 7~8, and temperature at 30°C. All experiments were conducted in duplicate. Symbols represent (◊) cultures exposed to 50 μM Cr(VI) at the beginning of incubation (arrow 3) – Cr(VI) was completely reduced within 12 hours; (□) cultures exposed to 100 μM Cr(VI) at the beginning of the incubation (arrow 3) – Cr(VI) was not completely reduced in these experiments; (■) cultures exposed to 100 μM Cr(VI) at an early stage of exponential growth phase (arrow 2) – Cr(VI) was completely removed within 2 hours; (♦) cultures exposed to 100 μM Cr(VI) in the middle stages of exponential growth (arrow 1) – Cr(VI) was completely removed with 20 minutes.

Figure 1

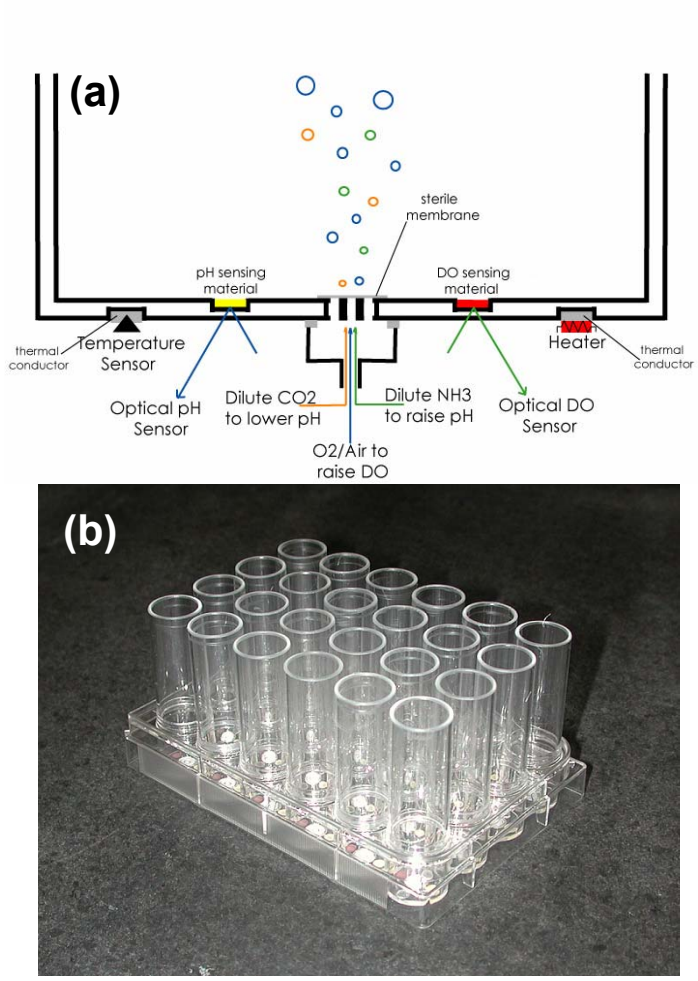
(a)
sterile membrane
pH sensing material
DO sensing material
thermal conductor
Temperature Sensor
Heater
thermal conductor
Optical pH Sensor
Dilute CO2 to lower pH
Dilute NH3 to raise pH
Optical DO Sensor
O2/Air to raise DO
(b)

Figure 2

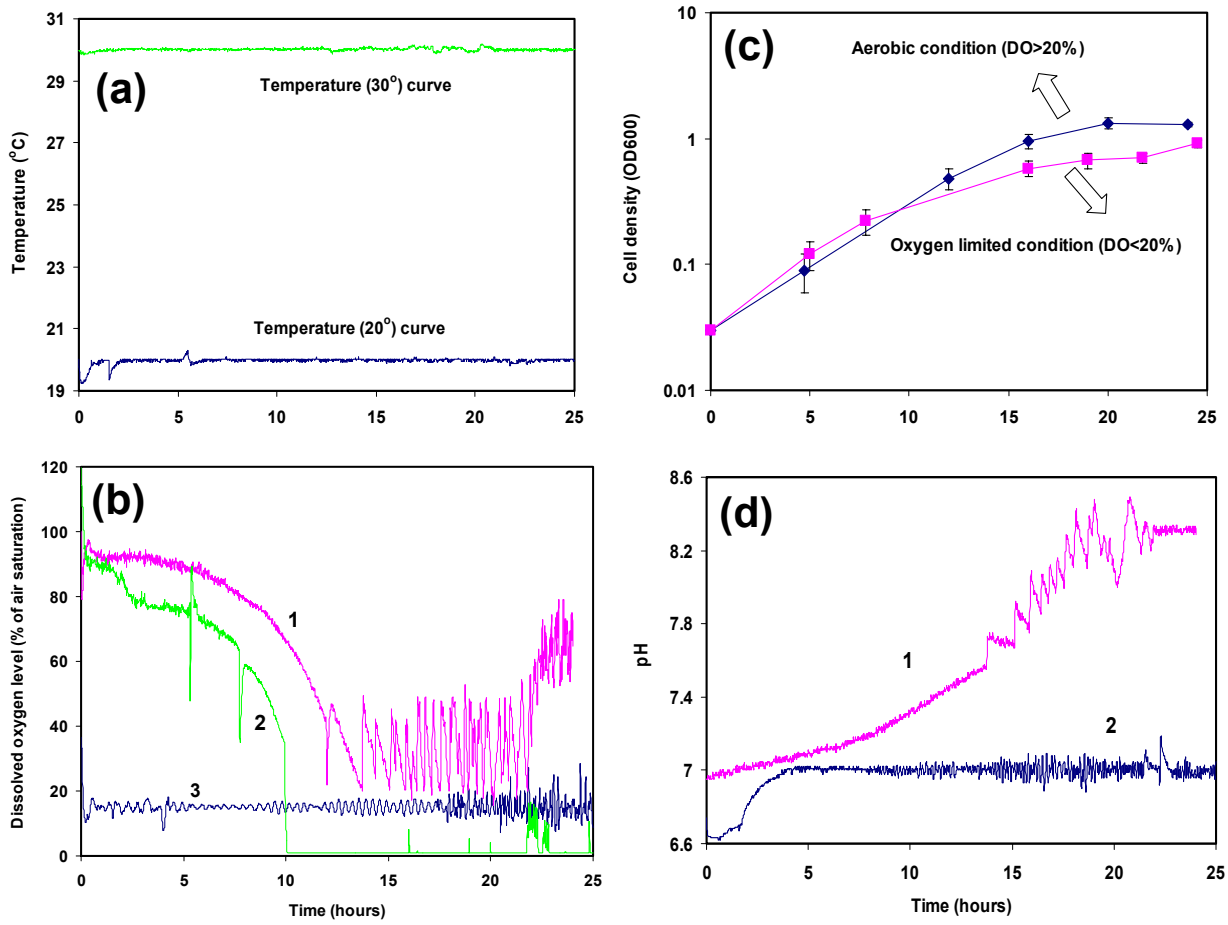
(a)
Temperature (30°) curve
Temperature (20°) curve
Temperature (°C)
(b)
1
2
3
Dissolved oxygen level (% of air saturation)
Time (hours)
(c)
Aerobic condition (DO>20%)
Oxygen limited condition (DO<20%)
Cell density (OD600)
(d)
1
2
pH
Time (hours)

Figure 3

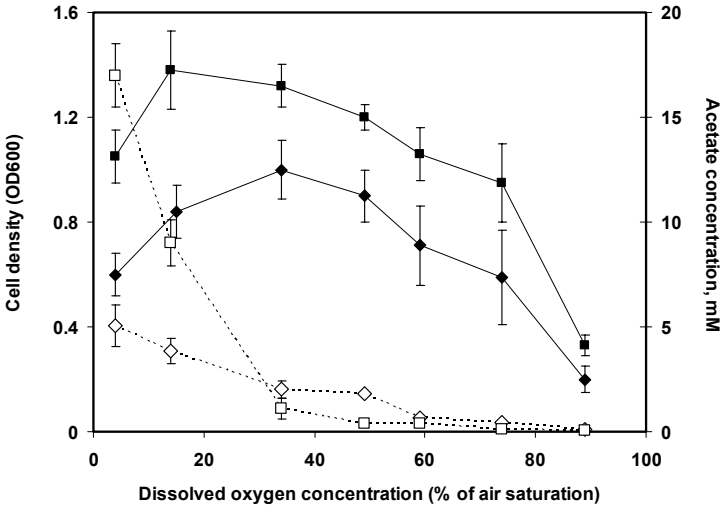
1.6
1.2
0.8
0.4
0
20
15
10
5
0
0
20
40
60
80
100
Cell density (OD600)
Acetate concentration, mM
Dissolved oxygen concentration (% of air saturation)

Figure 4

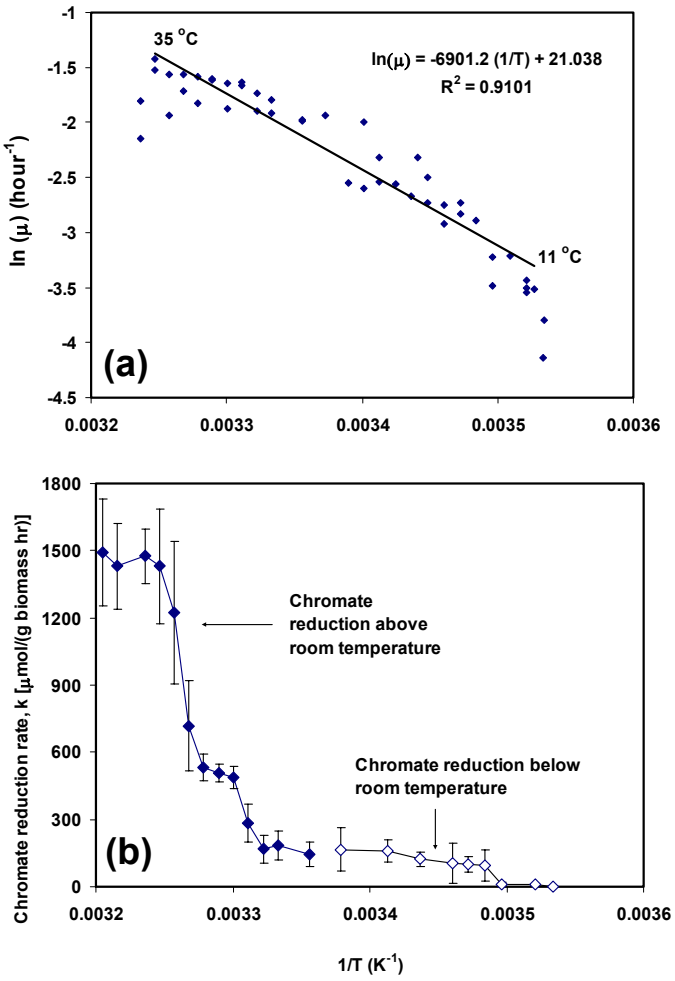
35 °C
ln(μ) = -6901.2 (1/T) + 21.038
R2 = 0.9101
11 °C
(a)
ln (μ) (hour-1)
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
0.0032
0.0033
0.0034
0.0035
0.0036
Chromate reduction above room temperature
Chromate reduction below room temperature
(b)
Chromate reduction rate, k [μmol/(g biomass hr)]
1800
1500
1200
900
600
300
0
1/T (K-1)

Figure 5.

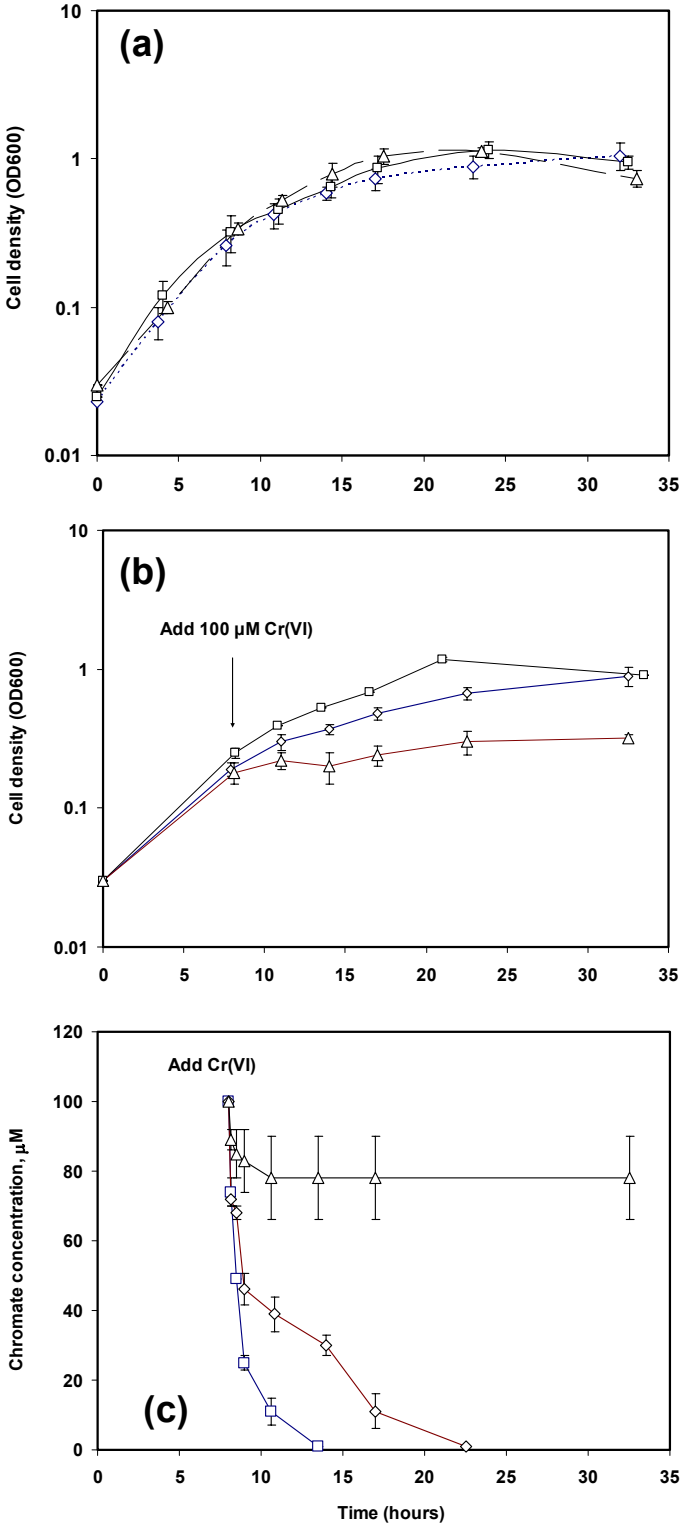
(a)
Cell density (OD600)
10
1
0.1
0.01
0
5
10
15
20
25
30
35
(b)
Add 100 µM Cr(VI)
Cell density (OD600)
10
1
0.1
0.01
0
5
10
15
20
25
30
35
Add Cr(VI)
Chromate concentration, µM
120
100
80
60
40
20
0
(c)
0
5
10
15
20
25
30
35
Time (hours)

Figure 6.

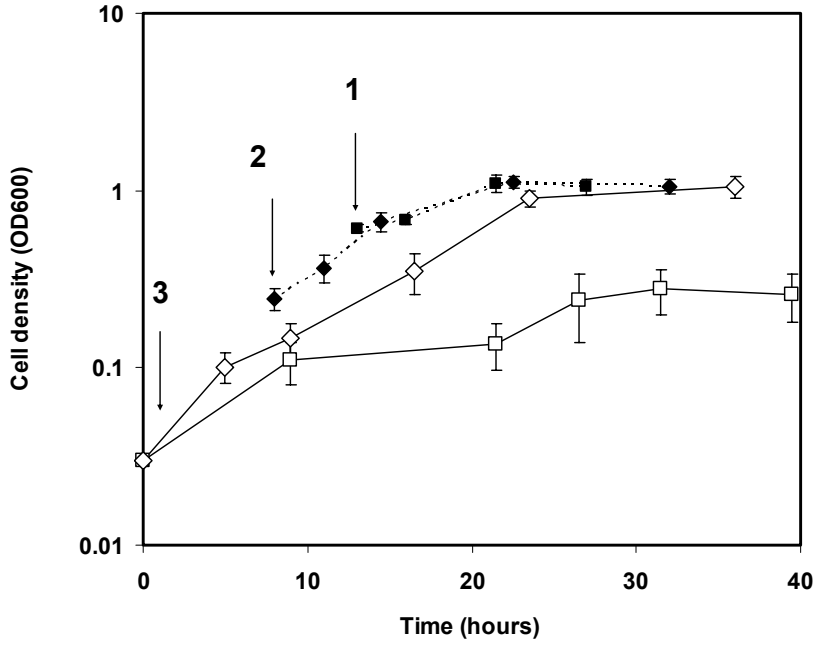

Table 1

Biomass production by *Shewanella oneidensis* MR-1 in defined mediums (n=4)

Medium formulation	Final cell density (OD_{600})*
Aerobic condition	
Minimal medium	1.31±0.08
Minimal medium with amino acid mix and vitamin mix	1.38±0.06
Minimal medium (Mg^{++} are removed);	0.35±0.13
Minimal medium (no trace elements)	0.14±0.05
Anaerobic condition (7 mM lactate and 30 mM fumarate)	
Minimal medium	<0.05
Minimal medium with amino acid mix and vitamin mix	0.19±0.06
Minimal medium + 100 mg/L glycine	0.21±0.03
Minimal medium + 100 mg/L serine	0.17±0.05
Minimal medium + 100 mg/L LB medium	0.20±0.04

*Under aerobic conditions, the OD_{600} was measured after 24 hours incubation. Under anaerobic conditions, the OD_{600} was measured after 48 hours incubation.