



Reductive Degradation of Organic Compounds Using Microbial Nanotechnology

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Members of the genus *Clostridia* can reduce Pd(II) ions to form metallic Pd nanoparticles (bio-Pd). Cultures of *C. pasteurianum* BC1 were used to generate bio-Pd, which is primarily formed on the microbial cell wall. Batch experiments using *C. pasteurianum* BC1 cells loaded with bio-Pd showed efficient reduction of the organic azo dyes, methyl orange and Evans blue, while little reduction of dyes was observed in control experiments using Pd-containing heat-killed microbial cultures or Pd-free viable bacterial cultures. Degradation of azo dyes was found to occur via reductive hydrogenation of the azo-linkage. Molecular hydrogen, which is concomitantly generated by *C. pasteurianum*, is used in the reduction reaction. The process described in this study is a potentially viable alternative to current groundwater and wastewater treatment technologies that fail to adequately degrade the large quantities of hazardous spent textile dyes that are discharged into the environment each year.

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Metal nanoparticles, by virtue of their catalytic properties, have the potential to be exploited for applications in environmental remediation.^{1–6} For instance, numerous studies have explored the use of Fe(0) nanoparticles to remove organic and inorganic contaminants.^{7–11} In the presence of a suitable electron donor, these nanomaterials can catalyze the reduction of pollutants, thereby rendering them insoluble.^{1,12,13} A variety of inorganic pollutants such as carcinogenic hexavalent chromium, and hexavalent uranium are soluble in their highly oxidized form and become sparingly soluble on reduction (to trivalent chromium and tetravalent uranium in the above case, respectively). On the other hand, several organic compounds such as organic dyes, fertilizers, pharmaceutical compounds and intermediates undergo reductive degradation. In all these cases, the contamination levels in the subsurface environment and their downstream flow can be significantly controlled and managed. However, these remediation strategies are limited by their ability to deliver catalytic nanoparticles, in addition to a suitable electron donor, to large treatment zones.

A new biologically inspired method to produce a nanoparticulate catalyst involves the precipitation of transition metals such as palladium, gold and iron on bacterial surface, resulting in the formation of bio-nano-Met, where Met = transition metal catalyst. Two gram-negative model organisms have been primarily used to reduce Pd(II) and subsequently induce precipitation of Pd(0) nano-particles: the metal-respiring bacterium *Shewanella oneidensis*,¹⁴ and the sulfate-reducing bacterium *Desulfovibrio desulfuricans*.¹⁵ It is known that Pd and bio-Pd can catalyze the reduction of various groundwater and soil pollutants.^{14,16,17} However, all these processes utilize two-steps, wherein bio-nano-Met nanoparticles are formed in a separate reaction before contaminant treatment, thus making these processes less suitable to treat subsurface contaminants. Recently, the authors described the proof of concept for a process in which Pd nanoparticles were formed at the zone of interest along with in-situ generation of molecular hydrogen leading to a reductive removal of hexavalent chromium.¹⁸ To our knowledge, a one-step nanoparticulate-based reductive process has not been demonstrated for organics. In this study, we present a proof of concept for such a process using two common azo dyes, methyl orange (MO) and Evans Blue (EB). EB is also known as Direct Blue 53. Azo dyes were chosen because they constitute the

largest group of synthetic colorants of the more than 280,000 tons of textile dyes that are improperly discharged into the environment each year.^{19,20} While, some microbes, including members of genus *Clostridium* are known to have azoreductases that can metabolize azo compounds,^{21–23} the kinetics of these natural processes are slow.

Experimental

Deionized water was used to make growth media and in analysis. Chemicals used in this study were analytical grade or better. All liquid culture experiments were performed in triplicate.

Culture.— *Clostridium pasteurianum* sp. BC1 (ATCC No. 53464) was grown as previously described.^{18,24} The medium contained (per liter) glucose, 5.0 g; NH₄Cl, 0.5 g; glycerol phosphate, 0.3 g; MgSO₄ · 7H₂O, 0.2 g; CaCl₂ · 2H₂O, 0.5 g; FeSO₄ · 7H₂O, 2.8 mg; peptone, 0.1 g; yeast extract, 0.1 g. NaOH was used to adjust the pH of the medium to 6.8. The medium was pre-reduced by boiling for 15 min with simultaneous purging using filtered ultra-high-purity (UHP) nitrogen gas. Growth medium was then cooled and transferred to an anaerobic chamber. 40 mL aliquots of the medium were dispensed into 60 mL serum bottles. The bottles were sealed with butyl rubber stoppers and aluminum crimps, and autoclaved. All manipulations of the samples were carried out inside an anaerobic glove chamber (Coy Laboratory products, MI) filled with nitrogen containing trace amount of hydrogen for catalytic removal of any oxygen. Cultures were grown in an incubator shaker at 28°C and 125 rpm.

Formation of Bio-Pd.— Cultures were grown until the end of the log phase of growth (O.D._{600nm} = 0.6), which typically required 18 hours. Aqueous Pd(II) (1 g/L Na₂PdCl₄ stock solution) was then added to the cultures to achieve an initial Pd(II) concentration of 100 mg/L. As reported previously, the medium turned black within one minute, due to the reduction of Pd(II) ions to Pd(0), which was confirmed with X-ray diffraction and X-ray absorption spectroscopy.¹⁸ Scanning electron microscopy was performed to confirm the nanoparticulate nature of the Pd particles. The bio-Pd morphology and spatial distribution within the biomass were examined using a LEO 1550 SEM equipped with a Schottky Field-Emission Gun.

Batch studies of azo dye reduction.— The native ability of *C. pasteurianum* BC1 to degrade the two dyes of interest was examined by studying the amount of dye degradation that occurs in the presence of live cells of *C. pasteurianum* BC1 and heat-killed cells of *C. pasteurianum*. MO and EB were added to bio-Pd suspensions using stock solutions to result in a final concentration of 450 μM and 48 μM,

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respectively. Dye concentrations and their breakdown products were analyzed using UV-Visible spectroscopy. These studies served as controls.

The following sets of experiments were conducted to monitor the effects of the addition of bio-Pd on the degradation of EB and MO. The bio-Pd was formed in-situ by the addition of requisite amount of Pd²⁺ from a stock of Na₂PdCl₄. The concentrations of bio-Pd used in this study were 1 ppm, 5 ppm and 10 ppm. These results were compared to sets containing no bio-Pd, which were used as control. Starting concentrations of 450 μM and 48 μM were used for MO and EB, respectively.

Analytical techniques.— The concentration of azo dyes and their breakdown products in the cultures at different time intervals was determined spectrophotometrically. Methyl orange and Evans Blue concentrations were monitored using UV-Visible spectroscopy at 479 nm and 610 nm, respectively. Further, the degradation product of MO was qualitatively and quantitatively observed at 244 nm.

Results and Discussion

C. pasteurianum BC1 glycolysis produces fermentation products including hydrogen, acetate, butyrate and formate that create a highly reducing environment.¹⁸ When a culture of *C. pasteurianum* BC1 was supplemented with aqueous Pd(II), the Pd(II) was immediately reduced forming bio-Pd. Supplementing the medium with an initial Pd concentration of 1, 5 or 10 mg/L changed the color of the medium to gray within one minute and the aqueous Pd concentration was found to be below the detection limit of 1 mg/L. The intensity of the gray color was directly related to added Pd concentration; higher concentrations led to a darker color. In contrast, no color change was observed in an abiotic control starting with aqueous Pd(II) in sterile growth media. Thus, the compounds in the growth medium did not significantly complex with Pd(II) ions or contribute measurably to Pd(II) reduction. The gray color change has previously been reported under comparable conditions with *Shewanella oneidensis*³ and *Desulfovibrio desulfuricans*⁴ as an indicator of reductive precipitation of metallic Pd nanoparticles. The reduction of over 99% Pd(II) by *C. pasteurianum* BC1 within one minute is notably faster than the rates reported for *S. oneidensis* and *D. desulfuricans*, where black precipitates first appear after 5–10 minutes and complete reduction is only observed after approximately one hour.^{3,4}

Scanning electron microscopy images showed bio-Pd to be precipitated mostly on the bacterial cell wall and to a lesser extent in the extracellular region. As shown earlier, particles in both regions were found to be present in nanoparticulate metallic form or Pd(0).¹⁸ An SEM image showing the nanoparticulate matter is shown in Figure 1.

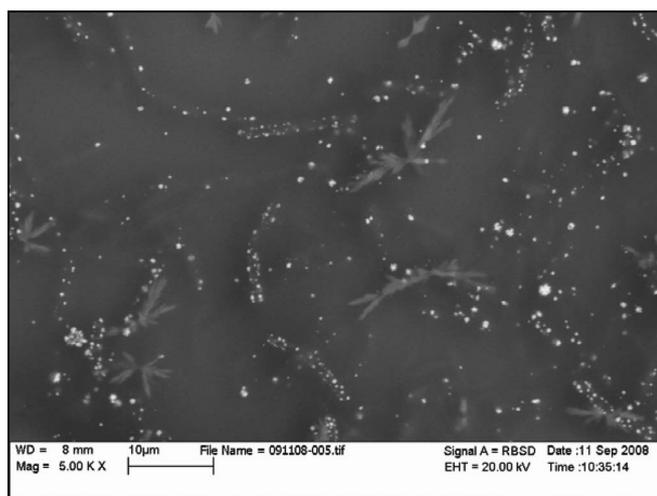


Figure 1. A scanning electron microscopic image of the nanoparticulate bio-Pd formed on the cells of *C. pasteurianum* BC1.

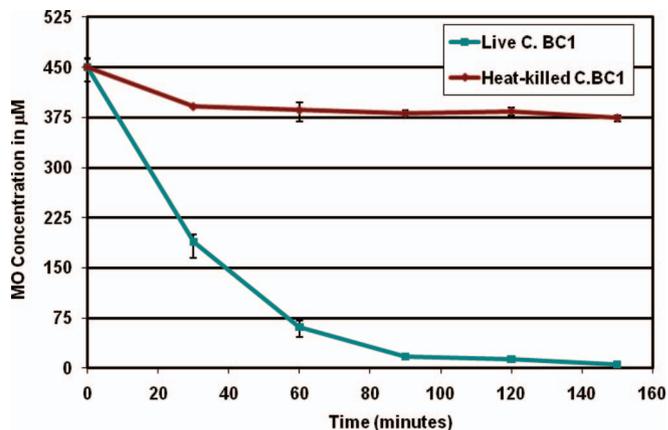


Figure 2. Concentration of methyl orange, determined at various times using UV-visible spectroscopy, showed that heat-killed cells of *C. pasteurianum* BC1 did not degrade the dye to any significant extent when compared to live *C. pasteurianum* BC1 culture.

Controls.— Heat-killed cells of *C. pasteurianum* BC1 did not degrade azo dyes to any significant extent as shown in Figure 2. Further, no degradation of azo dyes was observed in abiotic controls, which showed a constant dye concentration (data not shown). MO was degraded by a living culture of *C. pasteurianum* BC1. An initial MO concentration of 450 μM underwent almost complete reductive degradation in about 90 minutes.

On the other hand, EB was found to be extremely recalcitrant and was degraded only in insignificant amounts. The decomposition of Evans Blue was limited and *C. pasteurianum* BC1 culture containing no bio-Pd was unable to degrade EB at higher concentration. Thus, the amount of EB that was added was lowered to 4.8 μM; an almost 100-fold lower concentration compared to MO. As seen in Figure 3, even that concentration was not degraded completely by both heat-killed and living cultures of *C. pasteurianum* BC1 without bio-Pd.

Bio-Pd catalyzed reduction of azo dyes.— In the presence of bio-Pd, the degradation kinetics of azo dyes was significantly enhanced. Figure 4 shows the concentration of MO over time for a bio-Pd containing culture and a control without bio-Pd. It can be seen that the degradation rate of MO was accelerated over 10 times, as observed from the time taken to degrade 450 μM of MO (7 min with 10 ppm bio-Pd as opposed to nearly 90 min in the absence of bio-Pd).

In a similar manner, *C. pasteurianum* BC1 that was unable to entirely degrade 4.8 μM EB in the absence of bio-Pd, was found to be able to degrade 48 μM of EB in the presence of bio-Pd, as shown

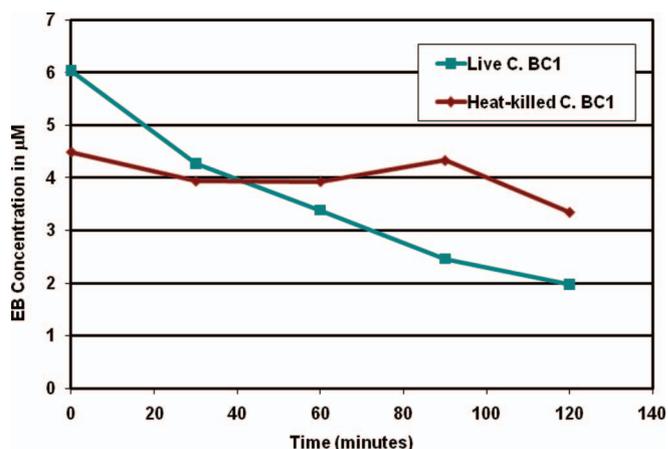


Figure 3. Concentration of Evans Blue, determined using UV-visible spectroscopy, showed that neither living culture or heat-killed cells of *C. pasteurianum* BC1 can degrade the dye to any significant extent.

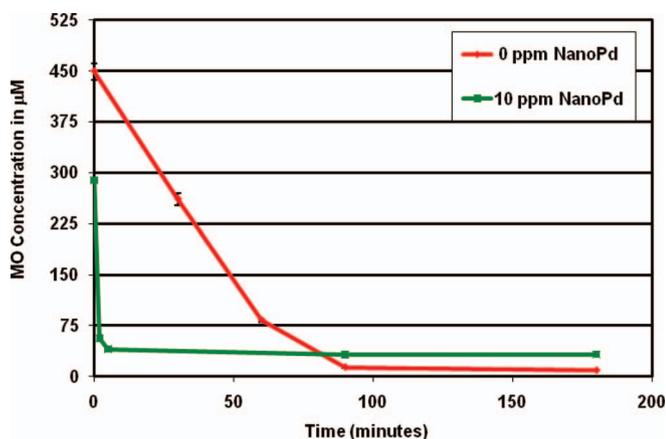


Figure 4. Concentration of methyl orange, determined using UV-visible spectroscopy, shows that the in-situ formed bio-Pd (10 ppm) acted as a catalyst for the reduction of MO in the presence of *C. pasteurianum* BC1.

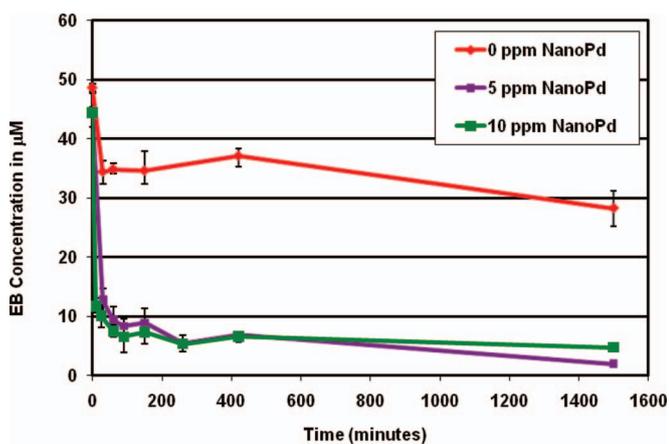


Figure 5. Concentration of Evans Blue, determined using UV-visible spectroscopy, shows that the in-situ formed bio-Pd (5 ppm and 10 ppm) acted as a catalyst for the reduction of EB in the presence of *C. pasteurianum* BC1.

in Figure 5. Further, it was seen that even a 5 ppm concentration of bio-Pd was sufficient to induce a near complete reduction of EB. However, 1 ppm concentration of bio-Pd was not found to lead to any significant reduction of EB (data not shown). Figure 6 shows the color changes that were observed through the course of this set of experiments. The initial addition of Pd in varying concentrations changed the color to varying levels of gray (Figure 6a). The addition of 48 μ M of EB led to the formation of an intense blue color at time $t = 0$ (Figure 6b). Visible reduction of EB was catalyzed by 5 ppm and 10 ppm of bio-Pd, as seen by the slow loss of color observed at 10 minutes (Figure 6c) and 6 hours (Figure 6d) after the addition of EB.

Degradation mechanism.— It is our hypothesis that the degradation of azo compounds occur via reductive breakage of the azo linkage. This proposed reaction mechanism is shown below. The N = N is broken down and hydrogenated, leading to the formation of amidated daughter compounds. In the case of methyl orange, the expected daughter products are sulfanilic acid and N,N-Dimethylbenzene-1,4-diamine as shown below in reaction 1. Breakdown of Evans Blue leads to three daughter products as shown in reaction 2, namely, 3,3-dimethyl-biphenyl-4,4-diamine, 6,8-diamino-7-hydroxy-naphthol-1,3-disulfanate and 4,7-diamino-6-hydroxy-naphthol-1,3-disulfanate and neither of which is identifiable using UV-Visible spectroscopy. In this proof-of-concept study, we only evaluated the breakdown of MO.

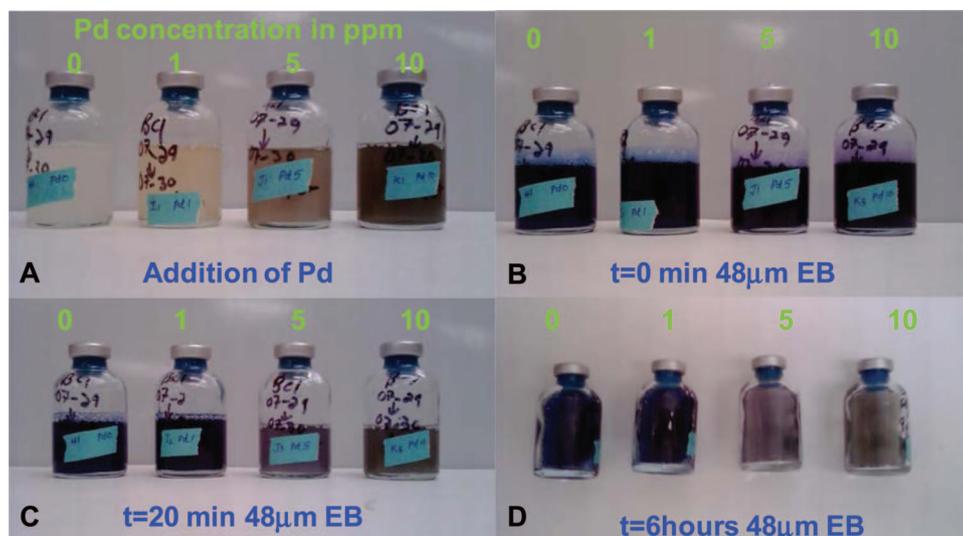
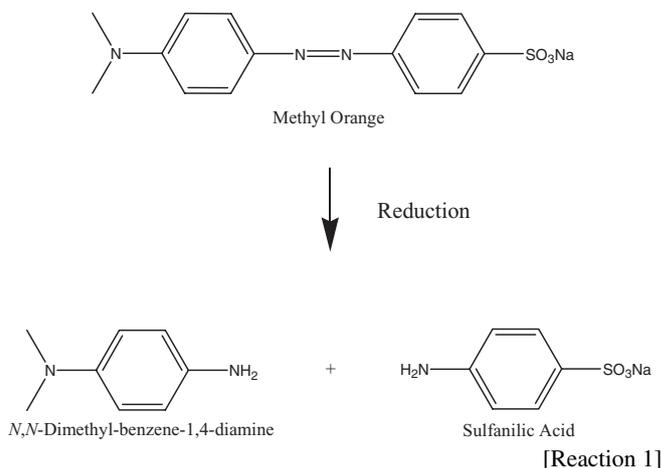


Figure 6. Photographs show the color changes observed through the course of this set of experiments. A: The initial addition of Pd in varying concentrations changed the color to varying levels of gray. B: The addition of 48 μ M of EB led to the formation of an intense blue color at time $t = 0$. Visible reduction of EB was catalyzed by 5 ppm and 10 ppm of bio-Pd, as seen by the slow loss of color observed at (C) 10 minutes and (D) 6 hours after the addition of EB.

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