Curr Microbiol (2008) 56:268–273
DOI 10.1007/s00284-007-9073-9

Influence of Reduced Electron Shuttling Compounds on Biological H_2 Production in the Fermentative Pure Culture *Clostridium beijerinckii*

Jennifer L. Hatch · Kevin T. Finneran

Received: 31 July 2007 / Accepted: 6 October 2007 / Published online: 1 January 2008

Abstract Several reports suggest that extracellular electron shuttles influence fermentative metabolism in a beneficial manner for bioremediation and biotechnology strategies. The focus of this research was to characterize the effects of reduced electron shuttling molecules on fermentative H_2 production. Reduced electron shuttles may provide reducing equivalents to generate H_2, which influences alternate cellular processes. Electron shuttling compounds cycle between reduced-oxidized states and influence fermentative physiology. *Clostridium beijerinckii* fermentation was altered using a physiological approach that resulted in H_2 production with the reduced extracellular electron shuttle anthrahydroquinone-2,6,-disulfonate (AH_2QDS) and biologically reduced humic substances as the primary electron donors. Cells were suspended in a buffer with an excess of the biological electron transfer molecule NAD^+, with AH_2QDS (100–1000 μM) or biologically reduced humic substances (0.01–0.025 g/L) as the sole electron source. Increasing concentrations of AH_2QDS and reduced humics increased H_2 production, while H_2 production was suppressed by Fe(III) hydroxides, which outcompeted the cells for electrons from the reduced shuttles, suggesting that the shuttles are in fact electron donors for H_2 production. Oxidized AQDS/humics did not increase H_2 production. Organic acid production shifted toward butyric acid in the presence of reduced electron shuttles, particularly with growing cells. Growth and hydrogen production rates in growing cells were initially faster in the presence of the reduced electron shuttles; however, the final biomass yield was inversely proportional to the starting AH_2QDS concentration, which suggests that reduced shuttles may compete with anabolic cell processes for available energetic resources or that the shift to excess butyrate becomes toxic to the cells.

J. L. Hatch · K. T. Finneran (✉)
University of Illinois at Urbana-Champaign, Environmental Engineering and Science Program, Civil and Environmental Engineering, 3221 Newmark Laboratory, 205 North Mathews Avenue, Urbana, IL 61801, USA
e-mail: finneran@uiuc.edu

Introduction

Extracellular electron transfer molecules are redox active compounds that cycle between oxidized and reduced states without being consumed and these reactions are catalyzed by many organisms [1]. Previous data suggest that fermentative cultures can reduce these compounds by an unspecified metabolic pathway without any benefit to the cells in terms of carbon or energy [2]. However, few if any data are available as to how the reduced form of the shuttle influences fermentative metabolism. This research is critical because recent reports suggest using electron shuttles with fermentative cultures in bioremediation and biotechnology applications [3, 4]. It is possible that reduced electron shuttles can provide electrons to fermentative cultures specifically for H_2 production, while also altering growth and metabolism.

The involvement of the quinone/hydroquinone redox couples in electron transfer has been investigated with both humic substances and the model compound anthraquinone-2,6-disulfonate (AQDS) primarily for metal reduction and bioremediation studies. AQDS is an exogenous electron shuttle with a redox potential of –184 mV that is both highly soluble and bioavailable [5]. AQDS is a small molecule with a single quinone moiety that is not sterically hindered and transfers two electrons per mole. Humic

substances are large, undefined molecules with varying quinone content whose individual quinone functional groups may be less accessible than single quinone moiety shuttles [6]. Humics along with quinone-containing humic analogues can transfer electrons from biomass [7], reduced inorganic compounds [8], and organic compounds [9] to contaminants. It is has been widely reported that reduced humic acids can donate electrons, and that humic substances have the ability to undergo reduction-oxidation, but the effect of reduced quinones and their electron shuttling capabilities has not yet been investigated with respect to fermentative H_2 production or their direct influence of fermentative metabolic activity [6, 10].

We have investigated the influence of reduced extracellular electron shuttles on a model fermentative pure culture, *Clostridium beijerinckii*. The reduced shuttles serve as electron donors for H_2 production, which may be an alternate method of H_2 production if appropriate conditions are met to generate reduced shuttles in the presence of fermentative cultures. Organic acid synthesis and growth yield were also influenced by the reduced electron shuttles. The results presented here are the first to suggest reduced electron shuttles as an electron donor specifically for H_2 production in fermentative metabolism, and demonstrate that electron shuttling molecules may alter fermentative physiology.

Materials and Methods

Cell Maintenance

Clostridium beijerinckii NCIMB 8052 (ATCC number 51743) was obtained from the College of Agricultural, Consumer and Environmental Sciences at the University of Illinois Urbana-Champaign and grown anaerobically in medium initially containing 30 g/L tryptone, 20 g/L glucose, 10 g/L yeast extract, and 1 g/L L-cysteine. The medium was made anaerobic using N_2 gas and sterilized by autoclaving at 121°C for 20 min. Previously described anaerobic and aseptic culturing techniques were used throughout. Cultures were incubated at 37°C (LabLine Imperial III Incubator).

Resting Cell Suspensions

Resting cell suspensions were performed to quantify H_2 production and other cellular reactions under nongrowth conditions in anaerobic bicarbonate buffer (0.05g/L sodium bicarbonate) adjusted to pH 6.0 using a 95:5 (vol:vol) gas mix of N_2:CO_2. *C. beijerinckii* cells were harvested in late log phase/early stationary phase of growth. One liter of cells was harvested by splitting it into four separate 250-mL centrifuge bottles on ice with a heavy N_2:CO_2 flow bubbling through the bottles while the medium was poured. Tubes were centrifuged and washed twice at 5000*g* for 15 min to form a dense cell pellet. Each resulting pellet was resuspended in 1.0 mL of 0.6 mM anaerobic bicarbonate buffer. The cells in the four centrifuge tubes remained on ice and under a heavy N_2:CO_2 flow throughout the procedure. The four separate tubes were pooled in one anaerobic pressure tube and sealed immediately under a N_2:CO_2 headspace. The experiments were initiated by taking aliquots from the anaerobic tube and injecting them into the various experimental tubes. Experimental tubes contained 0.15 mL of cells in a total volume of 10 mL of bicarbonate buffer with an anaerobic N_2:CO_2 headspace. Experimental tubes were incubated with either 100–1000 μM AH_2QDS, 0.01–0.025 g/L humic substances, or AH_2QDS in the presence or absence of 10 mmol/L Fe(III) oxide (as a competitor for electrons from the hydroquinone). All tubes contained 5 nM exogenous NAD^+ (to facilitate intracellular electron transfer for resting cell mass.) NAD^+ is expected to be sequestered in cell mass; it will not serve as an extracellular electron shuttle. Control tubes were either cells alone or mixtures of the chemicals alone. All experiments were run in triplicate and tubes were incubated at 37°C.

Chemicals

Anthraquinone-2,6-disulfonate (AQDS), nicotinamide adenine dinucleotide (NAD^+), and purified humic acids were purchased from Sigma Aldrich (Milwaukee, WI). Purified humics and AQDS were biologically reduced using *Geobacter metallireducens* strain GS-15 (ATCC 53774) and *Geobacter sulfurreducens* strain PCA (ATCC 51573), respectively. All metal-reducing cells were filtered prior to using the reduced humic substances and AQDS and, therefore, did not influence the results. Cellular lysate that may have been present was negligible compared to the AH_2QDS or reduced humics. Chemically reduced AH_2QDS was also tested (AQDS reduced by H_2 in the presence of palladium) and results were identical. Biogenic AH_2QDS was used for all remaining experiments. Fe(III) hydroxides (FeGel) were used as a competitive electron acceptor for AH_2QDS electrons in selected experiments. They were synthesized as previously described [11].

Sampling and H_2 Analysis

Gas headspace samples were collected (0.5 mL) and injected into a GC (Hewlett Packard 5890 or Shimadzu GC-14A) with a Carbosieve S-II Spherical Carbon column

(Supelco, Bellefonte, PA) equipped with a Reduction Gas Detector (RGD 2, Trace Analytical), as previously described [12]. All H_2 concentrations were quantified versus certified H_2 standard gases (Scotty gases; VWR Scientific).

Organic Acid Analysis

Liquid samples were filtered through 0.2-μm sterile PTFE filters into HPLC autosampler vials and organic acid production was monitored at 210 nm using HPLC and the Acclaim OA Column (Dionex) along with a photodiode array detector. The standards run for organic acid analysis included formic, acetic, lactic, butyric, and propionic acids.

Growth Experiments and Analysis

Experiments with growing cells were conducted in the same anaerobic test tubes as resting cell experiments with a 1:200 dilution of the original growth medium in a total volume of 10.0 mL. Tubes were monitored for cell density increases by optical density (OD) measured at 600 nm as previously described [13]. Hydrogen and organic acids were quantified as described above. Blanks consisted of uninoculated media.

Results

Cell suspensions were run with the reduced electron shuttling molecules as the sole electron donor source (i.e., no fermentable substrate). The intracellular electron carrier NAD^+ was added exogenously to the buffer at a concentration higher than normally present in cell mass (5 nM), to provide the cells with increased capacity for oxidizing the reduced quinone (NAD^+ alone did not alter metabolism). AH_2QDS concentrations were varied in the presence of NAD^+ and the highest concentration of AH_2QDS tested produced the most H_2 (Fig. 1). Increases in H_2 production were not linear with increases in AH_2QDS concentration, suggesting a possible limitation to using AH_2QDS oxidation as the sole electron donor for H_2 production. It is also possible that 7000–8000 nmol AH_2QDS was the saturation amount for the cell mass present. H_2 production continued to increase over 5 days with the exception of the 100 μM AH_2QDS concentration, in which day 2 had the highest H_2 yield. This may have been due to the subtraction of the cells-only H_2 mass from the experimental data. All data demonstrated an AH_2QDS-concentration dependent increase in H_2 mass.

Humic substances were tested and the high concentration of biologically reduced humics (0.025 g/L) produced

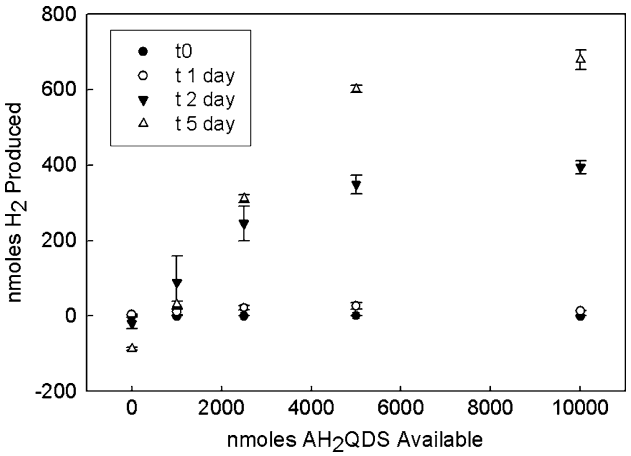

Fig. 1 Hydrogen production by *C. beijerinckii* in NAD^+-amended buffer (5 nM) with increasing concentrations of AH_2QDS (0–10,000 nmol) as the sole electron donor. Results are the means of triplicate analyses. Bars indicate one standard deviation. *Cells-only control* H_2 mass was subtracted from all data points

almost twice the amount of H_2 as the low concentration (0.01 g/L) of reduced humics (Fig. 2) when excess NAD^+ was present. Humic substances produced less H_2 than AH_2QDS over 4 days, but humics have multiple quinone groups on a larger molecule which may be less accessible than the single quinone group in AH_2QDS. Oxidized humic substances did not increase H_2 production, which demonstrates that the total humic molecule was not used as a fermentable substrate, but rather the reduced form was oxidized to generate H_2 directly.

An additional experimental series was run with Fe(III) hydroxides added to the suspensions with AH_2QDS as a competitive inhibitor for the hydroquinone electrons. Adding Fe(III) hydroxide inhibited H_2 production, as electrons from AH_2QDS are instantaneously shuttled to Fe(III) hydroxides as an electron acceptor (Fig. 2) [14]. This suggests that it is in fact electron transfer from the reduced shuttle to promote H_2 production, although the exact mechanisms have yet to be determined in detail.

Growing cells were amended with three concentrations of AH_2QDS (100, 250, and 500 μM) at the time of inoculation, and growth and H_2 production were monitored over time. The tubes without AH_2QDS followed the expected sigmoidal growth curve, with average hydrogen production reaching approximately 22 μmol (Fig. 3). However, growth in all AH_2QDS amended tubes was initially more rapid with immediate growth (no lag phase), and concomitant hydrogen production was also significantly faster (zero lag time) in tubes with the hydroquinone (Fig. 3). Final H_2 yield was approximately the same in all tubes; however, the final biomass yield was inversely proportional to the concentration of AH_2QDS added. Results were nearly identical when AH_2QDS was amended

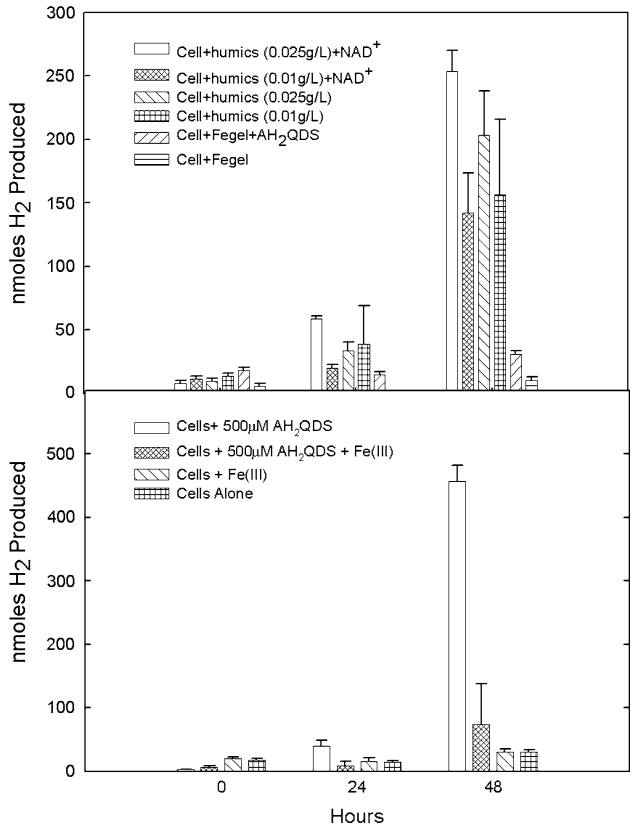

Fig. 2 Hydrogen production by *C. beijerinckii* amended with reduced humic substances (0.01 and 0.025g/L) and NAD^+ (5 nM) in the absence of Fe(III) (top). Poorly crystalline Fe(III) oxide (Fe Gel) was added at 10 mmol/L as a competitive electron acceptor for electrons from 500 μM AH_2QDS (bottom). Results are the means of triplicate analyses. Bars indicate one standard deviation. Glucose was not added in either experiment; the shuttles were the sole electron donor(s)

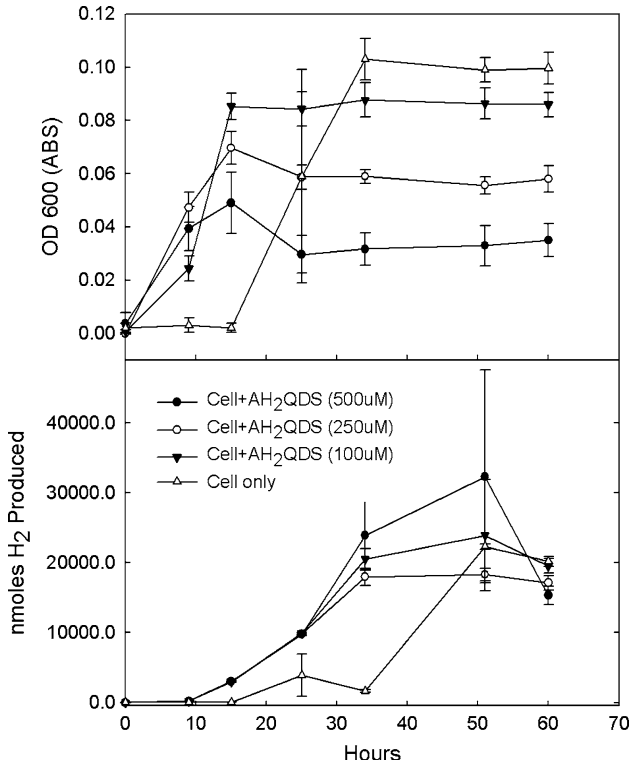

Fig. 3 Growth (top) and H_2 production (bottom) by *C. beijerinckii* amended with increasing concentrations of AH_2QDS (100, 250, 500 μM) in media with glucose as the primary fermentable substrate (500 μM); glucose was present in all treatments. Controls were incubated with cells + glucose alone (open triangles). Results are the means of triplicate analyses. Bars indicate one standard deviation

after 9 h of growth, with the exception that the final biomass yield with 100 μM AH_2QDS was the same as that with glucose alone (data not shown).

Organic acids were measured, as they are the primary expected end products (during the acidogenic phase) other than hydrogen. The average concentrations produced with 100 μM AH_2QDS are shown in Fig. 4 for growth phase experiments. Butyric acid concentrations significantly increased in the presence of AH_2QDS both during growth and in resting cell suspensions (data not shown); however, the growth phase butyric acid concentration increased considerably with 100 μM AH_2QDS (from 120 to 798 μM). A carbon mass balance could not be performed for the resting cells because of the endogenous respiration contribution; however, acetic acid production was stable and butyric acid production increased in the presence of AH_2QDS for all incubations tested. This increase in butyric acid production did not inhibit hydrogen production but may have contributed to the decreased cell yield in growth systems.

Discussion

These results suggest that fermentative micro-organisms are influenced at the physiological level with respect to H_2 production and organic acid synthesis, and they are the first data to suggest that extracellular electron shuttles act as direct electron donors for H_2 production in both resting and growing fermentative cultures. H_2 is produced naturally in fermentative systems despite its low redox potential, though the yield may be minimal compared to that of other end products. H_2-producing fermentative bacteria are often present in a syntrophic relationship with H_2-scavenging bacteria which use H_2 as an electron donor. H_2 oxidation has been linked to all anaerobic respiratory pathways [15], but it is typically methanogens that dominate as the H_2 utilizing microorganisms in engineered H_2-producing reactors because the inocula are from materials such as digester fluid or animal/agricultural waste mixtures [16].

The cells oxidized the electron shuttles to produce H_2 despite the more negative redox potential of the electron transfer pathway for hydrogen relative to the hydroquinones. Fermentative cells have no specific biochemical pathway for oxidation of the reduced electron shuttles;

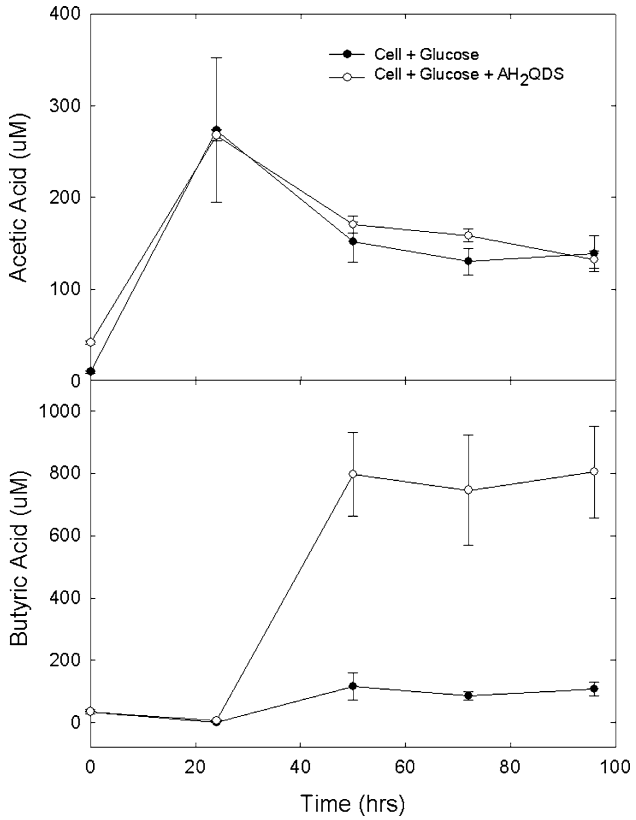

Fig. 4 Acetate production (top) and butyrate production (bottom) by *C. beijerinckii* amended with AH_2QDS (100 μM) in media with glucose as the primary fermentable substrate (500 μM); glucose was present in all treatments. Results are the means of triplicate analyses. Bars indicate one standard deviation

therefore, the electrons are likely channeled to the hydrogenase pathway because the reaction is a simple two-electron oxidation and it is likely the nearest biochemical analogue for AH_2QDS oxidation available to the cells. H_2 production increased with increasing concentrations of AH_2QDS (tested up to 1000 μM), and a maximum of 12% of the AH_2QDS was oxidized based on the H_2 produced. These data suggest that the exogenous, reduced shuttles likely interact with fermentative electron transport systems as a secondary electron donor molecule. Biologically reduced humic substances increased H_2 production, and similar to AH_2QDS, the higher the concentration of humics tested, the more H_2 was produced. It is possible that AH_2QDS directly transfers electrons to ferredoxin, which must be investigated further.

Fe(III) hydroxides inhibited H_2 production when added to the resting cell suspensions. Electron transfer from reduced shuttles to Fe(III) is instantaneous [14], and Fe(III) typically outcompetes all other electron acceptors present in anaerobic system for electrons from AH_2QDS based on thermodynamics and redox potentials [17, 18]. This suggests that it is in fact electron transfer from the reduced quinones promoting H_2 production in the fermentative system rather than an unidentified pathway in which the hydroquinones are themselves fermented. Oxidized humic substances and AQDS did not promote H_2 production, which negates the possibility that the quinones were fermented. It is possible that the reduced shuttles interfere with the cell's central metabolism (electron transport or ATP synthesis) based on growing cell yields in the presence of the electron shuttles, and more work is necessary to identify the mechanisms involved.

Organic acid production shifts were identified, particularly in growing cell incubations. However, the ratio of butyric to acetic acid always increased in the presence of AH_2QDS. NAD^+/NADH is directly linked to the butyrate pathway for fermentation, but it is only indirectly linked to the acetate pathway. AH_2QDS oxidation likely proceeds through the dinucleotide carrier and any increase in reduced NADH will increase butyrate production in addition to H_2 production. It is possible that the increased butyric acid caused the decrease in cell yield, as electrons from the tryptone-yeast extract portion of the media may have been redirected to organic acid synthesis rather than biomass production.

Several reports indicate that fermentative cultures can reduce quinones such as AQDS [19, 20]. This reduction is nonspecific and the cells do not gain energy from the reaction [2, 20]. Although AQDS reduction may not benefit the cells, it is possible that H_2 can be produced from the reduced electron shuttles. While these are the first studies of this nature and more work is necessary, the data are promising in that reduced electron shuttles generated H_2 in a fermentative culture as the sole exogenous electron donor.

Recent studies suggest that fermentative cultures have a role in the reductive transformation of several nitro-bearing contaminants and that electron shuttles increase the rate and extent of biodegradation [3, 4]. However, given the minimal capacity for fermentative cells to reduce the extracellular quinones, the total activity reported cannot be fully explained by just electron shuttle reactions between the cells and contaminants [3, 4]. It is possible that the slight excess hydrogen produced increased activity, as H_2 is a favorable electron donor for all of the reported reductive reactions. Although this reaction may not benefit the fermenter, it may benefit the remediation strategy by producing a favorable electron donor.

At this point, less than 12% of the available reducing power from the hydroquinone was recovered as biohydrogen (based on the H_2 generated and assuming that the remaining hydroquinone was not oxidized), which suggests that the reaction is inefficient; however, all data suggest that reduced quinones will directly produce H_2 in resting cells, and the reduced shuttles may alter the metabolism of growing cells. The growth data suggest that initial growth

and hydrogen production rates were faster than the traditional glucose fermentation pathway, but the eventual cell yield decreased. More research is needed to explore this unique metabolic phenomenon since electron shuttles have been suggested as a bioremediation amendment with fermentative cultures, and because it may possibly lead to alternate H_2 production strategies for engineered bioreactors.

Acknowledgments This work was supported by a SURGE Fellowship (to J.L.H.) and the University of Illinois. We would like to thank Man Jae Kwon for his laboratory assistance and both Hans Blaschek and Roderick Mackie at the College of Agricultural, Consumer and Environmental Sciences, University of Illinois at Urbana-Champaign, for providing the bacterial cultures used in the study.

References

1. Coates J, Ellis D, Blunt-Harris E, et al. (1998) Recovery of humic reducing bacteria from a diversity of environments. Appl Environ Microbiol 64:1504–1509
2. Lovley DR (1991) Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol Rev 55 (2):259–287
3. Bhushan B, Halasz A, Hawari J (2006) Effect of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by Clostridium sp EDB2. J Appl Microbiol 100(3):555–563
4. Borch T, Inskeep WP, Harwood JA, et al. (2005) Impact of ferrihydrite and anthraquinone-2,6-disulfonate on the reductive transformation of 2,4,6-trinitrotoluene by a gram-positive fermenting bacterium. Environ Sci Technol 39 (18):7126–7133
5. Hernandez ME, Newman DK (2001) Extracellular electron transfer. Cell Mol Life Sci 58 (11):1562–1571
6. Lovley DR, Coates JD, Blunt-Harris EL, et al. (1996) Humic substances as electron acceptors for microbial respiration. Nature 382:445–448
7. Kwon MJ, Finneran KT (2006) Microbially-mediated hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) biodegradation by extracellular electron shuttling compounds. Appl Environ Microbiol 72 (9):5933–5941
8. Schwarzenbach RP, Stierli R, Lanz K, et al. (1990) Quinone and iron porphyrin mediated reduction of nitroaromatic compounds in homogeneous aqueous solution. Environ Sci Technol 24 (10):1566–1574
9. Uchimiya M, Stone AT (2006) Aqueous oxidation of substituted dihydroxybenzenes by substituted benzoquinones. Environ Sci Technol 40(11):3515–3521
10. Lovley D, Phillips E (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl Environ Microbiol 54:1472–1480
11. Lovley DR, Phillips EJP (1987) Rapid assay for microbially reducible ferric iron in aquatic sediments. Appl Environ Microbiol 53(7):1536–1540
12. Anderson RT, Chapelle FH, Lovley DR (1998) Evidence against hydrogen-based microbial ecosystems in basalt aquifers. Science 281:976–977
13. Madigan M, Martinko J, Parker J, ed. (2003) Brock biology of microorganisms. 10th ed. Prentice Hall, Upper Saddle River, NJ
14. Lovely D, Coates J, Blunt-Harris E, et al. (1996) Humic substances as electron acceptors for microbial respiration. Nature 382:445–448
15. Lovley DR, Goodwin S (1988) Hydrogen concentrations as an indicator of the predominant terminal electron accepting reactions in aquatic sediments. Geochim Cosmochim Acta 52:2993–3003
16. Angenent LT, Kamir K, Al-Dahhan MH, et al. (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol 22(9):477–485
17. Scott DT, McKnight DM, Blunt-Harris EL, et al. (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. Environ Sci Technol 32 (19):2984–2989
18. Scott DT, McKnight DM, Blunt-Harris EL, et al. (1998) Quinones in humic substances as electron acceptors by humic-reducing microorganisms. Abstr Papers Am Chem Soc 216:074-ENVR
19. Benz M, Schink B, Brune A (1998) Humic acid reduction by *Propionibacterium freundenreichii* and other fermenting bacteria. Appl Environ Microbiol 64(11):4507–4512
20. Cervantes FJ, Duong-Dac T, Roest K, et al. (2003) Enrichment and immobilization of quinone-respiring bacteria in anaerobic granular sludge. Water Sci Technol 48(6):9–16