Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Original article doi: 10.12980/jclm.3.2015jclm-2015-0010

Dissimilatory reduction of perchlorate and other common pollutants by a consortium enriched from tidal flats of the Yellow Sea

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ARTICLE INFO

Article history:
Received 24 Feb 2015
Received in revised form 13 Jul 2015
Accepted 30 Jul 2015
Available online 11 Aug 2015

Keywords:
Perchlorate
Dissimilatory reduction
Consortium
Alternate electron acceptor
Electron donor

ABSTRACT

Objective: To enrich a facultative anaerobic bacterial consortium from the Yellow Sea and assess its ability to reduce perchlorate and other co-pollutants.
Methods: Bacterial consortium collected from the tidal flats of the Yellow Sea was enriched in an anoxic medium containing perchlorate as the electron (e^-) acceptor and acetate as the electron (e^-) donor. The enriched consortium was then tested for perchlorate reduction under different perchlorate concentrations and in the presence of nitrate by using standard anaerobic techniques. The complete enzymatic reduction of perchlorate to chloride was confirmed by chlorite dismutation. Ability of the consortium to grow with alternate e^- acceptors was also tested with acetate as the e^- donor.
Results: The enriched consortium could rapidly reduce perchlorate up to the initial concentration of 25.65 mmol/L. In the presence of nitrate, perchlorate reduction did not occur immediately and reduction of nitrate started after a lag phase, with concomitant accumulation of nitrite. The perchlorate-enriched consortium could reduce chlorate, oxygen, Cr (VI), and selenate as the alternate e^- acceptors but failed to utilize sulfate, thiosulfate, sulfite, and nitrite.
Conclusions: The consortium from the tidal flats of the Yellow Sea could reduce perchlorate and co-contaminants such as chlorate, nitrate, Cr (VI), and selenate under heterotrophic conditions with acetate as the e^- donor and carbon source. While perchlorate was completely dismutated into innocuous chloride and oxygen, accumulation of nitrite occurred during the reduction of nitrate.

1. Introduction

Perchlorate is an oxyanion of chlorine that is highly soluble, stable, and mobile in water. The greatest use of ammonium perchlorate is as an oxidizer in solid missile and rocket fuel. Other applications are in wet digestions, organic syntheses, explosives, pyrotechnics, fireworks and analytical chemistry. The only natural source of perchlorate is the Chilean nitrate deposits which were used as fertilizers[1]. Large-scale contamination with perchlorate came to light after the development of a highly sensitive analytical method in 1997[2,3], and came under US Environmental Protection Agency regulation as perchlorate is known to interfere with iodine uptake by human thyroid[4].

For the treatment of perchlorate containing groundwater, physico-chemical technologies such as ion exchange, carbon adsorption, and reverse osmosis have been tried. Biological treatment as an environment-friendly process has also received substantial attention during the past one decade[5,6]. For the *ex situ* treatment either fixed or fluidized-film bioreactors have been employed and treatment of both rocket propellant wash waters and contaminated ground waters have been achieved with acetate or hydrogen as the electron (e^-) donor[7-11]. The kinetics of perchlorate reducing mixed consortia and isolates have also been reported[12,13]. However, the metabolic diversity of the perchlorate reducing bacteria is still not fully understood. The present study reports on enrichment of a dissimilatory perchlorate-reducing consortium from the tidal flats of the Yellow Sea and demonstrates its metabolic ability to use several other pollutants commonly encountered in the ground waters. Since, ammonium salts of perchlorate get oxidized to nitrate under oxic conditions, both nitrate and perchlorate may often co-exist in the nature[14]. In the present study we also assessed feasibility of nitrate-independent perchlorate reduction or co-reduction of both pollutants. Additionally, the reductive metabolism of chlorate, sulfur anions, and heavy metals was investigated with the perchlorate-

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Foundation Project: Supported by INHA University (Research Grant No. 51787).

enriched consortium.

2. Materials and methods

2.1. Medium

Anoxic medium containing initial low concentration of chloride[7] was prepared with 10 mg/L of $MgSO_4 \cdot 7H_2O$ instead of 100 mg/L. The anoxic medium was prepared by boiling under oxygen-free nitrogen. The medium was dispensed in serum bottles (50 mL capacity), and the bottles were crimped with butyl rubber corks and aluminum caps prior to autoclaving (121 ℃; 15 min) under the same gas phase. Separately filter-sterilized (0.2 μm pore size) anoxic stocks were used for electron (e^-) acceptors and e^- donor. Resazurin was omitted from the medium for the analysis of cell density and Cr (VI) by spectrophotometer.

2.2. Inoculum, enrichment and characterization

The anaerobically collected liquid and mud samples from the tidal flats of the Yellow Sea, Inchon, South Korea were sub-cultured twice a week with 2.5% (by volume) of an inoculum and incubated at 30–34 ℃ under constant shaking at 150–200 r/min in anaerobically crimped serum bottle (50 mL capacity) containing 20 mL medium. The enriched consortium was tested for reduction under different perchlorate concentrations and in the presence of nitrate. The complete enzymatic reduction of perchlorate to chloride was confirmed by chlorite dismutation[15]. Additionally, possibility of heterotrophic growth with alternate e^- acceptors was tested with acetate following the methods reported earlier[16-18]. For all the experiments, sufficient acetate was used as the e^- donor in order to enable complete reduction of the e^- acceptors. For various analyses, samples were routinely withdrawn with gas tight disposable syringes.

2.3. Heterotrophic perchlorate reduction

For the growth curve, the consortium was grown on 5.8 mmol/L and 20 mmol/L of perchlorate and acetate, respectively. Changes in perchlorate, chloride, and cell density were monitored during the 72 h of incubation.

2.4. Effect of perchlorate concentration

To test the ability of the consortium towards increasing concentrations of perchlorate, various concentrations of perchlorate ranging from 0.98 to 39 mmol/L were tested. Changes in perchlorate and cell density were monitored during the 68 h of incubation.

2.5. Perchlorate reduction in the presence of nitrate

Due to similar thermodynamics of perchlorate and nitrate reduction, the effect of nitrate as an alternate e^- acceptor on perchlorate reduction was tested. For this purpose, consortium was grown on perchlorate (5 mmol/L) in the presence of nitrate (8 mmol/L) with acetate (17 mmol/L) as the e^- donor. Changes in perchlorate, nitrate, and nitrite concentrations with concomitant changes in cell density were monitored during 72 h of incubation.

2.6. Utilization of alternate e^- acceptors

The consortium was investigated for its ability to utilize chlorate (10 mmol/L), nitrate (10 mmol/L), nitrite (5 mmol/L), sulfate (10 mmol/L), thiosulfate (10 mmol/L), sulfite (5 mmol/L), selenate (2 mmol/L), and oxygen as the alternate e^- acceptors with acetate as the e^- donor. A pre-mixed and autoclaved gas mixture (N_2 + O_2) was used as the source of oxygen. The increase in cell density (measured as optical density at 600 nm) was monitored for various e^- acceptors after 48 and 72 h of incubation. For the reduction of Cr (VI), the consortium was first grown for 48 h with acetate and perchlorate (10 mmol/L and 17 mmol/L, respectively) to favor sufficient cell growth. After perchlorate was exhausted to undetectable levels, Cr (VI) was added at a level of 0.25 mmol/L and 0.30 mmol/L in two separate experiments along with an additional dose of either acetate (25 mmol/L) or acetate and glucose mixture (25 mmol/L and 8 mmol/L, respectively). The reduction of Cr (VI) and corresponding changes in the cell density were monitored for 96 h and 144 h, respectively for the two experiments.

2.7. Chlorite dismutation

After 48 h of growth on acetate and perchlorate, the acetate-depleted consortium (2 mL) was harvested by centrifugation in a Micrc 17TR centrifuge (Hanil Industrial Co., South Korea) equipped with a fixed rotor (10 000 r/min; 10 min; 4 ℃). After two washings, the pellet was re-suspended in 0.8 mL sterile anoxic saline (0.85% NaCl in deionized water). The presence of chlorite dismutase (CD) activity both in supernatant (0.8 mL) and cell suspension was tested in a 5 mL capacity vial by adding 0.2 mL of freshly prepared anoxic sodium chlorite solution (20 mmol/L) in the absence of acetate. Evolution of oxygen was confirmed with a dissolved oxygen meter (YSI Model 58, YSI Incorporation, Yellow Spring, Ohio, USA) pre-calibrated for the maximum dissolved oxygen at 26 ℃. Both the cell suspension and sodium chlorite solution were also pre-equilibrated at 26 ℃ for temperature compensation. Heat killed (80 ℃; 5 min) cell suspension and supernatant as controls were processed in the same manner as the test solutions.

2.8. Analytical methods

The samples were centrifuged as mentioned earlier (7 000 r/min; 10 min) and diluted in reverse osmosis pure water to less than 3 mg/L final concentrations of the analytes for analysis by ion chromatography (DX 500 Dionex). Perchlorate was analyzed with

IonPac AS11 analytical and IonPac AG11 guard columns. A 40 mmol/L NaOH eluent was used at a flow rate of 2 mL/min. Nitrate, nitrite, and chloride were measured with IonPac AS14 analytical and IonPac AG14 guard columns. An eluent consisting of 3.5 mmol/L Na_2CO_3 and 1 mmol/L $NaHCO_3$ buffer was used at a flow rate of 1.2 mL/min. Cell density (as optical density at 600 nm) and Cr (VI) concentration were analyzed with an Agilent 8453 spectrophotometer as per the methods described by Clesceri *et al.*[19].

3. Results

3.1. Heterotrophic perchlorate reduction

The enrichment of the consortium was first started at low concentration of perchlorate (1.2 mmol/L) that was subsequently increased to 5.8 mmol/L and 16 mmol/L during the second and third round of enrichment cycles, respectively. During the second enrichment cycle, with an initial inoculum cell density (OD_{600} nm) of 0.006, approximately 1.05 mmol/L of perchlorate was reduced in the first 35 h of incubation (Figure 1). The consortium utilized approximately 4.75 mmol/L perchlorate during the next 35 h. The average reduction rate during this period was 0.127 mmol/L/h with corresponding average increase in cell density by 0.011/h. An increase in Cl^- concentration up to 5.76 mmol/L could be detected upon reduction of 5.8 mmol/L of perchlorate. During the 3rd cycle of enrichment, the consortium could reduce over 35% of the 16 mmol/L of perchlorate in 36 h. A near complete reduction of perchlorate occurred in less than 120 h with corresponding increase in growth (data not shown).

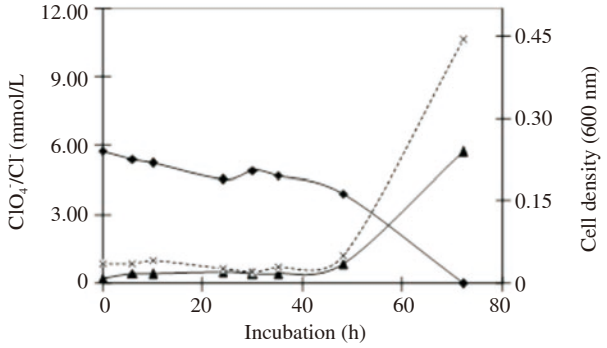

Figure 1. Perchlorate reduction by the consortium with acetate as the e^- donor.
♦: Concentration of ClO_4^-; ▲: Cl^-; ×: during growth on acetate as indicated by changes in the cell density.

3.2. Effect of perchlorate concentration

The perchlorate-reducing ability of the consortium was tested at various initial perchlorate concentrations ranging from 0.98 mmol/L to 39 mmol/L. Up to 11.87 mmol/L of perchlorate, no inhibition of the consortium in terms of perchlorate-reducing ability and growth were observed. Perchlorate concentration decreased linearly without any lag phase (Figure 2A), with corresponding increase in the cell density (Figure 2B). The complete reduction of 11.87 mmol/L of perchlorate was noticed in less than 44 h of incubation. An initial lag was observed in the onset of active perchlorate reduction at 25.65 mmol/L perchlorate concentration.

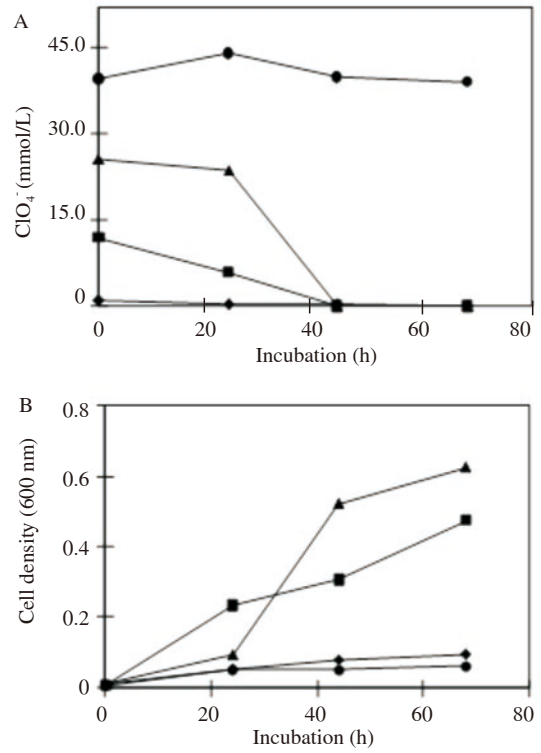

Figure 2. Effect of different perchlorate concentrations.
A: perchlorate reduction; B: growth. ♦: 0.98 mmol/L; ■: 11.87 mmol/L; ▲: 25.65 mmol/L; ●: 39 mmol/L.

3.3. Perchlorate reduction in the presence of nitrate

Presence of nitrate strongly suppressed perchlorate reduction by the consortium (Figure 3) compared to when only perchlorate was present (Figure 1). When a perchlorate-enriched consortium was used for the reduction of nitrate, reduction could start only after a lag phase of 48 h. In spite of acetate being provided to achieve stoichiometric conversion of both the e^- acceptors, complete reduction of the two e^- acceptors could not occur. In fact, during nitrate reduction, concomitant accumulation of toxic nitrite was observed. Interestingly however, despite nitrite accumulation, a linear increase in cell density was noticed.

3.4. Utilization of alternate e^- acceptors

Most of the perchlorate-contaminated sites in nature contain co-pollutants such as nitrate, chlorate and heavy metals, *etc*. The consortium therefore was further tested for its ability to utilize various alternate e^- acceptors. The increase in initial cell density with acetate as the e^- donor was monitored after 48 h and 72 h with oxygen, perchlorate, chlorate, and nitrate as the e^- acceptors. The cell density after 48 h incubation was the highest with oxygen (0.79) followed by perchlorate (0.66), chlorate (0.42), and nitrate (0.37), as illustrated in Figure 4. The other e^- acceptors were

tested but not utilized include sulfate, thiosulfate, sulfite, and nitrite. When the consortium was tested for growth with selenate, reddening of the medium occurred during extended period of incubation (> 240 h) with a concomitant increase in the cell density.

Since both perchlorate and Cr (VI) are used by electroplating facilities, the ability of the consortium was also tested for reduction of Cr (VI) with acetate (Figure 5A) or a mixture of acetate and glucose as the e^- donors (Figure 5B). With acetate, the consortium could reduce approximately 79% of the added Cr (VI) during 68 h, resulting in whitening of the medium contents. The cell density increased correspondingly, indicating respiration with Cr (VI) as the e^- acceptor. In a separate experiment, addition of glucose with acetate resulted in 96% of the added Cr (VI) being reduced during 72 h of incubation.

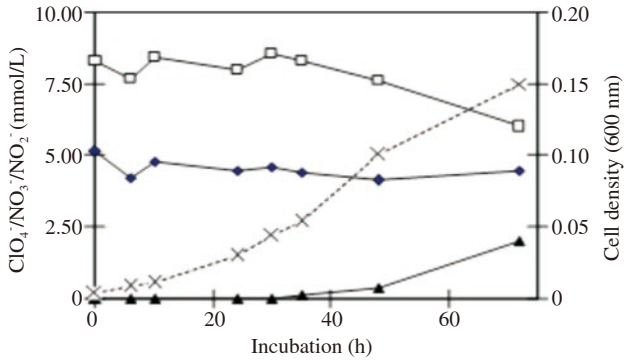

Figure 3. Effect of nitrate on perchlorate reduction and growth.
♦: Concentration of perchlorate; □: nitrate; ▲: nitrite; ×: during growth on acetate as indicated by changes in the cell density.

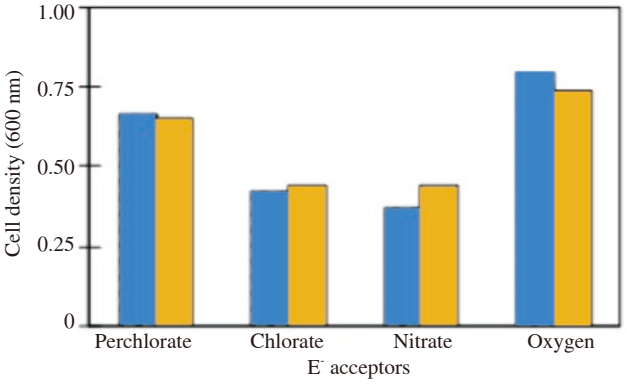

Figure 4. Increase in the cell density of the consortium during growth on alternate e^- acceptors.
■: 48 h; ■:72 h.

3.5. Chlorite dismutation

During the present study, CD activity in the consortium was confirmed by evolution of oxygen when whole-cell suspension was exposed to anoxic sodium chlorite in the absence of acetate. The oxygen content increased from 0.45 to 1.27 mg/L during the first 5 min as recorded by using a dissolved oxygen meter. The heat-killed whole-cell suspension (80 ˚C; 5 min) and supernatant were tested negative for the CD activity.

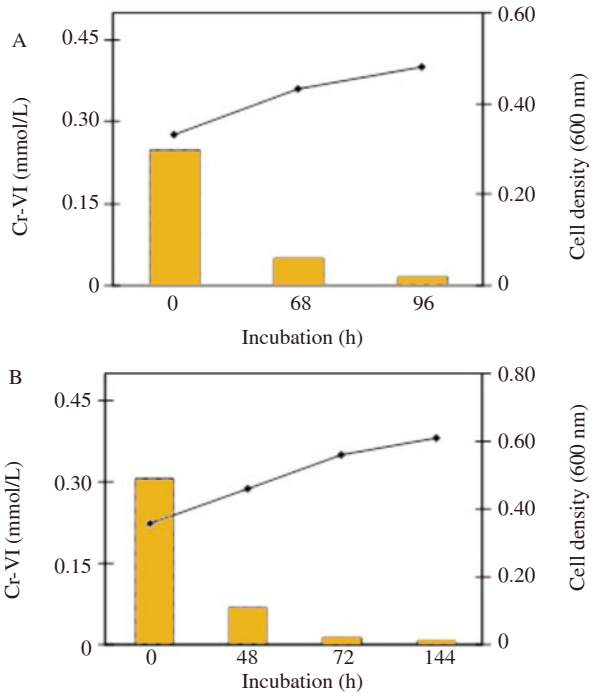

Figure 5. Growth and Cr (VI) reduction by the consortium with acetate and acetate-glucose mixture.
A: reduction of Cr (VI) with acetate; B: A mixture of acetate and glucose; ■: Changes in concentration of Cr (VI) with acetate and acetate-glucose mixture; ♦: Growth as indicated by changes in the cell density with acetate and acetate-glucose mixture.

4. Discussion

The perchlorate-reducing ability of the consortium improved progressively with enrichment cycles suggesting towards successful acclimatization and ability of the consortium to grow on perchlorate as the e^- acceptor. The reduction of perchlorate also coincided with increase in chloride concentration and cell density (OD_{600} nm). Even after the enrichment, an initial lag observed in the onset of active reduction at higher initial perchorate concentrations (*i.e.* 25.65 mmol/L and above) was suggested towards a toxic shock of perchlorate to the consortium. The information regarding the toxicity of perchlorate on mixed cultures and isolates has not been reported. However, optimum growth and perchlorate reduction is reported to occur between 10 and 20 mmol/L of perchlorate, which is in agreement with the present observations, although an exception of *Wolinella succinogenes* HAP-1 exists which is reported to grow at initial perchlorate concentration up to 70 mmol/L[8,20]. Similarly, strong suppression of perchlorate reduction in the presence of alternate e^- acceptors such as nitrate was observed which could be partly explained based on the marginal differences in the Gibbs free energy of nitrate reduction (ΔG°-792 kJ) and perchlorate reduction (ΔG°-801 kJ) with the oxidation of acetate in these bacteria. Conversely, a lag phase observed during the nitrate reduction by the perchlorate-enriched consortium further suggested towards mutual and competitive inhibitory effect of the two e^- acceptors.

Presence of nitrate during perchlorate reduction also caused incomplete utilization of both the e^- acceptors even in the presence of sufficient acetate. Despite the accumulation of nitrite from nitrate

reduction, a linear increase in cell density was observed which pointed out towards possibility of diversion of acetate by non-perchlorate and non-nitrate reducing acetate-oxidizers. A mixed consortium-based perchlorate and/or nitrate treatment system therefore may have an additional requirement of an organic e^- donor and carbon source such as acetate. Higher cell densities from (per) chlorate reduction compared to the nitrate reduction when individual e^- acceptors were tested could result from the utilization of oxygen generated from the complete reduction of (per) chlorate either by the aerobic population or the (per) chlorate reducers themselves since they are facultative anaerobes. During the dissimilatory (per) chlorate reduction, first chlorite is produced, which is then dismutated via a non-energy yielding reaction into oxygen and chloride by the enzyme chlorite dismutase (CD), the presence of which was also confirmed during the present study. Absence of CD has been shown to cause accumulation of chlorite and cessation of growth in several denitrifiers which could also explain the lowest cell densities observed from the partial reduction of nitrate and accumulation of toxic nitrite during the present study[21,22].

In the presence of heavy metals such as selenate and Cr (VI), an increase in the cell density with concomitant changes in the color of the growth media during extended incubation period suggested towards the biological reduction of these alternate e^- acceptors by the perchlorate-enriched consortium. A faster selenate and Cr (VI) reduction could possibly be achieved by building a higher cell mass of the consortium first on perchlorate followed by exposure of the consortium to the heavy metals for their reduction.

From the present study, following conclusions could be drawn:

1. After an initial acclimatization of the consortium, rapid perchlorate reduction could be achieved up to 25.65 mmol/L of perchlorate with acetate as the e^- donor.
2. Perchlorate was completely dismutated into chloride and oxygen.
3. Perchlorate concentration at 39 mmol/L resulted in total inhibition of perchlorate reduction.
4. Presence of nitrate suppressed perchlorate reduction.
5. Nitrate reduction by the perchlorate-enriched consortium could start only after an extended lag phase and with an accumulation of nitrite.
6. The consortium could also use Cr (VI) and selenate as the alternate e^- acceptors with acetate as the e^- donor.
7. Once the consortium was enriched on perchlorate, sulfate, thiosulfate, sulfite, and nitrite could not serve as the alternate e^- acceptors.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The financial support was provided by INHA University (Research Grant No. 51787).

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