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Aromatic compound degradation by iron reducing bacteria isolated from irrigated tropical paddy soils

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Abstract

Forty-six candidate phenol/benzoate degrading-iron reducing bacteria were isolated from long term irrigated tropical paddy soils by enrichment procedures. Pure cultures and some prepared mixed cultures were examined for ferric oxide reduction and phenol/benzoate degradation. All the isolates were iron reducers, but only 56.5% could couple iron reduction to phenol and/or benzoate degradation, as evidenced by depletion of phenol and benzoate after one week incubation. Analysis of degradative capability using Biolog MT plates revealed that most of them could degrade other aromatic compounds such as ferulic acid, vanillic acid, and hydroxybenzoate. Mixed-cultures and soil samples displayed greater capacity for aromatic degradation and iron reduction than pure bacterial isolates, suggesting that these reactions may be coupled via a consortia-based mechanism in paddy soils.

Key words: aromatic compounds; degradation; iron reducing bacteria; paddy soil

Introduction

The three most important redox reactions in anaerobic environments are the oxidation of organic matter with the reduction of Fe(III), the oxidation of organic matter with the reduction of sulfate, and the conversion of organic matter to carbon dioxide and methane (Takai and Kamura, 1966; Ponnamperuma, 1972; Reeburgh, 1983). Iron reducing bacteria are of interest because of their novel electron transfer capabilities and impact in natural environment. Various studies have shown that microbial Fe^{3+} reduction plays an important role in the decomposition of both natural and anthropogenic organic C in several diverse environments including marine, freshwater, and flooded rice soils (Lovley, 1993). A wide variety of organic C compounds were reported to be decomposed by different kinds of iron reducers, including acetate, lactate, sugars, amino acids, long-chain fatty acids, as well as a wide variety of monoaromatic compounds such as toluene, *p*-cresol, benzoate, and phenolic compounds (Lonergan and Lovley, 1991; Lovley, 1991, 1993; Lu *et al*., 2000, 2002; Myers and Nealson, 1990). Iron-reducing bacteria comprise a fairly wide range of phylogenetic types (Nealson and Little, 1997). *Geobacter metallireducens*, found in fresh water, represents the first organism coupling oxidation of aromatic contaminants to reduction of Fe^{3+} (Lovley, 1993; Lovley and Lonergan, 1990). The great potential of this organism for bioremediation of contaminated environments has been intensively investigated.

In this study, we screened and isolated iron reducing bacteria on phenol, a contaminant of environmental concern, and on an analogous compound, benzoate. The capacity of the isolated bacteria to degrade aromatics coupled to ferric reduction was characterized in an attempt to develop effective inocula for the bioremediation of organic pollutants in anaerobic reactors.

1 Materials and methods

1.1 Isolation and characterization of iron reducing bacteria (IRB) from irrigated paddy soils

In a previous study, bacteria capable of phenol/benzoate degradation coupled to iron reduction were isolated and identified from irrigated paddy soils at the International Rice Research Institute (Lu *et al*., 2003). In this study, the terminal positive MPN (Most Probable Number) tubes were used as inocula to isolate iron reducers on selective media as described in the same article (Lu *et al.*, 2003). The isolates were then cultured anaerobically on nutrient agar (NA) and purified after several repeated outstreakings and colony picking on new agar plates before storing on slant at 4°C for further study. To measure the iron-reducing capacity, bacterial isolates were grown aerobically or anaerobically in a broth media with the following components (g/L): K_2HPO_4 (3.0), KH_2PO_4 (0.8), KCl (0.2), yeast

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extract (0.5), asparagine (5.0), glucose (20.0) or acetate (10.0), and Fe_2O_3 (1.0). A colorimetric method based on the oxidation of 2-2'-dipyridyl was then used to measure the produced Fe(II) in culture media (Kumada and Asami, 1958).

1.2 Phenol/benzoate degradation test by the isolates

The medium used for the measurement of phenol/benzoate degradation has the same components as that mentioned above, except that 2.0 mmol/L phenol or 10 mmol/L benzoate, respectively, were used as the sole carbon source. After 3 weeks of anaerobic incubation, a 2.0-ml aliquot of the supernatant of the centrifuged culture solution was passed through a 0.45-μm pore filter (Millipore, Bedford, MA) and stored at –85°C before analysis. Phenol or benzoate degradation by pure or mixed culture was determined by High-performance liquid chromatography (HPLC) (Shimadzu, Japan) as described in Häggblom *et al*. (1993). 100 μl of the samples (pH adjusted to 3.0 by HAc) were analyzed with a 250 mm × 4.6 mm C-18 reverse phase column of 5-μm particle size (Supelco, Inc., Bellefonte, PA, USA) with a methanol-water-acetic acid (60:38:2) running solvent flowing at 1.0 ml/min. The variable UV detector was set at 280 nm to measure phenol or benzoate with retention times of 4.68 or 5.47 min, respectively. A linear standard curve served to quantify the compounds.

1.3 Test of the isolates' capability for degrading other aromatics

Biolog MT plates (Biolog, Inc., Hayward, USA) are miniaturized substrate utilization testing plates that exploit the colorimetric change in tetrazolium dyes as they are reduced in the presence of respiring bacteria (Fulthorpe and Allen, 1994). The capability of the iron reducers to degrade six aromatic compounds, i.e., ferulic acid, vanillic acid, hydroxybenzoate, phenol, and benzoate, in different concentration ranges was tested in Biolog MT plate (Table 1). An aliquot of 50 μl bacterial suspension with a density equivalent to an absorbency of 0.2 at 620 nm was pipetted into each well containing 100 μl of the appropriate concentration of the aromatic compounds, with an equivalent amount of sterile water or cell suspension being added to control wells. The inoculated plates were covered with caps and incubated at 30°C for 24–72 h in anaerobic incubator before reading the absorbency at 550 nm with a Bio-Tek EL311S automatic plate reader.

Table 1 Aromatic compounds used in MT microplates and their concentration ranges

Aromatic substrate	Concentration levels (mmol/L)
Ferulic acid	2.0, 5.0, 10, 15, 20, 30
Hydroxybenzoate	5.0, 8.0, 10, 15, 18, 20
p-Cresol	0.5, 1.0, 2.0, 2.5, 5.0, 6.0
Phenol	0.5, 1.0, 2.0, 2.5, 5.0, 6.0
Benzoate	5.0, 10, 15, 20, 25, 30
Vanillic acid	2.0, 5.0, 10, 15, 20, 30

2 Results and discussion

2.1 Isolation and characterization of predominant iron reducing bacteria in irrigated paddy fields

Forty-six bacterial strains were enriched and isolated from long term irrigated tropical paddy soils as candidate iron reducing bacteria (IRB). Table 2 shows the ferric iron reduction capability on different carbon sources of these strains. Some of the isolated IRB could couple iron reduction with degradation/utilization/oxidation of all the tested carbon sources, i.e., glucose, phenol, benzoate and acetate whereas some could only metabolize the smaller carbon substrates. Only 11.1% of the isolates were unable to use glucose as the sole carbon source to reduce iron. Of those that can reduce iron coupled to glucose utilization, 66.7% could reduce iron either aerobically or facultatively, whereas 22.2% showed obligate aerobic reduction. The results also showed that when incubated with aromatics, 23% of the pure isolates could reduce iron in media with phenol as the sole carbon source, with 36% of these using benzoate as the sole carbon source to reduce iron, and 37% could produce Fe(II) at the expense of acetate. Mixed cultures of these isolates showed higher ability of iron reduction in both rich carbon media and aromatics media.

Some bacterial strains showed very strong iron reducing capacity at the expense of oxidation of glucose in anaerobic condition, such as S3, S9, S12, S14, S21, S30, S33, S35, S42, and mixed cultures of these isolates. The highest concentration of Fe(II) detected in culture media was 0.99 mmol/L (Fig.1). In the study of the time course of reduction of ferric iron by isolates, Fe(II) in culture media started to increase significantly after 5 or 6 d of

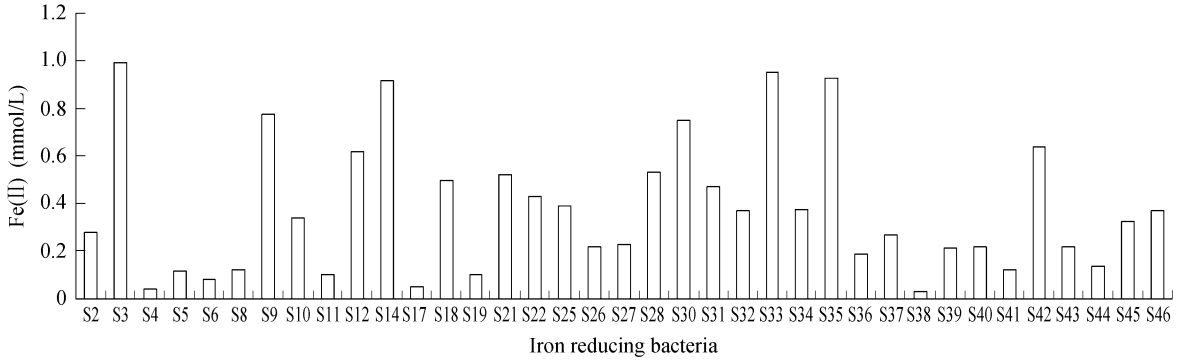

Fig. 1 Anaerobic Fe_2O_3 reducing capacity of the isolates from irrigated paddy soils.

Table 2 Characteristics of the ability for iron reduction in the isolates using different carbon sources as electron donor

Isolate	Ferric iron reduction in enriching media with different carbon sources				Isolate	Ferric iron reduction in enriching media with different carbon sources			
	Glucose (g/L)	Phenol (mmol/L)	Benzoate (mmol/L)	Acetate (mmol/L)		Glucose (g/L)	Phenol (mmol/L)	Benzoate (mmol/L)	Acetate (mmol/L)
*S1	–	–	+	–	S24	–	–	–	+
S2	+	–	–	+	S25	+	–	–	+
S3	+	+	+	+	S26	+[a]	–	–	–
S4	+	–	+	–	S27	+[b]	–	–	–
S5	+	+	+	–	S28	+[b]	–	–	–
S6	–	–	+	–	S29	–	–	–	+
S7	–	–	+	–	S30	+[a]	+	+	+
S8	+[a]	–	+	+	S31	+[a]	–	-	–
S9	+[a]	–	+	+	S32	+[b]	–	+	–
S10	+[a]	–	–	–	S33	+[b]	–	+	–
S11	+[a]	–	–	+	S34	+	+	–	–
S12	+	–	+	+	S35	+	+	–	–
S13	+[b]	+	–	–	S36	+	–	–	–
S14	+[a]	–	+	+	S37	+	–	–	–
S15	+[b]	–	–	–	S38	+	–	–	–
S16	+[b]	–	–	–	S39	+	+	–	–
S17	+[a]	–	–	–	S40	+	–	–	–
S18	+[b]	–	–	–	S41	+	–	+	–
S19	+[a]	–	–	–	S42	+	+	–	+
S20	+[b]	–	–	–	S43	+	+	–	+
S21	+[a]	–	+	+	S44	+	+	–	+
S22	+[a]	–	+	–	S45	+[a]	+	+	+
S23	+[b]	–	+	–	S46	+[a]	+	–	–
					Mix-all	+[a]	+	+	+

* Pure isolates are indicated as numbers; [a] capable of iron reduction both in aerobic and anaerobic conditions; [b] capable of iron reduction in aerobic condition.

incubation, and increased steadily thereafter. The different bacteria had different growth rates in the iron media and showed different rates of Fe reduction as evidenced by the various slopes of the reduction curves (Fig.2).

2.2 Degradation of phenol and benzoate by pure isolates and mixed cultures

Both biochemical and HPLC analysis revealed almost complete disappearance of phenol in media, when incubat-

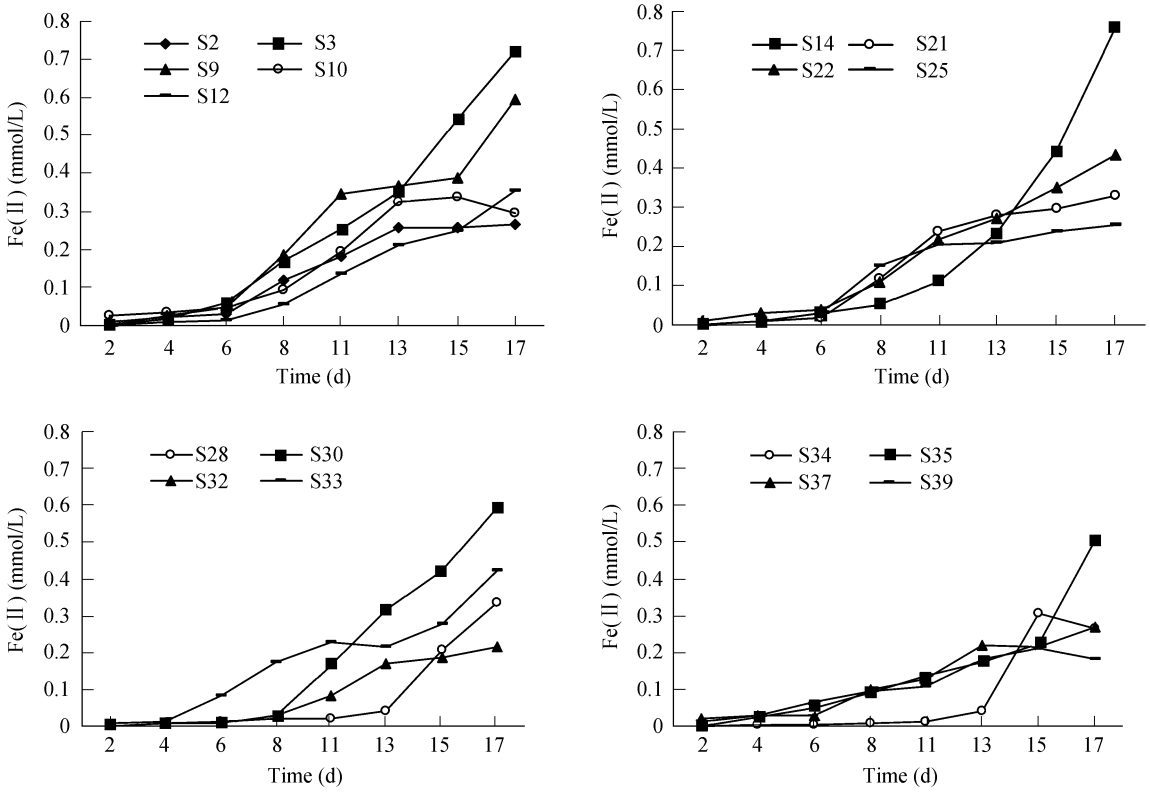

Fig. 2 Time course of Fe_2O_3 reduction by the iron reducing bacteria isolated from irrigated paddy soils.

ed with pure isolates of S40, S41, S42, S43, S44, S45, S46, as well as different combinations of these strains (Fig.3a). HPLC analysis of phenol degradation by S45 demonstrated it to be a highly efficient phenol degrader (Fig.4 a1–d1). It showed that the remaining isolates could degrade phenol, although the amounts were considerably lesser than that of the highly efficient species S45. Nevertheless, mixtures/co-cultures of these strains had higher activity in phenol degradation (Fig.5).

In one anaerobic phenol degradation pathway, 4-

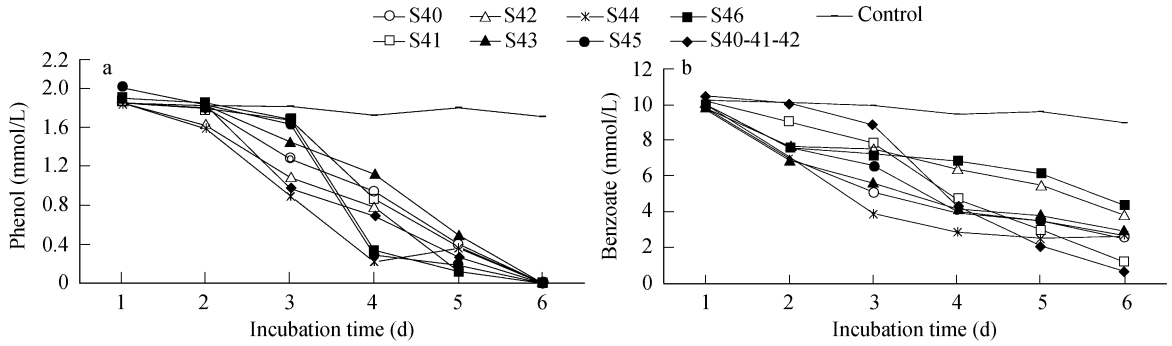

Fig. 3 Time courses of phenol (a) and benzoate (b) degrading by some pure isolates and their co-cultures/mixtures.

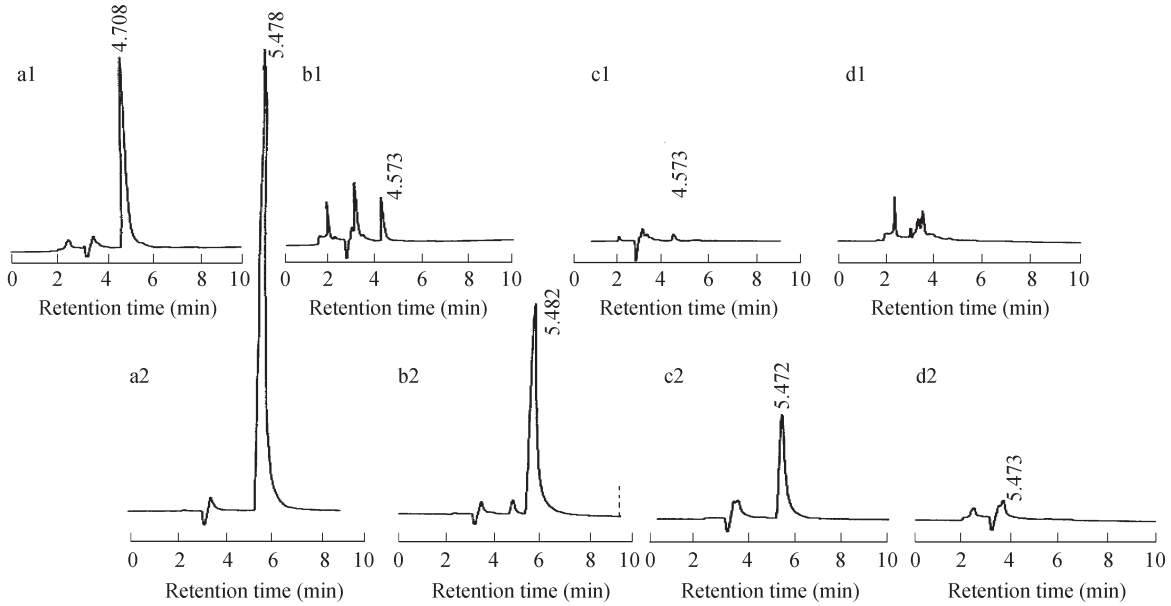

Fig. 4 Complete phenol degradation by iron reducer S45 (a1–d1) and complete benzoate degradation by iron reducer S41 (a2–d2). a1, b1, c1, and d1 indicate day 0, 3, 7, and 10 after incubation, respectively; and a2, b2, c2, and d2 indicate day 0, 3, 7, and 10 after inoculation, respectively.

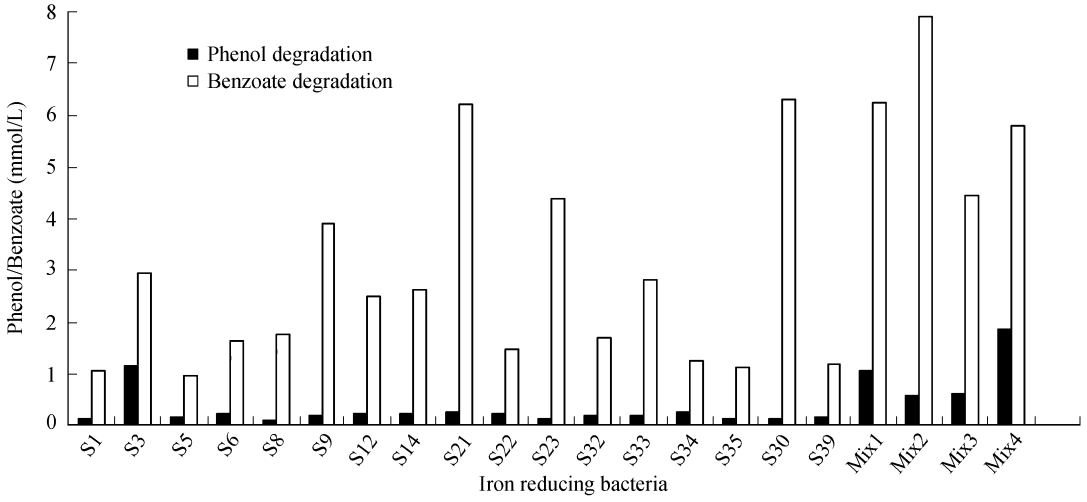

Fig. 5 Phenol and benzoate degrading capability of some iron reducers isolated from irrigated paddy soils.

hydroxybenoate and 4-hydroxybenoate-CoA are intermediates (He and Wiegel, 1995). In our HPLC chromatogram, there were products eluted at retention time of 2.3 and 3.7 min, which are the typical retention time of the derivatives of hydroxybenzoate such as *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde in the standards analyzed under the same condition (Fig.4 a1–d1, Fig.6 a1–e1). We therefore postulate that degradation of some isolates utilized the same pathway of phenol metabolism as proposed by He and Wiegel (1995).

As seen in Fig.5, most of the isolates displayed benzoate degrading ability. An almost complete degradation of benzoate (10 mmol/L) was detected for strains S41 (Fig.4d2), S42, and the co-culture of S43 and S44, which proved them to be highly efficient benzoate degraders (Fig.3b).

Bacterial identification using a Biolog system, reported previously, showed that the majority of these isolates belong to two families–Pseudomonaceae and Enterobacteriaceae, with most of them belonging to the genus *Pseudomonas* and *Klebsiella*, while others were not identified by this approach suggesting the diverse nature of the bacterial population involved in iron reduction in this environment (Lu *et al*., 2002). The capacity of common *Pseudomonas* species to grow on xenobiotics as sole sources of carbon and energy is well established (Ottow and Glather, 1971; Nealson and Saffarini, 1994; Sanders and Sanders, 1997; Xie *et al*., 1999) and *Pseudomonas* play an important role in aromatic degradation in the environment. Therefore, it is not surprising to see *Pseudomonas* species isolated as the predominant type of iron-reducing-bacteria (IRB) in this study.

2.3 Degradation of phenol and benzoate in MPN tubes

HPLC analysis showed that phenol was totally removed after 14 d of anaerobic incubation in the trials with inocula of a soil dilution of 5×10^{-4} or higher (Fig.6). The higher the dilution, the more remaining phenol in the media. The same trend was seen in benzoate media, i.e., less benzoate remained in tubes inoculated with higher concentration of soil dilutions. Removal of benzoate was observed in tubes inoculated with a soil dilution of 5×10^{-5} or higher. We postulated that degradation of aromatics coupled with ferric iron reduction in paddy soil is achieved by metabolizing mixotrophic consortia. An important clue was that active phenol/benzoate degraders such as IRB40, IRB46 were isolated at the forepart of the incubation, followed by different isolates of *Pseudomonas aeruginosa*, *Klebsiella* sp., and other species after longer periods of incubation. We assumed that the active phenol/benzoate degraders were mainly responsible for initiating phenol/benzoate degradation and the *Klebsiella* and other species isolated later were responsible for consuming the intermediates and concomitant ferric iron reduction. However, this model may be oversimplified as some *Klebsiella* sp. have been reported to be able to degrade benzoate and some *Pseudomonas* display iron reducing activity (Fulthorpe *et al*., 1993).

2.4 Degrading capabilities of the IRB for other aromatic compounds

Aromatic compounds are important building blocks of humus in soil. In irrigated paddy soils, ferulic acid, vanillic acid, hydroxybenzoate together with phenol and benzoate are the dominant monoaromatic compounds as detected by HPLC (Makoto, 2000; Naik *et al*., 1993). As seen in Table 1, different concentration levels of each compound ranging from 0.5 to 30 mmol/L were used according to their toxicity to microorganisms. The majority of the isolates were strongly capable of aromatic compounds utilization as evidenced by the intense dye reduction in

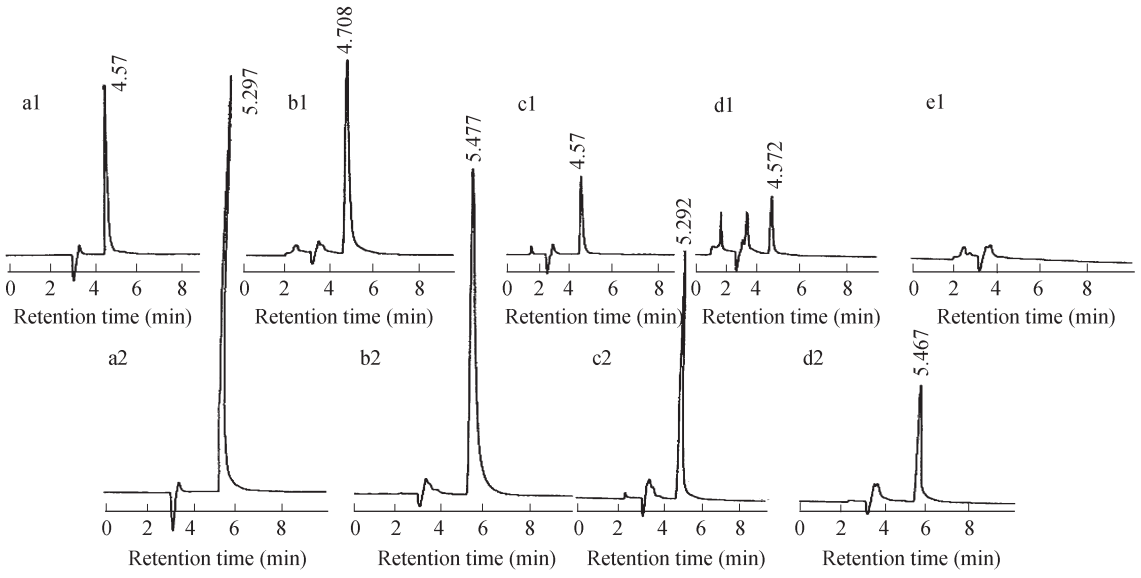

Fig. 6 Phenol degradation (a1–e1) and benzoate degradation (a2–d2) in positive MPN tubes inoculated with different levels of soil diluents in 14 d anaerobic incubation (samples were 6 times diluted for phenol detection and 15 times diluted for benzoate). a1, a2 indicate phenol (0.2 mmol/L) and benzoate (0.4 mmol/L) standard, respectively; b1, c1, d1, e1 indicate inoculation with 5×10^{-8}, 5×10^{-6}, 5×10^{-5}, 5×10^{-4} soil diluent, respectively; b2, c2, d2 indicate inoculation with 5×10^{-8}, 5×10^{-6}, 5×10^{-5} soil diluent, respectively.

microplates containing the tested compounds. Twenty-six isolates were extremely promising with regard to the aromatic degradation in pure cultures (Table 3). The strains showed no or very weak degradation of the tested substrates, indicating that these iron reducing bacteria were unable to couple Fe_2O_3 reduction to aromatic degradation. This study demonstrates time advantage of utilizing MT microplates in screening a large number of isolates for a given biochemical activity.

The presence of diverse bacterial populations in the soil isolates indicates that the activities of different metabolic types may occur during aromatic degradation coupled to the reduction of Fe(III). Thus, a consortia-based aromatic degrading – iron reducing mechanism was proposed, supported by the fact that mixed cultures had stronger activity in the degradation of aromatics and iron reduction than single isolates. Nevertheless, some bacteria capable of coupling aromatics degradation to iron reduction in pure culture were isolated from irrigated paddy soils in our study, and the most promising candidates were *P. aeruginosa* and *Acinetobacter calcoaceticus*. It is of great interest to study these two species further for degradation of organic compounds in bioremediation reactors owing to their important roles in carbon recycling in the wetlands, where, Fe serves as a predominant electron acceptor.

Moreover, these isolates can also be proper inocula for the reactors of Micoribal Fule Cells (MFC) since they use the same function of anerobic communities with chemical-electrical metabolic activities (Rabaey, 2004). Further study must be done to prove that these bacterial isolates

Table 3 Test of aromatic compounds utilization by the iron reducers from irrigated paddy soils using Biolog MT microplate

Aromatic compounds	Ferulic acid		Hydroxyben zoate		*p*-Cresol		Phenol		Benzoate		Vanilic acid	
Concentration (mmol/L)	2.0–15	16–30	5–10	11–20	0.5–3.0	3.5–6.0	0.5–3.0	3.5–6.0	5–15	16–30	2.0–15	16–30
Isolate												
S1	–	–	–	–	–	–	–	–	+	–	–	–
S2	/	–	+	+	+	+	+	/	+	/	+	/
S3	+	+	+	+	+	+	+	+	+	+	+	+
S4	+	–	+	+	+	–	+	/	+	/	+	–
S5	–	–	+	–	–	–	+	–	/	–	+	–
S6	–	–	–	–	–	–	–	–	+	–	–	–
S7	–	–	–	–	–	–	–	–	+	–	–	–
S8	+	–	+	+	+	+	+	+	+	+	+	+
S9	+	+	+	+	+	+	+	+	+	+	+	+
S10	–	–	+	–	–	–	+	–	+	–	+	–
S11	–	–	+	+	+	–	-	–	-	–	/	–
S12	+	+	+	+	+	/	+	+	+	+	+	–
S13	+	–	+	+	+	–	+	+	+	+	+	+
S14	+	+	+	+	+	/	+	+	+	+	+	+
S15	–	–	+	–	+	+	+	+	+	+	+	/
S16	+	+	+	+	+	/	+	+	+	/	+	+
S17	+	–	+	/	+	/	+	+	+	+	+	–
S18	+	–	+	/	+	–	+	+	+	–	+	–
S19	+	–	+	/	–	–	+	+	/	–	+	–
S20	–	–	–	–	–	–	–	–	–	–	–	–
S21	+	+	+	+	+	+	+	+	+	+	+	+
S22	+	–	+	+	+	+	+	+	+	+	+	–
S23	/	–	/	/	+	–	/	/	+	/	+	–
S24	–	–	+	–	–	–	–	–	–	–	–	–
S25	+	+	+	+	+	+	+	+	+	+	+	+
S26	–	–	+	–	+	–	+	+	+	+	/	/
S27	+	+	+	+	+	+	+	+	+	+	+	+
S28	+	+	+	+	+	+	+	+	+	+	+	+
S29	–	–	–	–	–	–	–	–	–	–	–	–
S30	+	/	+	+	+	+	+	+	+	+	+	+
S31	+	–	+	+	+	+	+	+	+	+	+	+
S32	+	/	+	+	+	+	+	+	+	+	+	+
S33	+	+	+	+	+	+	+	/	+	+	+	+
S34	+	+	+	+	+	/	+	+	+	+	+	+
S35	+	+	+	+	+	+	+	+	+	+	+	+
S36	–	–	+	/	+	/	+	+	+	+	+	/
S37	+	–	+	/	+	/	+	+	+	/	+	/
S38	+	–	+	/	+	/	+	+	/	/	+	/
S39	–	–	–	–	–	–	+	–	+	–	/	–
S40	+	+	+	+	+	+	+	+	+	+	+	+
S41	+	+	+	+	+	+	+	+	+	+	+	+
S42	+	+	+	+	+	+	+	+	–	–	+	+
S43	+	+	+	+	+	+	+	+	+	+	+	+
S44	+	+	+	+	+	+	+	+	+	+	+	+
S45	+	+	+	+	+	+	+	+	+	–	+	+
S46	+	+	+	+	+	+	+	+	+	+	+	+

/ indicates very weak growth.

can function as another environmental alleviation strategy, which will not only remove the organic contaminants but will also yield energy (electric) from the process.

3 Conclusions

The degradation capacity of the aromatic compounds by 46 bacterial isolates under iron reducing conditions revealed that most of them can couple degradation of one or two aromatic compounds to iron reduction. Bacteria capable of coupling these reactions may be major contributors to the microbial cycling of large molecule carbon substrates and are thus promising tools for bioremediation and elimination of organic pollutants in Fe mediated anaerobic simulators.

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