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Anaerobic degradation of phenanthrene by a newly isolated humus-reducing bacterium, *Pseudomonas aeruginosa* strain PAH-1

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Abstract
Purpose The anaerobic degradation of polycyclic aromatic hydrocarbons (PAHs) has great significance to PAHs' natural attenuation in contaminated sites. Previous studies mainly focused on anaerobic PAH degradation by mixed cultures with nitrate, sulfate, or Fe(III) oxides as electron acceptors, and the roles of pure cultures in the process was rarely reported. The aim of this paper is to isolate a pure culture that is capable of degrading phenanthrene anaerobically with anthraquinone-2,6-disulphonate (AQDS) as the sole electron acceptor and evaluate its environmental functions.
Materials and methods A strain of pure culture was isolated from anodic solution of a microbial fuel cell via enrichment procedure with phenanthrene and AQDS under anaerobic conditions. Using the single colony isolation technique, a distinct colony was obtained and identified by the phenotypic and phylogenetic analysis. Its ability to reduce AQDS and degrade phenanthrene was conducted in serum bottles by standard anaerobic techniques (purged with 80% N_2–20% CO_2). The concentration of AH_2QDS and fructose was quantified by UV-vis spectrophotometer at 450 and 210 nm, respectively. Cells number was determined by direct plate counting on aerobic LB agar medium. The concentration of phenanthrene was determined using HPLC.
Results and discussion Strain PAH-1 was identified as *Pseudomonas aeruginosa*. It could oxidize fructose or glucose to reduce AQDS and can support microbial growth by conserving energy from fructose to AQDS. It has the ability to degrade phenanthrene directly with AQDS as the sole electron acceptor (46.5% removal), and the microbial process may be AQDS dependent. The addition of small organic substances (fructose) could enhance the anaerobic biodegradation of phenanthrene from 46.5% to 56.7%. The anaerobic degradation of phenanthrene fits the pseudo-first-order kinetics, giving the rate constants of 0.0233/day (R^2=0.934) and 0.0328/day (R^2=0.933) for non-fructose set and fructose set, respectively.
Conclusions We successfully isolated a facultative anaerobe, *P. aeruginosa* strain PAH-1. This study was the first paper reporting that a pure culture (strain PAH-1) has the ability to anaerobically degrade phenanthrene with AQDS as the sole electron acceptor. The finding also explores the environmental significance of the *Pseudomonas* genus.

Keywords Anaerobic degradation · Anthraquinone-2, 6-disulphonate · Phenanthrene · *Pseudomonas aeruginosa*

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are aromatic hydrocarbons with two or more fused benzene rings and ubiquitously exist in the natural and anthropogenic environments (Chang et al. 2008; Wang et al. 2009). Due to their potential toxicity, carcinogenicity, tumorigenicity, and low water solubility, approximately 16 PAHs have been designated as priority pollutants by the European Union and the United States Environmental Protection Agency

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(Rockne and Strand 2001). Consequently, their environmental fates have gathered significant research interests (Haritash and Kaushik 2009).

A key parameter affecting the loss fate of PAHs in the natural environment is microbial activity. It was thought that microbial degradation of PAHs is an oxidation–reduction reaction, in which the hydrocarbons are oxidized coupling with electron flow to electron acceptors (Lovley et al. 1994; Lovley et al. 1996a). Numerous aerobic PAH-degrading bacteria have been isolated and the degradation pathways have been successfully elucidated (Cerniglia 1984; Meckenstock et al. 2004; Haritash and Kaushik 2009). However, the PAHs-contaminated sites are usually anaerobic, and anaerobic degradation may make a more important contribution to PAHs natural attenuation. Previous literatures have widely studied the anaerobic biodegradation of PAHs by mixed cultures under sulfate- (Rothermich et al. 2002), nitrate- (Coates et al. 2001), Fe(III)-, or humic substances (HS)- (Ramsay et al. 2005) reducing conditions and methanogenic conditions (Chang et al. 2008) in subsurface soils, sediments, and waste disposal sites, but the reports on PAHs degradation by pure bacterial strains is relatively rare. Only a few pure anaerobic bacteria were isolated, which have the capability to degrade PAHs with nitrate, sulfate, or CO_2 serving as the terminal electron acceptors (McNally et al. 1998; Galushko et al. 1999; Rockne et al. 2000; Yuan and Chang 2007; Chang et al. 2008). Among the above reported electron acceptors, HS that are more abundant and active components in the natural environments have great potentials to serve as terminal electron acceptors in microbial respiration (Van Trump et al. 2006; Perminova et al. 2005; Straub et al. 2005). Furthermore, due to its important role in the anaerobic biodegradation of organic pollutants, the microbial-mediated HS reduction has been attracted significant research concerns (Bradley et al. 1998; Cervantes et al. 2000; Cervantes and Dijksma 2001). Though HS are highly expected to play an important role in the PAHs natural degradation, there has no research studying PAHs anaerobic degradation coupling with HS reduction by pure culture.

In this study, we selected anthraquinone-2,6-disulphonate (AQDS, humic substance analog) as the terminal electron acceptor and phenanthrene as the target pollutant of PAHs (electron donor). A strain of pure culture, identified as *Pseudomonas aeruginosa* PAH-1, was successfully isolated from the anodic solution in a microbial fuel cell via enrichment procedure with AQDS and phenanthrene. Its capability of reducing AQDS and degrading phenanthrene under anaerobic conditions was demonstrated to be novel functions for the *Pseudomonas* genus. As well, the effect of the alternative carbon sources on anaerobic degradation of phenanthrene by strain PAH-1 was studied. The potential role of strain PAH-1 in degrading PAHs in the contaminated sites in the abundance of HS was also studied with respect to phenanthrene and AQDS.

2 Methods

2.1 Enrichment and isolation procedure

All the experiments were followed by standard anaerobic techniques and sterilized by standard autoclaving procedure (121°C, 20 min). The inoculum source for enrichment was the anodic solution obtained from a well-operated microbial fuel cell (MFC) in our laboratory (pH 7.0). An aliquot of 5 ml anolyte was transferred to a 100 ml sterile serum bottle, which contained 50 ml basal medium (pH 7.0) added with 1 mmol l^{-1} AQDS (electron acceptor) and 20 mg l^{-1} phenanthrene (dissolved in 100% methanol). The basal medium consisted of the following components (amounts per liter of deionized water): 2.50 g $NaHCO_3$, 0.68 g $NaH_2PO_4{\cdot}2H_2O$, 0.25 g NH_4Cl, 0.10 g KCl, 10 ml vitamin stock solution, and 10 ml trace mineral solution (Lovley and Phillips 1988). The bottle was purged with 80% N_2–20% CO_2 for 30 min and sealed with butyl rubber stopper and aluminum cap. Meanwhile, the culture medium without inoculum (anodic solution of MFC) was conducted under the same experimental conditions as the control assay. All the bottles were incubated at 25°C, in dark. After 1–2 months, as the color of the solution changed to dark yellow and the concentration of phenanthrene was reduced below 10 mg l^{-1}, 10% (*v*/*v*) inoculum was transferred to a serum bottle containing fresh medium. The transfer processes were repeated at least three times. To obtain pure cultures, the final enriched solution was serially diluted and plated onto agar plates (the same enriched medium added with 0.20 gl^{-1} yeast extract). Following the single colony isolation technique, distinct colonies were picked and streaked three times on agar plates.

2.2 Identification of the strain

The bacterial isolate was tested for key physiological–biochemical characteristics by standard procedures according to Bergey's Manual of Determinative Bacteriology (9th edition, 1994). The 16S rRNA gene of the isolate was amplified by PCR as described previously (Li et al. 2007). The obtained sequences were then aligned with related 16S rRNA sequences from GenBank data libraries. Multiple alignments with corresponding nucleotide sequences of representatives of the genus *Pseudomonas* retrieved from GenBank were carried out using Clustal X program (Thompson et al. 1997). The phylogenetic tree was constructed with the software package MEGA version 4.0 using the neighbor-joining method according to bootstrap analyses based on 1,000 replicates.

2.3 AQDS reduction and phenanthrene degradation

HPLC-grade methanol and ultrapure water were used in the phenanthrene degradation experiment. Other chemical reagents used were of analytical grade. To prevent chemical contamination, all the bottles were soaked in chromic acid overnight, rinsed with ultrapure water, and dried at 110°C for 2 h.

Harvested cells of the obtained strain were used for the AQDS reduction and phenanthrene degradation experiments. Cells were grown aerobically in Luria–Bertani (LB) medium at 30°C on a rotary shaker (180 rpm), and harvested at the late log phase by centrifugation at 8,000 rcf for 10 min at 4°C. The cell suspension was washed twice and resuspended in sterile fresh basal medium to an optical density of about 1.4 (λ=600 nm).

To test the effect of different electron donors on AQDS reduction, each 25.2 ml serum bottle contained 20 ml basal medium with 1 ml cell suspension (1×10^7 CFU ml^{-1}), 0.5 mmol l^{-1} AQDS and one of the following electron donors (10 mmol l^{-1}): acetate, glucose, fructose, and methanol. The medium without cells served as the control sets.

For anaerobic degradation of phenanthrene, each 100 ml serum bottle contained 20 ml basal medium with 1 mmol l^{-1} AQDS, 1 mmol l^{-1} fructose (as necessary), and 1 ml 1.0×10^7 CFU ml^{-1} cells. The bottles were purged with 80% N_2–20% CO_2 for 30 min and 0.1 ml methanol-based stock phenanthrene solution was injected into the bottles using a sterilized syringe to give a concentration of 20 mg l^{-1}. Finally, the serum bottles were immediately sealed with butyl rubber bungs and crimped with aluminum caps. As well, an abiotic control without bacterial cells and a biotic control without alternative carbon sources (fructose) were operated under the same conditions.

All the treatments were conducted in triplicate and all bottles were incubated in the dark at 25°C.

2.4 Chemical analysis

In the fructose-fed set, the concentration of reduced AQDS (AH_2QDS), growth of the cells and consumption of fructose were monitored at the same time. The concentration of AH_2QDS was quantified by UV-vis spectrophotometer (TU1800-PC, Beijing) at a wavelength of 450 nm compared with the controls using $Na_2S_2O_3$ as chemical reductant ($OD_{sample}/OD_{Na2S2O3}$) (Liu et al. 2007). Cells number was determined by direct plate counting on aerobic LB agar medium. The consumption of fructose was determined by the method from Garrett and Blanch (1967) with small modifications: added 3 ml HCl to 1 ml sample solution, supplemented with deionized water to 10 ml, then underwent a boiling bath for 8 min, cooled the sample to room temperature, and finally measured using a UV-vis spectrophotometer (TU1800-PC, Beijing) at a wavelength of 282 nm. The detection range of the method is 0–30 mg l^{-1}.

For phenanthrene analysis, the sample bottles were immediately added with 20 ml methanol and the mixtures were extracted in an ultrasonic bath for 20 min at the controlled temperature below 10°C by using ice. Before analyzed by HPLC (Waters 1527/2487), the obtained extraction was passed through a 0.22 μm syringe filter (Polyvinylidene fluoride, Millipore Inc.). The conditions of HPLC were: a reverse-phase C_{18} column (4.6×250 mm); a flow rate of mobile phase, 1 ml min^{-1}; UV detector wavelength, 254 nm; the isocratic mobile phase, methanol: ultrapure water=90:10 (*v*/*v*); and injector volume, 20 μl.

3 Results and discussion

3.1 Characteristics of strain PAH-1

Strain PAH-1 was gram-negative, straight rods (1.0–2.0 μm in length, 0.3–0.5 μm in width), motile, non-fermentative, and a facultative anaerobe. The colonies were wet, smooth, and cream in color on aerobic LB agar plates after a 48-h incubation and secreted green or dark brown pigment called as pyocyanin. It could grow at 41°C, and not grow at 4°C. The strain can utilize glucose and fructose as carbon source. Other biochemical features were summarized in Table 1. According to the phenotypic and phylogenetic analysis, strain PAH-1 was identified as *P. aeruginosa*. The 16S rRNA gene sequence of the strain was submitted to the GenBank database with the accession number HQ615870. Based on 16S rRNA gene sequences analysis, strain PAH-1 displayed a close relationship with the members of *Pseudomonas* sp. P1 (GU113071) and *Pseudomonas* sp. Pyr41 (GU951459) in the phylogenetic tree. Its position in the 16S rRNA phylogenetic tree is shown in Fig. 1.

3.2 Effect of electron donors on AQDS reduction by strain PAH-1

Four types of organic substrates were tested as the alternative electron donors for AQDS reduction (Fig. 2). The color change of the culture liquid from colorless to yellow was a good indicator for AQDS reduction. After 10-d incubation, there was a significant increase in the production of AH_2QDS as glucose or fructose was serving as electron donor. Approximately 26.4% and 10.7% AQDS were reduced to AH_2QDS in the sets fed with fructose and glucose, respectively. Similar to the control sets (data not shown), almost no AH_2QDS was detected in both acetate- and methanol-fed batches. It is clear that the AQDS reduction by strain PAH-1 was highly dependent on the

Table 1 Phenotypic characteristics of strain PAH-1

Characteristic	Results	Characteristic	Results
H_2S	−	Methyl red	−
Catalase	+	V-P	−
Oxidase	+	Gelatin hydrolysis	+
Phenylalanine dehydrogenase	−	Amylolysis	−
Lysine decarboxylase	−	Nitrate reduction	−
Arginine digydrolase	+	Citrate	+

+ positive, − negative

type of electron acceptors, and fructose and glucose seemed to be the favorable electron donors.

Figure 3 represents the AH_2QDS production along with microbial growth and fructose consumption in the fructose-fed sets during the 10-d incubation. In the inoculated batches served with electron donor (fructose) and electron acceptor (AQDS), strain PAH-1 achieved the maximum cell density of 9.1×10^7 CFU ml^{-1}, which was almost five times greater than the initial cell density. The microbial growth of strain PAH-1 was negligible in the sets without the addition of cells, AQDS, or fructose. These results indicated that (a) there was no abiotic factor in the medium that could support the redox reaction between AQDS and fructose; (b) the strain grew at the expense of AQDS and fructose simultaneously; and (c) the strain cannot ferment fructose.

As known, the microorganisms with the capacity to conserve energy from humus reduction are phylogenetically dispersed, the most representative strains are *Desulfuromonas acetexigens* (Lovley et al. 1998), *Corynebacterium humireducens* sp. nov (Wu et al. 2010), and *Geobacter metallireducens* (Lovley et al. 1996b). To the best of our knowledge, this study, for the first time reported a strain of *P. aeruginosa* could utilize AQDS as the sole electron acceptor for their growth under anaerobic conditions. This finding defines a new characteristic of the *Pseudomonas* genus, which also expands the environmental significance of the genus. It is well-known that the *Pseudomonas* genus comprises many hydrocarbon-degrading bacteria both in aerobic and anaerobic conditions (Haritash and Kaushik 2009). On the other hand, microbial-mediated humus reduction may highly be related to the anaerobic biodegradation of organic or inorganic pollutants (Gu et al. 2005; Wang et al. 2009). Previous literatures have reported that AQDS (and humic substances) can be utilized by microbes as effective electron acceptors or shuttles for the oxidative degradation of organic pollutants, such as 1,2-dichloroethylene, vinyl chloride, toluene, and phenolic compounds (Bradley et al. 1998; Cervantes and Dijksma 2001; Cervantes et al. 2000). Therefore, the newly isolated humus-reducing strain of *P. aeruginosa* holds great potentials for microbial remediation of organic pollutants, especially in the contaminated soil enriched with HS.

3.3 Anaerobic degradation of phenanthrene

Figure 4 showed the anaerobic degradation of phenanthrene by strain PAH-1 with AQDS as the sole electron acceptor. Data showed that the concentration of phenanthrene remained almost constant in the uninoculated controls. In the inoculated batches, phenanthrene started to degrade after a lag phase of about 10 days. Such acclimation period resulted from the time required for a small population of

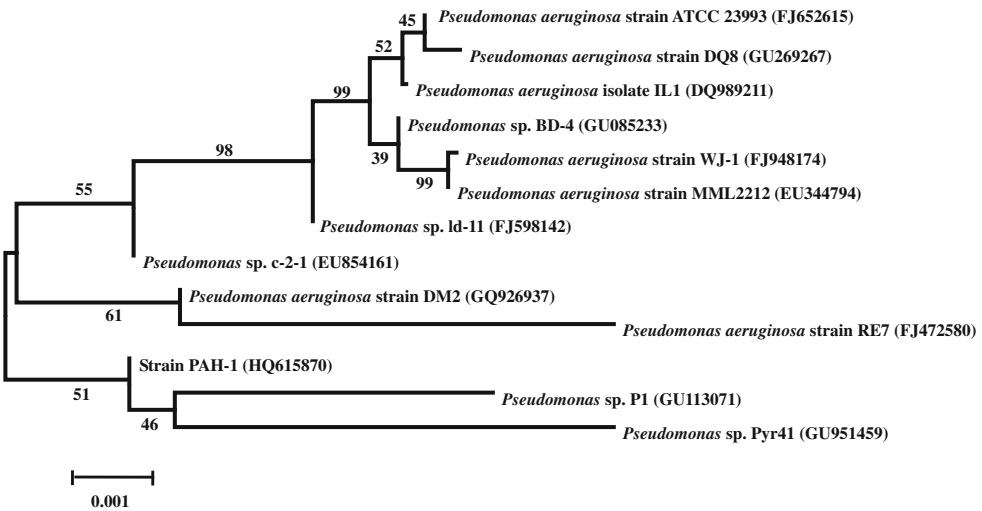

Fig. 1 Phylogenetic tree obtained by neighbor-joining analysis based on 16S rRNA gene sequences showing the position of strain PAH-1 among phylogenetic neighbors

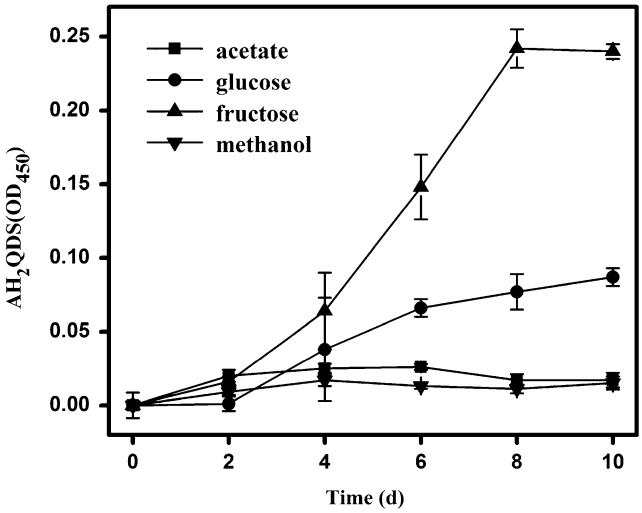

Fig. 2 Reduction of AQDS by strain PAH-1 with different organic substances as electron donors under anaerobic condition. *Error bars* represent SD of the mean (n=3)

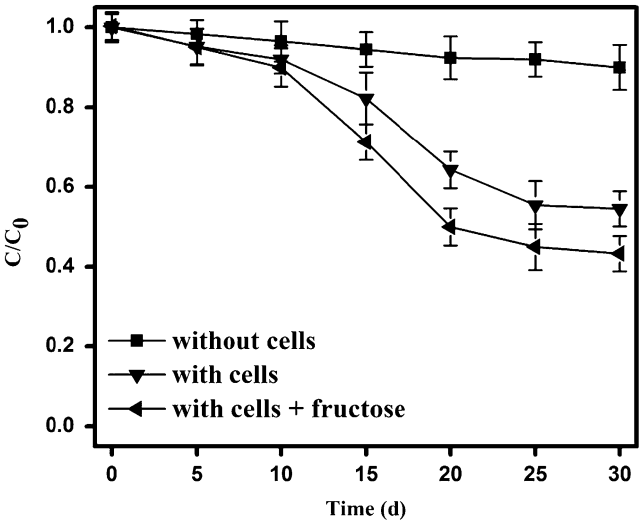

Fig. 4 Anaerobic degradation of phenanthrene by strain PAH-1 over 30-d incubation. *Error bars* represent SD of the mean (n=3)

microorganisms to attain sufficient density to exhibit detectable PAHs degradation. This was consistent with the previous findings that pure or mixed cultures isolated from non-contaminated sites required an apparent adaption period for degrading PAHs under anaerobic conditions (Mihelcic and Luthy 1988; McNally et al. 1998).

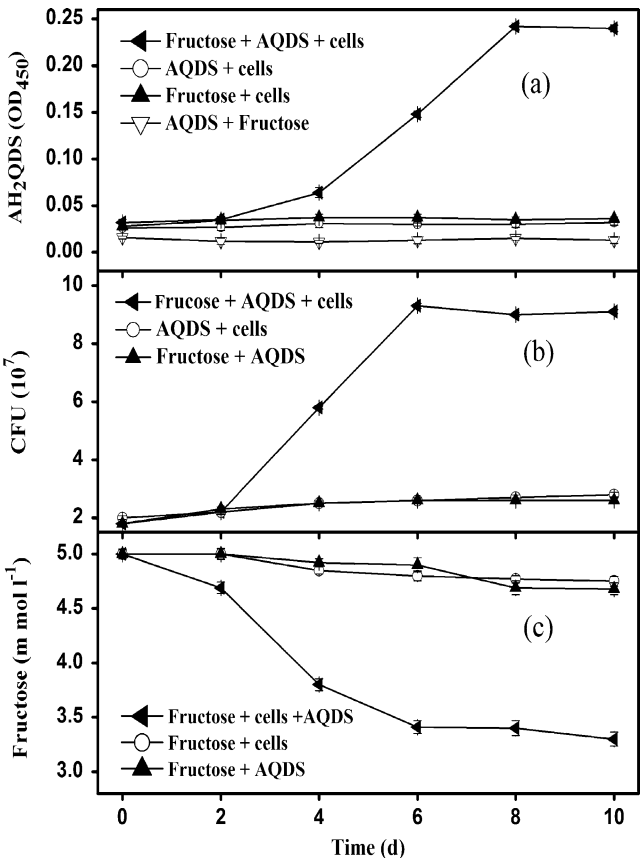

Fig. 3 Anaerobic growth of strain PAH-1 with fructose as the electron donor and AQDS as the electron acceptor: **(a)** AH_2QDS production; **(b)** cells number with fructose; and **(c)** fructose consumption during this experiment. *Error bars* represent SD of the mean (n=3)

After 30 days, approximately 46.5% of phenanthrene (20 mg l^{-1}) was degraded in the inoculated sets. When 1 mmol l^{-1} fructose was added, the degradation rate was enhanced to 56.7%, and 17.4% of fructose was consumed simultaneously. More than 53.6% and 19.6% AQDS was reduced in the active sets with and without fructose, respectively. As seen from Fig. 3b and c, strain PAH-1 could consume fructose and AQDS to attain energy for stimulating cells number. Thus, the enhanced removal of phenanthrene in the fructose sets resulted from an increase in the microbial growth of PAH-degrading bacteria. There was no significant loss of phenanthrene and nearly no AH_2QDS was detected in the cell-free or non-AQDS controls (data not shown). The results suggested that anaerobic degradation of phenanthrene by strain PAH-1 was a biotic and an AQDS-dependent process. In this study, the phenanthrene degradation data fit the pseudo-first-order kinetics, giving the degradation rate constant of 0.0233/day (R^2=0.934) and 0.0328/day (R^2=0.933) for the non-fructose and fructose sets, respectively. The rate constant was similar to the phenanthrene degradation by *Clostridium pascui* MSA3 under methanogenic condition (0.032/day, Chang et al. 2008).

It is known that the addition of an alternative carbon source would effectively enhance PAHs degradation under both aerobic and anaerobic conditions. Previous studies have demonstrated that the addition of carbon sources (such as acetate, pyruvate, and lactate) could enhance PAHs (acenaphthene, fluorine, and phenanthtene) degradation by two to three times in contaminated soils (Chang et al. 2002; Chang et al. 2008). The proposed explanation was that

these carbon sources promoted the growth of particular consortium and the production of more electrons to accelerate the process (Chang et al. 2002; Chang et al. 2008). For pure cultures, Ye et al. (1995) found that benzo (a)pyrene mineralization by *Sphingomonas paucimobilis* strain EPA 505 (an aerobic strain) was increased from 5% to 28% when glucose was amended in the liquid culture. Rockne et al. (2000) demonstrated that pre-growth of *Pseudomonas stutzeri* NAP-3 on acetate and *Vibrio pelagius* NAP-4 on pyruvate resulted in a much higher cell mass production than when grown on naphthalene alone under nitrate-reducing condition, and the naphthalene degradation was increased by 50–60% with significant removal of nitrate. In our study, the anaerobic degradation of phenanthrene was increased by 10.2% with the addition of fructose, and the enhancement was expected to be greater if a higher concentration of fructose was applied. With the addition of fructose, strain PAH-1 may prefer to utilize fructose for significant increase in cells number that can further oxidize phenanthrene coupling with AQDS reduction. The strain may also directly oxidize phenanthrene as the sole carbon sources for growth.

Though the *Pseudomonas* genus has many PAHs-degrading bacteria, such as *Pseudomonas fluorescens* (McNally et al. 1998), *Pseudomonas putida* (McNally et al. 1998), and *P. stutzeri* (McNally et al. 1998; Rockne et al. 2000), *P. aeruginosa* strain PAH-1 was the first strain that was capable of anaerobic phenanthrene degradation coupling with AQDS reduction. Due to the environmental significance of AQDS reduction (humus reduction), the strain may have great potential for microbial degradation of other organic pollutants.

4 Conclusions

In this study, the newly isolated bacterium, *P. aeruginosa* strain PAH-1, could oxidize fructose to conserve energy for microbial growth coupled with AQDS reduction. It was reported for the first time that a strain of the *Pseudomonas* genus was able to degrade phenanthrene under anaerobic conditions with AQDS serving as the terminal electron acceptor. Such findings explore the new environmental functions of the *Pseudomonas* genus and also provide opportunity for the development of a PAHs-bioremediation technology in the contaminated sites in the abundance of HS. To employ this technology, a better understanding of the metabolic pathways of anaerobic phenanthrene degradation is required. Additionally, knowledge on the biochemical reactions, enzymes, and genetics of anaerobic PAH biodegradation with the isolated strain is also needed to make purposeful application of biodegradation possible.

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