**BTEX biodegradation and its nitrogen removal potential by a newly isolated *Pseudomonas thivervalensis* strain MAH1**

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<th>Canadian Journal of Microbiology</th>
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<td>Manuscript ID:</td>
<td>cjm-2015-0152.R2</td>
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<td>Manuscript Type:</td>
<td>Article</td>
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<td>Date Submitted by the Author:</td>
<td>01-Jul-2015</td>
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<td>Complete List of Authors:</td>
<td>Qu, Dan; Jilin University, Zhao, Yongsheng; Jilin University, Sun, Jiaqiang; Jilin University, Ren, Hejun; Jilin University, Zhou, Rui; Jilin University,</td>
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<td>Keyword:</td>
<td>BTEX, Biodegradation, Bioremediation, Nitrogen removal, Pseudomonas thivervalensis MAH1</td>
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BTEX biodegradation and its nitrogen removal potential by a newly isolated *Pseudomonas thiivalensis* strain MAH1

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Abstract  Benzene, toluene, ethylbenzene, and xylene (BTEX) are of great environmental concern because of their widespread occurrence in groundwater and soil, which pose an increasing threat to human health. The aerobic denitrifying BTEX-degrading bacterium *Pseudomonas thivervalensis* (MAH1) was isolated from BTEX-contaminated media under nitrate-reducing conditions. The degradation rates of benzene, toluene, ethylbenzene, and xylene by MAH1 were 4.71, 6.59, 5.64, and 2.59 mg L\(^{-1}\) d\(^{-1}\), respectively. The effects of sodium citrate, nitrate, and NaH\(_2\)PO\(_4\) on improving BTEX biodegradation were investigated, and the optimum concentrations were 0.5 g L\(^{-1}\), 100 mg L\(^{-1}\), and 0.8 mM, respectively. Moreover, MAH1, which has *nirS* and *nosZ* genes, removed ammonium, nitrate, and nitrite at 2.49 mg L\(^{-1}\) NH\(_4\)\(^+\) N h\(^{-1}\), 1.50 mg L\(^{-1}\) NO\(_3\)\(^-\) N h\(^{-1}\), and 0.83 mg L\(^{-1}\) NO\(_2\)\(^-\) N h\(^{-1}\), respectively. MAH1 could help in mitigating the pollution caused by nitrogen amendments for biostimulation. This study highlighted the feasibility of using MAH1 for the bioremediation of BTEX-contaminated sites.

Keywords  BTEX · Biodegradation · Bioremediation · Nitrogen removal · *Pseudomonas thivervalensis* · MAH1
Introduction

Benzene, toluene, ethylbenzene, and xylene, collectively called BTEX, form the major aromatic components of petroleum. They are widely found in groundwater because of frequent leakages from underground storage tanks and pipelines, improper waste disposal practices, inadvertent spills, and leaching from landfills (Jo et al. 2008). Given their high motility, toxicity, carcinogenicity, and teratogenicity, BTEX are listed as priority pollutants by the United States Environmental Protection Agency (Bowlen et al. 1995).

Bioremediation, including bioaugmentation and biostimulation, has been commonly adopted in contaminated sites because it provides a non-disruptive, cost-effective, and highly efficient approach for treatment (Atlas and Hazen 2011). Bioaugmentation can be used when degrading bacteria do not occur naturally at a site or occur at too low of a population to be effective (EPA 2006). In a bioaugmentation project, efficient and environment-friendly degrading bacteria are often inoculated underground. Pure cultures of bacteria, such as *Rhodococcus rhodochrous* (Deeb and Cohen 1999), *Alcaligenes xylosoxidans* Y234 (Yeom and Yoo 2002), *Ralstonia* sp. YABE411 (Lin and Cheng 2007), *Pseudomonas putida* F1 (Kim et al. 2013), and *Janibacter* sp. SB2 (Jin et al. 2013), that are capable to degrade BTEX components have been isolated. Biostimulation involves supplying to the environment with nutrients, such as carbon substrates, nitrogen, phosphorus, and electron acceptors, which can favor the growth of degrading bacteria to accelerate contaminant degradation (Alexander 1999; Chaineau et al. 2005; Vidali 2001; Wu et al. 2008). Appropriate concentrations of amendments are important for the efficient degradation of BTEX (Knezevich et al. 2006). Jin et al. (2013) reported that the percentage of BTEX degradation by *Janibacter* sp. SB2 increases under the optimum concentrations of NH₄Cl and NaH₂PO₄. Kim et al. (2013) investigated the impact of nitrate dose on toluene degradation by *P. putida* F1, and an optimum ratio of nitrate to toluene was reported to yield the highest degradation rate of toluene.

Nitrate is commonly added in bioremediation projects, because microorganisms can utilize it as both nitrogen source and electron acceptor. Some of nitrates are utilized by microorganisms for BTEX degradation, whereas others are left in the groundwater and transported with flow. The rest of nitrates could be transformed to ammonium through dissimilatory nitrate reduction to ammonium (DNRA) (Knowles 1982; Ma and Aelion 2005; Rokosz et al. 2009) or nitrite through denitrification (Long et al. 2011; Wang et al. 2013). The excess nitrogen compounds may cause secondary pollution of groundwater.
groundwater. Adopting a new treatment process to remove nitrogen increases the operation cost. Hitherto, exploration of the bacteria which are able to both degrade BTEX and remove nitrogen is necessary. Although BTEX biodegradation using pure bacteria has been investigated intensively, the nitrogen removal potential of the same BTEX-degrading microorganism remains unclear. In this study, the BTEX-degrading strain MAH1 was isolated from BTEX-contaminated media under nitrate-reducing conditions. The ability of the isolate to degrade BTEX was investigated. The nitrogen removal potential of MAH1 was also assessed. This isolate, which is capable of both degrading BTEX and removing nitrogen, may be an alternate microbial resource for bioremediation of BTEX-contaminated sites.

**Materials and methods**

**Media and screening of BTEX-degrading bacterium**

For the isolation of BTEX-degrading bacterium, BTEX-contaminated sediment under nitrate-reducing conditions were obtained from the column experiment described previously (Zhao et al. 2015). 1 g of sediment was transferred to 250-mL Erlenmeyer flasks containing 100 mL of sterile bromothymol blue (BTB) medium (Qu et al. 2015) and cultured at 10 °C in a shaking incubator (120 rpm) for 72 h. The enriched cultures were sampled using gradient dilution. The diluent was streaked onto mineral salt medium (MSM) plates and cultured at 10 °C in an incubator filled with BTEX vapor until visible colonies surrounded by yellow (positive result) were formed. The colonies surrounded by yellow were transferred to 250-mL headspace vials containing MSM (100 mL) and BTEX [200 mg L\(^{-1}\), benzene, toluene, ethylbenzene, and o-, m-, p-xylene (3:3:3:1:1)]], and incubated at 10 °C with shaking at 120 rpm. The predominant BTEX-degrading isolates with the highest degradation efficiency were then selected and observed morphology on Luria Bertani (LB) plates.

**LB media contains** 10.0 g L\(^{-1}\) peptone, 5.0 g L\(^{-1}\) yeast extract, 10.0 g L\(^{-1}\) NaCl, and 2 % agar for solid medium. The MSM with pH 7–7.3 was composed of the following: 0.36 g L\(^{-1}\) KNO\(_3\), 0.12 g L\(^{-1}\) NaH\(_2\)PO\(_4\), 2.0 g L\(^{-1}\) MgCl\(_2\), 1.0 g L\(^{-1}\) CaCl\(_2\), 1 mL of trace element solution, and 2 % agar for solid medium. The modified MSM components were the same as MSM above except for KNO\(_3\) and NaH\(_2\)PO\(_4\). The components of trace element solution at pH 7.0 were as follows: 1.0 g L\(^{-1}\) EDTA, 0.2 g L\(^{-1}\) ZnSO\(_4\)\(\cdot\)7H\(_2\)O, 0.5 g L\(^{-1}\) FeSO\(_4\)\(\cdot\)7H\(_2\)O, 0.02 g L\(^{-1}\) Na\(_2\)MnO\(_4\)\(\cdot\)2H\(_2\)O, 0.02 g L\(^{-1}\) CuSO\(_4\)\(\cdot\)5H\(_2\)O, 0.04 g L\(^{-1}\) CoCl\(_2\)\(\cdot\)6H\(_2\)O, and 0.01 g L\(^{-1}\) MnCl\(_2\)\(\cdot\)2H\(_2\)O. All the chemical reagents used were of analytical grade.
Identification of the isolates

Total DNA was prepared using a genomic DNA extraction kit (Sangong, Shanghai, China). The bacterial 16S rRNA gene sequence was amplified from the extracted genomic DNA using PCR with universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GATTACCTTGTACGACTT) (Heuer et al. 1997). The 1.5 kb PCR products were purified using Agarose Gel DNA Purification Kit (Takara, Dalian, China), cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and sequenced by Sangon Co. Ltd. (Shanghai, China). The sequences were searched against the GenBank database (National Center for Biotechnology Information) using BLAST. The closest matching sequences were obtained, and a phylogenetic tree was constructed using MEGA 5.2.1 by applying the neighbor-joining (NJ) method and maximum likelihood analysis on various data sets.

BTEX degradation tests

The strains were pre-cultured in MSM with 200 mg L$^{-1}$ BTEX [benzene, toluene, ethylbenzene, and o-, m-, p-xylene (3:3:3:1:1)] for 72 h at 10 °C and 120 rpm. The bacterial suspension was centrifuged for 10 min at 4 °C and 8000 × g. The pellets were washed thrice with sterile water to purify the bacterial suspension. 2 mL of washed pre-culture [optical density (OD$_{600}$) = 1.0] was inoculated into 100 mL of MSM containing 51.81 mg L$^{-1}$ benzene, 50.69 mg L$^{-1}$ toluene, 49.51 mg L$^{-1}$ ethylbenzene, and 52.05 mg L$^{-1}$ xylene [o-, m-, p-xylene (1:1:1)], respectively. Meanwhile, control experiments were also conducted without inoculations and with inactivated biomass by sterilization. Sample tests were performed in a rotary shaker at 10 °C and 120 rpm. A periodic sampling for each culture set-up was performed to determine BTEX concentration and OD$_{600}$. All tests were performed in triplicate.

Effect of sodium citrate, nitrate, and NaH$_2$PO$_4$ on BTEX biodegradation

2 mL of pre-culture (OD$_{600}$ = 1.0) was inoculated into 100 mL of modified MSM containing 200 mg L$^{-1}$ BTEX [benzene, toluene, ethylbenzene, and o-, m-, p-xylene (3:3:3:1:1)], and different concentrations of sodium citrate, nitrate, and NaH$_2$PO$_4$ were added into the vials. Five levels for each of the three variable factors were considered and arranged as follows: sodium citrate (0, 0.1, 0.5, 1, and 2 g L$^{-1}$), NO$_3^-$-N (20, 50, 100, 150, and 200 mg L$^{-1}$), and NaH$_2$PO$_4$ (0.4, 0.6, 0.8, 1.0, and 1.2 mM). In each experiment, one variable factor was changed, whereas the other variable factors were fixed to a constant value (sodium citrate, 0 g L$^{-1}$; NO$_3^-$-N, 100 mg L$^{-1}$; NaH$_2$PO$_4$, 1.0 mM). A periodic sampling was performed to measure OD$_{600}$, NO$_3^-$-N, and BTEX concentration for each time point. All
tests were performed in triplicate. Vials without inoculation were used as sterile controls.

**Nitrogen removal performance of MAH1**

For functional gene detection, fragments of the nirS and nosZ genes were amplified using primer pairs nirS-F/nirS-R for nirS and nosZ-F/nosZ-R for nosZ developed by Rosch et al. (Rösch et al. 2002). The primer pairs were nirS-F: 5′-CAC GGY GTB CTG CG C AAG GGC GC-3′, nirS-R: 5′-CGC CAC GCG CGG YTC SGG GTG GTA-3′, nosZ-F: 5′-CGY TGT TCM TCG ACA GCC AG-3′, and nosZ-R: 5′-CAT GTG CAG NGC RTG GCA GAA-3′. Takara Taq hot-start polymerase (Takara, Dalian, China) was used for the PCR reaction. Negative controls without DNA template were simultaneously performed. The PCR products were separated by 1 % agarose gel electrophoresis and stained with SYBR Safe DNA gel stain (Molecular Probes, USA). Bands were visualized by UV excitation. The methods of purifying and sequencing the PCR products of nirS and nosZ genes were as same as that of bacteria 16S rRNA gene. The nirS and nosZ sequences were analyzed for similarity to other published sequences in the GenBank database by using BLAST program.

The strain was pre-cultured in 100 mL of MSM with 200 mg L\(^{-1}\) BTEX [benzene, toluene, ethylbenzene, and o-, m-, p-xylene (3:3:1:1:1)] for 4 days at 10 °C and then harvested by centrifugation (8000 × g, 10 min at 4 °C) to assess the nitrogen removal abilities of MAH1. After washing the pellets thrice with sterile water, 1 mL of pre-culture (OD\(_{600}\) = 1.0) was inoculated into 100 mL of denitrification medium (Qu et al. 2015) containing 121.01 mg L\(^{-1}\) NH\(_4\)+N, 95.98 mg L\(^{-1}\) NO\(_3\)−N, and 97.59 mg L\(^{-1}\) NO\(_2\)−N, respectively. Batch experiments were carried out using a gyratory incubator shaker at 10 °C and 120 rpm to allow better gas exchange. Samples were withdrawn at a regular time interval during the experiments to determine OD\(_{600}\) and centrifuged to obtain supernatants for the determination of residual ammonium, nitrite, and nitrate. All the experiments in this study were performed in triplicate.

**Analytical methods**

Cell growth was monitored by measuring the absorbance at 600 nm (OD\(_{600}\)) using a spectrophotometer. The concentrations of ammonia, nitrite, and nitrate were determined according to the standard methods (Apha 1998) of Nessler’s reagent, N-(1-naphthalene)-diaminoethane, and diphenylamine spectrophotometry, respectively. BTEX were analyzed using a gas chromatograph (GC-2012; Shimadzu, Japan) equipped with a flame ionization detector and a HP-1 column (30 m × 0.25 µm × 0.25 mm). The column temperature was programmed from 40 °C (2 min) to 250 °C (3 min).
at a rate of 10 °C min⁻¹. The detector temperature was 300 °C, the injection port temperature was 250 °C and the 1-µL samples were loaded with an auto-sampler in the split ratio of 10:1.

Results and Discussion

Isolation and phylogenetic analysis of the aerobic denitrifying bacterium MAH1

In the screening process using selective plates exposed to BTEX vapor, yellow coloration around the colonies of 10 strains was observed. Among these colonies, a strain designated as MAH1, which exhibited the highest BTEX degradation efficiency at 10 °C after 6 days, was selected for further characterization. The colonies were white and circular, with semi-transparent, slabby, wet, and smooth surfaces on LB plates. The complete 16S rDNA sequences (1472 bp, accession number: KP742980) of MAH1 were deposited to GenBank, and the database search result showed that MAH1 was nearly (up to 99 %) identical to the genus *Pseudomonas*. The phylogenetic tree was constructed employing the NJ method (Fig. 1). The tree clearly showed that MAH1 was closest to *Pseudomonas thivervalensis* (accession number: AF100323.1) based on the evolutionary relationship along with the morphological and biochemical characteristics. Therefore, MAH1 was proposed to be *P. thivervalensis*.

BTEX degradation ability of the isolates

To investigate the degradation ability of the isolates for BTEX, each component compound (benzene, toluene, ethylbenzene, and xylene) was added as the sole carbon source. BTEX concentration barely changed in the control experiments either without inoculations or with inactivated biomass (supplementary Fig. S1). It was demonstrated that BTEX removal in the microcosm was caused by biodegradation rather than volatilization or adsorption. Strain MAH1 can degrade all BTEX compounds as shown in Fig. 2. Fig. 2A illustrates that benzene was slowly degraded with the growth of MAH1, which slowly increased during the first 4 days and entered the rapid growth stage from the 4th day to 10th day. Benzene was completely degraded from 51.86 mg L⁻¹ within approximately 11 days, corresponding to a degradation rate of 4.71 mg L⁻¹ d⁻¹. Simultaneously, OD₆₀₀ increased from 0.02 to 0.11. In the first 6 days, a significant decrease in toluene and ethylbenzene was observed which correlated well with the rate of cell growth (Fig. 2B and Fig. 2C). On the 8th day of cultivation, toluene was already undetectable, but ethylbenzene had still a residual of 4.42 mg L⁻¹. The degradation rates of toluene and ethylbenzene were 6.59 mg L⁻¹ d⁻¹ and 5.64 mg L⁻¹ d⁻¹, respectively. The degradation tests also showed that xylene could not be completely degraded and the final degradation percentage was
only 39.20 % (Fig. 2D). The degradation rate of xylene was only 2.59 mg L$^{-1}$ d$^{-1}$. A similar result was reported in the previous studies using *Janibacter* sp. SB2 (Jin et al. 2013). Compared with other BTEX compounds, xylene was markedly persistent (Deeb and Cohen 1999). Initial oxidative attack of BTEX converting the compound into a catechol structure and the cleavage of the catechol structure are key steps in aerobic BTEX degradation (Hendrickx et al. 2006). Initial oxidative attack consists of direct oxidation of the aromatic ring via a mono-oxygenase (Kahng et al. 2001) or a dioxygenase attack (Furukawa et al. 1993; Zylstra and Gibson 1989) or oxidation of the alkyl side chain which is catalyzed by mono-oxygenases (Burlage et al. 1989). Xylene was harder attacked by oxygenase due to two methyl groups. So it was not easily degraded completely.

**Effects of sodium citrate, nitrate, and NaH$_2$PO$_4$ on BTEX degradation**

Biostimulation by adding carbon substrates and nutrients (such as nitrogen or phosphorus) was the most widely accepted approach in bioremediation process (Margesin and Schinner 2001). Appropriate concentrations of amendments in the contaminated sites result in optimal degradation performance of degrading microorganisms (Mohajeri et al. 2010; Zahed et al. 2010). Sodium citrate and NaH$_2$PO$_4$ were the best sources of carbon and phosphorus based on the preliminary tests (supplementary Fig. S2). Nitrate, both a nitrogen source and electron acceptor, is a favorable amendment added into the groundwater to promote pollutant biodegradation (Cunningham et al. 2001; Ha et al. 2011). Hence, effects of sodium citrate, nitrate, and NaH$_2$PO$_4$ on BTEX biodegradation were further investigated.

Fig. 3A shows the percentages of BTEX degradation by MAH1 with different amounts of sodium citrate supplementation. On the 8th day, BTEX degradation percentage for the treatment without sodium citrate was 52.64 %, which was higher compared with that of treatments supplemented with 0.1, 1.0, and 2.0 g L$^{-1}$ sodium citrate. The highest BTEX degradation percentage (91.17 %) was achieved with 0.5 g L$^{-1}$ sodium citrate supplementation. Adding sodium citrate, the biomass increased but BTEX did not decrease at the beginning (data not shown). OD$_{600}$ corresponding to treatment with 0.1 g L$^{-1}$ sodium citrate supplementation was 0.28, which was almost as same as that of the treatment without sodium citrate addition (OD$_{600} = 0.25$). However, the degradation of BTEX was far worse. It indicates that the bacteria preferably use citrate instead of BTEX, because BTEX was toxic to microorganisms. These conforms with many studies that addition of a carbon source as a nutrient will increase the biomass of microorganisms, but may inhibit the biodegradation of contaminants and result in diauxic growth (Alexander 1999; Lee et al. 2003). In addition, the extremely high sodium citrate concentrations

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(1.0 and 2.0 g L$^{-1}$) resulted in a large population of degrading bacteria, and promoted a great competition among them. Adding more carbon substrates did not improve the removal of pollutant. This observation did not agree with the results in the previous studies (Teng et al. 2010; Thompson et al. 2008). Therefore, optimizing the amount of additional carbon substrates for each degrading bacterium is important. When 0.5 g L$^{-1}$ sodium citrate was added, the degradation rate of each component was accelerated. Notably, almost complete removal of xylene was observed on the 8th day, but only a small amount of xylene was degraded in the treatment without sodium citrate (supplementary Fig. S3). Adding appropriate amount of sodium citrate had a significant effect in promoting biodegradation of xylene. The experiments showed that the addition of carbon substrates improved the biodegradation of persistent aromatic hydrocarbons.

The effect of the initial concentration of nitrate in the biodegradation of BTEX was illustrated in Fig. 3B. On the 8th day, the degradation percentages of BTEX with the initial concentrations of 20 and 50 mg L$^{-1}$ NO$_3^-$-N were only 5.99 % and 7.88 %, respectively, which illustrates that sub-optimal nitrate addition did not significantly promote BTEX biodegradation. The BTEX degradation percentage significantly increased when 100 mg L$^{-1}$ NO$_3^-$-N was supplied. However, a decrease in BTEX degradation percentage was observed at higher concentrations (150 and 200 mg L$^{-1}$) of NO$_3^-$-N. Nitrate has inhibitory effect on microorganisms and excess nitrate will inhibit microbial growth and hinder BTEX degradation (Blackmer and Bremner 1978; Hernandez et al. 1991; Qu et al. 2015; Thomas et al. 1994). While oxygen is mainly consumed, a small amount of nitrate can be used as electron acceptor for BTEX biodegradation (Kim et al. 2004). Comparing to other biodegradation added NO$_3^-$-N, most of BTEX was degraded and the remaining nitrate was the least at the initial concentration of 100 mg L$^{-1}$ NO$_3^-$-N. Whereas 91.45 % of nitrate was still left in the solution. The results showed that some nitrate was reduced during BTEX degradation, but majority remained in the solution.

Concentration of phosphorus is another important factor with significant effects on the biodegradation of contaminants in marine habitats (Yao et al. 2009). The effect of phosphorus (NaH$_2$PO$_4$) on BTEX degradation was evaluated as shown in Fig. 3C. Insufficient amount of phosphorus (0.4 and 0.6 mM) limited the growth of degrading bacteria, whereas a high concentration was undesirable because of the toxicity of NaH$_2$PO$_4$ (Mohajeri et al. 2010). The optimal NaH$_2$PO$_4$ dose was 0.8 mM.
Amplification of the denitrification genes

The nirS and nosZ genes are often used as functional markers to identify aerobic denitrifying bacteria. NIR enzyme (nitrite reductase) encoded by the nirS gene could reduce NO$_2^-$ to NO, and NOS enzyme (nitrous oxide reductase) encoded by the nosZ gene could convert N$_2$O to N$_2$ (Veraart et al. 2014; Wallenstein et al. 2006). In this study, the genes of nirS (702 bp) and nosZ (707 bp) were successfully amplified from the strain MAH1 (Fig. 4). After sequencing, GenBank’s BLAST program was used. nirS (671 bp) gene fragment is 91 % identical to that of Pseudomonas fluorescens (accession number: AF197466.1) and nosZ (669 bp) gene fragment is 91 % identical to that of Pseudomonas sp. PD 22 (accession number: DQ377794.1). These results indicated that the potential occurrence of denitrification in MAH1 can be reasonably considered.

Nitrification and denitrification potential of MAH1

A percentage of added nitrate, which was not used up during BTEX biodegradation, could be transformed to ammonium or nitrite (Long et al. 2011; Wang et al. 2013). Hence, removing nitrogen, including ammonium, nitrate, and nitrite, in the groundwater by inoculated bacteria of bioaugmentation through nitrification and denitrification is necessary. To clarify the nitrification and denitrifying abilities of MAH1, three N-compounds (NH$_4$Cl, NO$_3^-$, and NO$_2^-$) and sodium citrate were used as nitrogen and carbon sources, respectively. Ammonium (121.01 mg L$^{-1}$ initial NH$_4^+$-N) was nearly undetectable after 48 h with a removal rate of 2.49 mg L$^{-1}$ NH$_4^+$-N h$^{-1}$ (Fig. 5A). Nitrate was completely reduced from 95.98 mg L$^{-1}$ within 64 h at the rate of 1.50 mg L$^{-1}$ NO$_3^-$ h$^{-1}$ (Fig. 5B). NO$_2^-$-N was removed more slowly with the rate of 0.83 mg L$^{-1}$ h$^{-1}$ because of its inhibitory effect to microbial growth (Fig. 5C). These phenomena were also reported for Pseudomonas sp. yy7 (Wan et al. 2011). Moreover, it is an advantage that denitrifying products were few and the isolates can reduce secondary pollution after adding nitrogen nutrients for biostimulation.

Conclusion

MAH1, a new bacterium capable of BTEX degradation and nitrogen removal, was isolated from BTEX-contaminated media under nitrate-reducing conditions. The degradation rates observed for benzene, toluene, ethylbenzene, and xylene by the strain were 4.71, 6.59, 5.64, and 2.59 mg L$^{-1}$ d$^{-1}$, respectively. The most efficient biodegradation of BTEX occurred when supplemented with 0.5 g L$^{-1}$ sodium citrate, 100 mg L$^{-1}$ NO$_3^-$-N, and 0.8 mM NaH$_2$PO$_4$. Especially, the xylene biodegradation percentage was significantly increased by adding sodium citrate. In addition, the genes of nirS and
nosZ were detected on the genome of MAH1, and the strain could remove ammonium, nitrate, and nitrite at the rates of 2.49 mg L\(^{-1}\) NH\(_4\)\(^+\) h\(^{-1}\), 1.50 mg L\(^{-1}\) NO\(_3\)\(^-\) h\(^{-1}\), and 0.83 mg L\(^{-1}\) NO\(_2\)\(^-\) h\(^{-1}\), respectively. These results indicate that MAH1 is a promising candidate to remove BTEX and excess nitrogen simultaneously in BTEX-contaminated sites.

**Acknowledgements**

This work was partly supported by the Doctoral Education Fund (new teachers) [grant number: 2012061120074], Specific Research of the Environmental Nonprofit Industry [grant number 2013A073], Promotion of Innovation Ability of Beijing Municipal Universities Project by Beijing Municipal Education Commission [grant number: TJSHG201310772028], and Graduate Innovation Fund of Jilin University [grant number: Project 2015112]. Key Laboratory of Groundwater Resources and Environment of Ministry of Education (Jilin University) is acknowledged for providing support to the work.

**References**


Figure captions

Fig. 1. NJ phylogenetic tree of 16S rDNA gene sequence of strain MAH1 and most closely related species. The GenBank accession numbers for the corresponding sequences are given in parenthesis after the strain name.

Fig. 2. Degradation of BTEX by *P. thivervalensis* MAH1 at 10 °C. (A) Benzene, (B) toluene, (C) ethylbenzene, and (D) xylene.

Fig. 3. BTEX degradation capability of *P. thivervalensis* MAH1 at various sodium citrate (A), NO$_3$-N (B) and NaH$_2$PO$_4$ (C) concentrations at 10 °C. Symbols: closed columns, BTEX degradation percentage; columns with leftward steak, OD$_{600}$; closed circles, remaining nitrate percentage.

Fig. 4. Amplification profiles of nir$S$ and nos$Z$ genes from *P. thivervalensis* MAH1.

Fig. 5. Growth of *P. thivervalensis* MAH1 and changes in nitrogen compound at 10 °C. (A) Ammonium (B) nitrate, and (C) nitrite.
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