Characterization of naphthalene degradation by *Streptomyces* sp. QWE-5 isolated from active sludge

Peng Xu, Wencheng Ma, Hongjun Han, Baolin Hou and Shengyong Jia

ABSTRACT

A bacterial strain, QWE-5, which utilized naphthalene as its sole carbon and energy source, was isolated and identified as *Streptomyces* sp. It was a Gram-positive, spore-forming bacterium with a flagellum, with whole, smooth, convex and wet colonies. The optimal temperature and pH for QWE-5 were 35 °C and 7.0, respectively. The QWE-5 strain was capable of completely degrading naphthalene at a concentration as high as 100 mg/L. At initial naphthalene concentrations of 10, 20, 50, 80 and 100 mg/L, complete degradation was achieved within 32, 56, 96, 120 and 144 h, respectively. Kinetics of naphthalene degradation was described using the Andrews equation. The kinetic parameters were as follows: q_{max} (maximum specific degradation rate) = 1.56 h^{-1}, K_s (half-rate constant) = 60.34 mg/L, and K_I (substrate-inhibition constant) = 81.76 mg/L. Metabolic intermediates were identified by gas chromatography and mass spectrometry, allowing a new degradation pathway for naphthalene to be proposed. In this pathway, monooxygenation of naphthalene yielded naphthalen-1-ol. Further degradation by *Streptomyces* sp. QWE-5 produced acetophenone, followed by adipic acid, which was produced as a combination of decarboxylation and hydroxylation processes.

Key words | Andrews equation, biodegradation, metabolites, naphthalene, *Streptomyces* sp.

Peng Xu
Wencheng Ma
Hongjun Han (corresponding author)
Baolin Hou
Shengyong Jia
State Key Laboratory of Urban Water Resource and Environment,
Harbin Institute of Technology,
Harbin 150090,
China
E-mail: *han13946003379@163.com*

INTRODUCTION

Naphthalene and its derivates are an important class of polycyclic aromatic hydrocarbons (PAHs). PAHs are primarily formed during incomplete combustion of organic materials and are emitted into the environment mainly by anthropogenic activities such as coal combustion and refining processes as well as through contamination associated with the transport and use of fossil fuels and derivatives (Chang *et al.* 2009; Horng *et al.* 2009; Xin *et al.* 2010). Extensive works are directed toward the adverse effects of PAHs, being carcinogenic and mutagenic, on living objects (Kaushik *et al.* 2012). Due to their hazardous effect on plants, aquatic organisms and human lives, the US Environmental Protection Agency has included them in its priority pollutant list.

Various physico-chemical methods such as adsorption, ozonation, photocatalysis, flocculation, electrocoagulation and membrane processes are available for the efficient removal of naphthalene from industrial wastes and other contaminated streams (Andersen *et al.* 2008; Heidmann & Calmano 2008). However, these processes are energy intensive, require high capital and operating costs and also generate secondary waste streams. Alternatively, biological treatment methods can provide a better option in view of their low capital and operating costs and the treatment resulting in formation of innocuous products (Haritash & Kaushik 2009). Many studies have shown that naphthalene could be removed by a variety of bacteria. These include *Rhodococcus* sp., *Desulfobacterium indolicum*, *Burkholderia pickettii*, *Comamonas* sp., and white rot fungus (Mollea *et al.* 2005; Filonov *et al.* 2006; Lin *et al.* 2010; Shah *et al.* 2012). Farjadfard *et al.* (2012) successfully isolated a *Streptomyces* strain from oil and creosote contaminated soils to degrade phenanthrene. Cao *et al.* (2009) have reported three *Streptomyces* strains from the Kuwait Burgan oil field with the ability to utilize naphthalene as sole carbon and energy sources. Results of the early studies on catabolic metabolism of naphthalene by *Pseudomonas* sp. confirmed the presence of naphthalene dioxygenase, known as an oxidoreductase enzyme that catalyzes ring hydroxylation by incorporating two atoms of O_2 molecule (Shah *et al.* 2012). Dioxygenase and monooxygenase enzymes were reported as major degrading enzymes in the oxidizing degradation of PAHs. Lee *et al.* (2003) found that

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the initial step for degradation of naphthalene in strain ERI-CPDA-1 involves hydroxylation by 1,2-dioxygenase to give *cis*-1,2-naphthalenedihydrodiol. This undergoes extra diol cleavage and further degradation to give benzaldehyde. Further degradation takes place by the tricarboxylic acid cycle.

The objective of the present study was to isolate and characterize a bacterial strain capable of degrading a high concentration of phenanthrene. In addition, the degradation kinetics model and metabolites produced by this strain were also investigated. The results will help to provide the basic information and optimum conditions for the treatment of naphthalene-containing wastewater by bioaugmentation technology.

MATERIALS AND METHODS

Chemicals and media

Naphthalene was of chromatographic grade, and obtained from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). All other chemicals used were of analytical grade, and purchased from the local suppliers.

Luria–Bertani (LB) medium was used for bacterial enrichment and maintenance. A basic mineral salt medium (MSM), as described by Lin *et al.* (2010), was used for bacterial acclimation and degradation experiments. Solidified naphthalene MSM was prepared by the addition of 19 g/L (1.9%) agar to the medium. All media were sterilized in an autoclave at 121 °C for 15 min prior to their use.

Isolation and identification

Samples of activated sludge were obtained from the coking wastewater treatment plant of the China Coal Longhua Harbin Coal Chemical Industry Co., Ltd, Harbin, China. Two hundred millilitres of samples were put into a 500 mL Erlenmeyer flask. In order to deflocculate and mix thoroughly, two drops of 0.01% sodium pyrophosphate and several glass beads were added; then the mixture was shaken at 30 °C, 180 rpm for 20 min. The mixture was centrifuged at 3000 *g* for 10 min. One gram of the centrifuged deposit was transferred into the MSM containing 50 mg/L naphthalene and incubated at 35 °C, 180 rpm. When naphthalene was removed completely from the medium, 10 mL of the culture was transferred to 90 mL of the fresh medium. After five successive transfers, the serial dilutions of suspensions (10^{-1}–10^{-7}) were spread onto the solidified naphthalene MSM. Colonies of naphthalene-degrading bacterium were purified through three cycles of single colony isolation. For preservation, strain QWE-5 was cultivated aerobically with the LB liquid medium containing 50 mg/L naphthalene, and then preserved in 15% glycerol at −10 °C in a freezer.

The isolated strain was identified by color and morphology. Further identification was done using the 16S rDNA sequencing. Genomic DNA was isolated using standard procedures as mentioned in the earlier study (Deveryshetty & Phale 2010). The following primers were used for polymerase chain reaction (PCR) amplification of the 16S rDNA: forward 63F: AGGCCTAACACATGCAAGTC; backward 1387R: GGGCGGAGTGTACAAGGC. PCR amplification was performed in a PCR thermocycler (TaKaRa, Japan) with an initial denaturation of the template DNA at 94 °C for 2 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension performed at 72 °C for 15 min, and then the sample kept at 4 °C. The PCR product was separated by agarose (0.8%) gel electrophoresis. The sequence data of the amplified 16S rDNA were determined and compared with known 16S rDNA sequences using the BLAST program of the National Center for Biotechnology Information database.

Batch biodegradation study

In the present study, to represent the degradation kinetics of naphthalene, degradation experiments were carried out in a series of 250 mL Erlenmeyer flasks with 100 mL MSM. Each flask was inoculated with 2 mL of enriched culture (optical density $OD_{600} = 1.2$). The 250 mL flask was supplemented with naphthalene at different initial concentrations (10–200 mg/L). The pH value of the media was adjusted by sterile HCl (1 mol/L) and NaOH (1 mol/L) to 7.0 and samples were incubated at 35 °C and 180 rpm. The control experiments were conducted with the same sterilized medium but without addition of the strain QWE-5. Samples were withdrawn at regular intervals, centrifuged ($8000 \times g$ for 15 min) and filtered. The filtrates were used to determine residual naphthalene concentration. Each result was reported as an average of three independent experiments.

To study the degradation kinetics of naphthalene, the experimental data obtained from the batch degradation experiments were fitted into the Andrews kinetic model. The Andrews model is as follows (Collina *et al.* 2003):

$$q = \frac{q_{\max} S_0}{K_s + S_0 + (S_0^2/K_I)} \quad (1)$$

where q is the specific degradation rate (l/h), S_0 is the initial substrate concentration (mg/L), q_{max} is the maximum degradation rate (l/h), K_s is the half-rate constant (mg/L) and K_I is the substrate-inhibition constant (mg/L). The values of the kinetic parameters for Andrews equation were obtained using nonlinear regression analysis using Orgin 8.6 SR1.

Identification of metabolites of naphthalene

To trace the metabolites during the degradation of naphthalene, the experiments were carried out in a series of 250 mL Erlenmeyer flasks containing 100 mL of the MSM, supplemented with naphthalene at 50 mg/L and inoculated with 2 mL of enriched culture ($OD_{600} = 1.2$). All flasks were sealed with sealfilm and incubated at 35 °C under shaking at 180 rpm. The flask that contained the same concentration of naphthalene without strain QWE-5 was used as a negative control under the same condition. At regular time intervals (12 h), a flask was taken out for the analysis of concentrations of possible metabolites. In the present study, five intermediates were identified and confirmed during naphthalene degradation. These metabolites were not detected in sterile controls. Gas chromatography retention times and mass spectral data of these metabolites are shown in Table 1.

Analysis methods

The concentration of naphthalene was analysed by high performance liquid chromatography (HPLC) (HP7950, Agilent). Culture samples for HPLC analysis were filtered through a 0.25 μm membrane. Five microlitres of the filtered solution was injected into the HPLC system equipped with a reversed-phase C18 column (Diamonsil, 200 × 5.1 mm, 5 μm). The mobile phase was composed of methanol and water (65:35, v/v) and the flow rate set at 1.0 mL/min. The initial column temperature was 50 °C and temperature was increased from 50 to 250 °C, with column temperature being held for 10 min. Naphthalene was detected at 300 nm.

Naphthalene metabolites were identified using gas chromatography (GC)/mass spectrometry (MS) (Thermo, USA) with EI mode (70 eV). For GC/MS analysis, a 30 m × 0.25 mm TR-35MS capillary column with a film thickness of 0.25 μm was used for the separation. The carrier gas was helium maintained at a column flow of 1.0 mL/min (at a pressure of 105 kPa). A 1.0 μL sample of the extract was injected and the column temperature was maintained at 60 °C for 2 min followed by temperature programming at 10 °C/min to 160 °C for 2 min. This was raised to 240 °C at a rate of 5 °C/min for 2 min, and finally to 290 °C at a rate of 30 °C/min for 2 min (Jajuee *et al.* 2007). The mass spectrometer and transfer line was held at 290 °C.

Table 1 | Gas chromatography retention times and mass spectral data of metabolites

Metabolites	Retention time (min)	Prominent fragment ion (m/z)
Naphthalen-1-ol	6.8	142(M^+), 128 (M^+-14), 108 (M^+-20),
2-propylphenol	7.4	118(M^+), 96 (M^+-22), 88 (M^+-30)
Acetophenone	11.3	106(M^+), 88 (M^+-18), 72 (M^+-34)
Benzaldehyde	13.2	92 (M^+), 74 (M^+-18), 50 (M^+-42)
Adipic acid	15.1	84 (M^+), 68 (M^+-16)

RESULTS AND DISCUSSION

Isolation and identification of naphthalene-degrading strain

A naphthalene-degrading microbe was isolated by enrichment shaking culture at 35 °C and named QWE-5. The strain QWE-5 obtained was rod shaped and formed whole, smooth, convex, rounded and wet colonies that became black-gray in color and circular with a diameter of 1.5–2 mm within 7 days. This strain was Gram-positive, spore-forming, with a flagellum as well as a capsule, but it was negative for catalase and gelatin liquefaction and was unable to reduce/restore nitrate. A partial 16S rDNA sequence (about 964 bp) was obtained and sequence alignment revealed that strain QWE-5 was closely related to the species in genus *Streptomyces*. Strain QWE-5 also exhibited the highest similarity (99.9%) to *Streptomyces* sp. SZA (DQ101053). According to both gene sequence analysis and cell characteristic information above, the isolated strain QWE-5 was identified as *Streptomyces* sp. The optimum temperature and pH for strain QWE-5 was 35 °C and 7.0, respectively.

Naphthalene biodegradation kinetics

Naphthalene degradation experiments were carried out for a wide range of its initial concentration from 10 to 200 mg/L. Figure 1(a) shows the naphthalene degradation profile with time for part of its initial concentrations. As Figure 1(a) shows, in the MSM with an initial pH of 7.5,

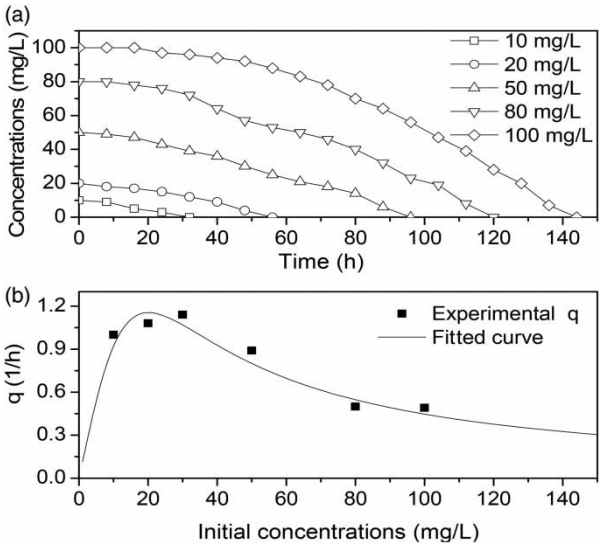

Figure 1 | (a) Naphthalene degradation profiles for its various initial concentrations; (b) experimental q and fitted curve by Andrews equation.

QWE-5 strain was capable of completely degrading naphthalene at a concentration as high as 100 mg/L. At initial naphthalene concentrations of 10, 20, 50, 80 and 100 mg/L, complete degradation was achieved within 32, 56, 96, 120 and 144 h, respectively. The lag phase was observed even at relatively low concentrations. In addition, with the increase of initial naphthalene concentration, the lag phase was extended, although well-acclimatized inoculum was used during the experiments. A similar phenomenon was observed by others in the biodegradation systems for naphthalene removal, which could be attributed to the recalcitrance and the toxicity of naphthalene (Shah *et al.* 2012).

The relationship between the experimental specific degradation rates (q) and initial naphthalene concentrations is shown in Figure 1(b). Figure 1(b) shows a typical trend in which specific degradation rates first increased with the increase in initial naphthalene concentrations up to a certain concentration level, and then decreased with increase in the initial naphthalene concentrations. It is a fact that naphthalene displays the inhibitory nature at high concentrations. Cell growth (and consequently naphthalene degradation) may dramatically reduce as a result of increase in naphthalene concentration. The rate of cell growth reaches a maximum level at a specific naphthalene concentration and any increase beyond this value diminishes the growth rate. The Andrews equation was used here to express the kinetics of naphthalene degradation. By using a nonlinear least squares regression analysis, the kinetic parameters were determined as follows: $q_{max} = 1.56$ (l/h), $K_s = 60.34$ (mg/L) and $K_I = 81.76$ (mg/L). The value of R^2 was 0.9716, which demonstrated that the experimental data were well correlated by the Andrews equation. The value of maximum experimental specific degradation rate was found to be equal to 1.13 l/h, which was achieved at an initial naphthalene concentration of 30 mg/L. The declining trend of specific degradation rates beyond the initial naphthalene concentration of 30 mg/L confirmed that naphthalene was an inhibitory type of substrate, and the inhibition effect of naphthalene became predominant above 50 mg/L.

Many research works have reported the degradation of naphthalene by microbes under aerobic condition. Farjadfard *et al.* (2012) found the value of q_{max} was 0.73 l/h, when *Achromobacter* sp FBHYA2 was used to degrade naphthalene. Maillacheruvu & Pathan (2009) reported that the values of q_{max} and K_I were 0.62 l/h and 93 mg/L, respectively, when using *Burkholderia pickettii* to degrade naphthalene. The naphthalene degradation by *Streptomyces* sp. was also tested. Mollea *et al.* (2005) found the value of q_{max} was 0.509 l/h, when *Streptomyces* sp. ZG7 was used to degrade naphthalene. The maximum q_{max} value obtained for *Streptomyces* sp. was 0.86 l/h reported by Collina *et al.* (2003). Compared with the calculated q_{max} from data given in the literature, q_{max} obtained for *Streptomyces* sp. QWE-5 in this study was the highest, which is an advantage of *Streptomyces* sp. QWE-5 for the treatment of naphthalene-containing wastewater. The large q_{max} value indicated that naphthalene could be degraded by *Streptomyces* sp. QWE-5 more rapidly than by other microorganisms. However, the q_{max} value of *Streptomyces* sp. QWE-5 was lower compared with microorganisms degrading other inhibitory substrates, such as phenol, which typically ranged between 0.87 and 1.4 l/h. The low q_{max} value observed in the naphthalene biodegradation system was probably due to the high toxic and inhibitory effects of naphthalene toward microorganisms.

Identification of metabolites of naphthalene

In the present study, during the course of naphthalene degradation, the formation of pink pigment (later identified to be acetophenone) in the medium was observed, which suggested that a colorful intermediate was formed. As the degradation went on, the medium became colorless, which suggested the further degradation of the intermediate. Pigment production during naphthalene degradation was also previously observed in other reports. Horng *et al.* (2009) reported pink pigment formation by a naphthalene-biodegrading strain of *Pseudomonas* sp. O'Loughlin *et al.*

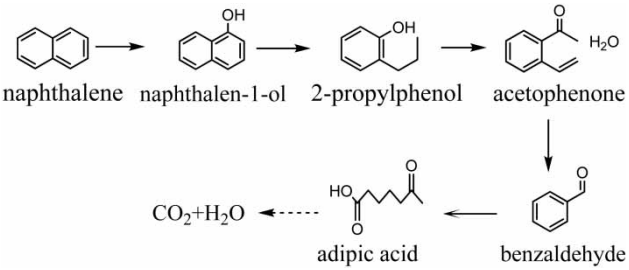

Figure 2 | Proposed pathway for the degradation of naphthalene by strain QWE-5.

(1996) observed the green pigment and brown pigment production by several *Rhodococcus* sp. The formation of different pigment observed by researchers revealed that degradation of naphthalene by different microorganisms is different.

In order to trace the metabolites during the degradation of naphthalene, the experiments were carried out in a series of 250 mL Erlenmeyer flasks containing 100 mL of the MSM, supplemented with naphthalene at 50 mg/L and inoculated with 2 mL of enriched culture ($OD_{600} = 1.2$). Five intermediates were identified and confirmed during naphthalene degradation. These metabolites were not detected in sterile controls. Based on the identified intermediates, the pathway for the degradation of naphthalene is proposed as shown in Figure 2, where monooxygenation of naphthalene yields naphthalen-1-ol. Further degradation by *Streptomyces* sp. QWE-5 produced acetophenone, followed by adipic acid, which was produced through a combination of decarboxylation and hydroxylation processes. Dioxygenase and monooxygenase enzymes were reported as major degrading enzymes in the oxidizing degradation of PAHs. Benzaldehyde is proposed as the decarboxylation product of acetophenone, as described in other studies (Filonov *et al.* 2004). This pathway differs from a typical naphthalene degradation showed by bacteria, which will cleave naphthalene at carbon number 1 and 2 (C1 and C2) to produce 1,2-dihydro-1,2-dihydroxynaphthalene and 1,2-dihydroxynaphthalene (Jegan *et al.* 2010).

CONCLUSIONS

A novel strain that is capable of degrading naphthalene at high concentrations was isolated from acclimated activated sludge. This strain was identified with the method of DNA sequencing, and designated as *Streptomyces* sp. QWE-5. The QWE-5 strain was capable of completely degrading naphthalene at a concentration as high as 100 mg/L. The experimental data were fitted well to the Andrews model using nonlinear regression analysis. Compared with the calculated q_{max} from data given in the literature, q_{max} obtained for *Streptomyces* sp. QWE-5 in this study was the highest. Finally, on the basis of GC/MS analysis, the possible pathway for the degradation of naphthalene using strain QWE-5 is proposed. Taken together, the results suggested that QWE-5 would be an excellent candidate with a strong potential for future application in the bioremediation of naphthalene-contaminated sites.

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