Characterization and application of a newly isolated pyrene-degrading bacterium, *Pseudomonas monteilii*

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Abstract *Pseudomonas monteilii* PL5 (PL5) was newly isolated from soil sample and was identified by 16S rDNA sequence analysis. The strain PL5 had a high potential to degrade pyrene (PYR) in both liquid solution and soil and was able to degrade 51.8% of PYR at 25 °C and pH 7.0 condition within 10 days. At 25 °C, the ability of strain PL5 to degrade PYR at different pH values followed the following order pH 6.0 > pH 7.0 > pH 8.0 > pH 9.0. Degradation of total PYR was 56.5 and 51.8% after 10 days at pH 6.0 and 7.0 with PYR half-lives of 8.8 and 9.2 days, respectively. The ability of strain PL5 degraded PYR under different temperatures was 35 > 25 > 15 °C at pH 6.0. Among the tested soils contaminated by PYR, the best degradation of PYR by strain PL5 occurred in paddy soil where the degradation was 57.5% after 10 days, and the half-life of PYR was reduced 19-fold in the presence of strain PL5. This study suggested that *P. monteilii* PL5 could be used for the bioremediation of the contaminated soil and water through the degradation of PYR.

Keywords Microbial degradation · Soil · Pyrene · *Pseudomonas monteilii*

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are contaminants of great concern due to their persistence and toxicity (Marusenko et al. 2011). 16 PAH compounds have been recommended as priority pollutants by the United States Environmental Protection Agency (USEPA), seven of which have been placed on the black list of priority pollutants in China. PAHs are derived primarily from anthropogenic activities, such as biomass burning, incomplete combustion of fossil fuels (coal and petroleum), and certain industrial processes (Rogula-Kozłowska et al. 2016). Concentrations of PAHs in environment have gradually increased over the last 150 years (Ortiz et al. 2012; Abdolapur Monikh et al. 2014) with the development and use of petroleum, coal, natural gas and other resources. Due to the toxicity and widespread distribution of PAHs, there is an increasing interest in developing methods to remove PAHs from the environment (Lors et al. 2012; Chen et al. 2015). Soil clean-up can be accomplished using different remediation technologies, where the biological degradation is the primary method of environmental PAH removal. Recent studies have focused on bioremediation as a low-cost tool for the restoration of contaminated soil, due to its effectiveness and overall promise compared to alternative physical and chemical techniques (Shahsavari et al. 2013; Zhu et al. 2016). Commonly, bioremediation refers to the use of microorganisms to transform or degrade a contaminant into harmless product(s) (Antizar-Ladislao 2010; Owabor et al. 2010; Kumar and Maitra 2016). Biodegradation of PAHs has attracted widespread attention, and various bacteria with the ability to degrade individual PAHs have been isolated and identified (Chen et al. 2015; Zafra et al. 2015).

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PAHs with four or more rings are relatively stable and cannot degrade well in the environment. To biodegrade such PAHs efficiently, it is urgent to screen for microorganisms with significant degradation abilities. In the list of 16 USEPA priority control PAHs, pyrene (PYR) with four ring accounts for a large proportion of PAHs in the environment (Kastner et al. 1998; Ping et al. 2007). Though there are several reports about the microbial PYR degradation (Arulazhagan and Vasudevan 2011; Fu et al. 2014; Ping et al. 2017), PYR-degrading microorganisms still need to be further isolated and studied for efficient biodegradation of PYR.

This research was aimed to screen for an efficient PYR-degrading microorganisms and provide an alternative for the biodegradation of PYR in the environment. In this study, PYR-degrading bacteria in long-term hydrocarbon polluted soil were screened. The identified bacterium was analyzed and named *Pseudomonas monteilli* PL5 by its physiological characteristics and 16S rDNA sequence. The characteristics of strain PL5 identified in this study make it potentially useful in degradation of PYR.

Materials and methods

Materials

Pyrene (PYR, purity 92.2%) was purchased from Supelco Corporation (Bellefonte, PA, USA). All other chemicals used were of analytical grade and purchased from J&K Scientific Ltd (Beijing, China). The primers p16S-8 and p16S-1541 were designed and synthesized by Sangon Biotech Company (Shanghai, China) with the respective sequences 5'-aga gtt tga tcc tgg ctc ag-3' and 5'-agg gag gtg atc cag ccg ca-3'. Gel, plasmid, DNA extraction kits, and PCR reagents were provided by Sangon Biotech Company. A 1000-mg L⁻¹ PYR solution was prepared in acetone and kept in a brown bottle at 4 °C to avoid any light exposure prior to use. Isolation of bacterial strains from soil samples and the degradation experiments were described by Ping et al. (2017).

The degradation medium (DM) contained 1.0 g of (NH₄)₂SO₄, 2.0 g of K₂HPO₄, 0.5 g of MgSO₄·7H₂O, 0.1 g of NaCl, 0.5 g of yeast, 0.5 g of CaCO₃, and 1000 mL of distilled water. DM agar plate contained 20.0 g L⁻¹ of agar. The LB medium was composed of 10.0 g NaCl, 10.0 g peptone, 5.0 g yeast extract, and 1000 mL of distilled water. The LB agar plate contained 15.0 g L⁻¹ agar.

Methods

Strain isolation and identification

Screening and isolation of bacterial strains was done according to the methodology and procedures described by Ping et al. (2014). The sequences obtained in this study were compiled and compared with sequences deposited at GenBank using the BLAST program. The sequences were aligned using multiple sequence alignment software (CLUSTAL W 1.81) (Thompson et al. 1994). A phylogenetic tree was constructed using MegAlign software (DNASTAR Inc., Madison, WI, USA) based on the partial 16S rDNA sequences and using similarity as the index. Bootstraps were calculated by 1000× of random sampling to assess the confidence of the phylogenetic tree.

Degradation experiment

Two sets of degradation experiment including a control and a PL5 treatment were incubated at 25 °C with shaking (180 rpm) in darkness. The control consisted of boiled PL5 cells inoculated in liquid with 20 mg L⁻¹ PYR. Two milliliters of PL5 pre-culture (approximately 2.0 × 10⁸ cells mL⁻¹) inoculated into 8 mL of degradation culture with 20 mg L⁻¹ PYR was used as the PL5 treatment. Bacterial cell amounts and PYR concentrations in each sample were analyzed 10 times between the 12th and 240th hour of growth. To investigate the effects of pH on the biodegradation of PYR, degradation solutions were adjusted to different pH values (ranging from 6.0 to 9.0) using 0.1 M HCl or NaOH and were incubated as described above. Cultures were grown at 15, 25, and 35 °C to assess the effects of temperatures on PYR biodegradation. For each sample, 20 g of soil was placed in an Erlenmeyer flask and autoclaved at 121 °C for 20 min. Two sets, including a control (no inoculant) and treatment (10% inoculant), were used to investigate the biodegradation efficiency. The final PYR concentration in the soil samples was 10 mg kg⁻¹ for each treatment. After the acetone was allowed to evaporate at room temperature overnight, the inoculants were added to the soil samples. The soils contained in flasks were placed at 30 °C in a culture incubator in darkness. Soil moisture contents were kept at 65% by the addition of sterile water. Soil samples from each treatment were collected after 0, 12, 24, 36, 48, 60, 72, 96, 144, 192, and 240 h to analyze PYR concentrations. All treatments were performed in triplicate unless otherwise noted.

Detection of PYR from medium and soil

The extraction of residual PYR from the medium was performed as follows. PYR from 10 mL of the degradation

solution was extracted by three additions of 30 mL of dichloromethane. After each addition, the separatory funnel was shaken for 30 s, and then the organic phase was collected and saved. The extracts were dried at 40 °C under reduced pressure and redissolved in acetonitrile. Extraction of PYR from soil was performed according to previously reported method (Ping et al. 2007). The concentrations of PYR in acetonitrile were analyzed by HPLC (Waters Corporation, Milford MA, USA) using a PAH C18 column (4.6 mm × 250 mm, 5-μm particle size; Waters Corporation) under running conditions of 1.0 mL min⁻¹ using acetonitrile–water as the mobile phase (v:v = 7:3). The mobile phase solvents were distilled and passed through a 0.45-μm filter before use. The HPLC detector was set at 250 nm for the excitation wavelength and 385 nm for emission wavelength. For each sample, an injection volume of 20 μL was used. The retention time of PYR was approximately 10 min under these conditions. The PYR extraction and analytical methods were subject to strict quality control procedures. The total recovery of PYR from samples was within the acceptable ranges, with mean recovery values ranging from 96 to 101% in 2.0 and 20.0 mg L⁻¹. The mean recovery value of PYR from the soil samples was 89 to 101% in 1.0 and 10 mg L⁻¹.

The numbers of bacteria were counted using the spread plate technique. For each sample, 1.0 mL of degradation solution was serially diluted in 9 mL of distilled water eight times, and 0.1 mL of the 10⁻⁴, 10⁻⁵, and 10⁻⁶ dilutions was spread on LB plates. The plates were incubated at 30 °C for 24 h to prepare bacterial colonies.

Results and discussion

Identification of isolated strain based on 16S rDNA sequence analysis

For the bioremediation of contaminated environments, one of the most important steps is to screen for dominant microorganisms that efficiently degrade pollutants from long-term polluted environments (Tao et al. 2007; Liang et al. 2014; Wilcke 2000). During the domestication process, the aqueous phase was clarified, and strains with strong degradation abilities were continuously enriched, forming a relatively stable microbial community. In this study, a PYR-degrading strain was isolated using a streak plate separation technique and was named strain PL5. Plates were incubated for 48 h to obtain isolated colonies. Strain PL5 colonies were milky white and had a smooth, wet, and sticky surface with a flat projection. Total DNA was extracted from strain PL5 and used as a template for PCR amplification (Ping et al. 2014; Liu et al. 2014). A 16S rDNA fragment with length of 1.5 kb was amplified

using primers p16S-8 and p16S-1541. The 16S rDNA fragment was ligated into a T-vector and the exact length of the fragment was determined to be 1453 bp by sequencing. The 16S rDNA sequence obtained in this study has been submitted to GenBank database with GenBank accession no. MF538661. Using BLAST analysis, the 16S rDNA sequence of strain PL5 had the highest homology with *Pseudomonas* sp. The phylogenetic analysis (Fig. 1) indicated that strains PL5 and *P. monteilii* CIP (AF064458) were most closely related using the neighbor-joining method. The genetic analysis indicated that the strain PL5 belongs to *P. monteilii* and was named as *P. monteilii* PL5.

Ability to degrade PYR and modeling of biodegradation kinetics

P. monteilii PL5 exhibited a good ability to degrade PYR in the liquid system, as shown in Fig. 2. 51.8% of PYR was degraded by the strain PL5 after 10 days, where the initial conditions were 20 mg L⁻¹ of PYR at 25 °C and pH 7.0. According to the kinetic model (El-Mansi et al. 2007), the first-order kinetic equation is $-dc/dt = Kc$, where c is the concentration, t is the reaction time, and K is the kinetic constant. The PYR degradation reaction of strain PL5 was fitted by a first-order reaction kinetics equation as follow $c = 18.617e^{-0.075t}$ ($R^2 = 0.9308$) with a PYR half-life of 9.2 days. The degradation rate of PYR by strain PL5 was faster from 0 to 4 days than that after 4 days. Possible reasons for this observation include an initial higher concentration of PYR, formation of intermediate metabolites, reduction in bacterial growth nutrients, and changes in environmental conditions (Ping et al. 2014). After being cultivated with 50 mg L⁻¹ of PYR and BaP, strain PL5 could remove 34% of PYR and 30% of BaP after 30 days (Song et al. 2011). After inoculation with mixed PAHs for 7 days, *Stenotrophomonas* sp. P1 degraded 98.0% NAP, 83.1% FLR, 87.8% PHE, 14.4% PYR, and 1.6% BaP, and *Pseudomonas* sp. P3 degraded 95.3% NAP, 87.9% FLR, 90.4% PHE, 6.9% PYR, and negligible BaP (Zhu et al. 2016). After 2 weeks, *Novosphingobium* and *Microbacterium* sp. strains degraded 98 and 71% of the PYR, respectively (Wongwongsee et al. 2013). Ping et al. (2014) showed that strain PL1 could degrade 63.4% of PYR and 55.8% of BaP after 10 days in the presence of 20 mg L⁻¹ PYR and 10 mg L⁻¹ BaP.

Effect of initial pH and culture temperature in liquid matrix

As shown in Fig. 3, the pH order of biodegradation by strain PL5 was pH 6.0 > pH 7.0 > pH 8.0 > pH 9.0, where the degradation was best at pH 6.0–7.0. Degradation rate were 45.8 and 38.3% on the 4th day and 56.5 and

Fig. 1 Phylogenetic tree of strain PL5. The accession numbers of published sequences are given in parentheses. The bootstrap values were based on 1000 iterations

Fig. 2 Ability to degrade PYR by strain PL5 in degradation medium. CK, the control consisted of boiled PL5 cells; PYR, the PL5 treatment

Fig. 3 PYR concentration after degraded by PL5 under different pHs

51.8% on the 10th day, respectively. Degradation kinetic equations are presented in Table 1 with PYR half-lives of 8.8, 9.2, 10.7, and 18.7 days at pH values of 6.0, 7.0, 8.0, and 9.0, respectively. Under the condition of pH 6.0, the temperature order of biodegradation by PL5 was $35 > 25 > 15$ °C after 10 days. As shown in Fig. 4, the PYR degradation rate by PL5 was the highest at 35 °C, and PYR was degraded by 50.7% after 3 days. Kinetic equations for these reactions were $c = 18.998e^{-0.049t}$ ($R^2 = 0.9030$), $c = 17.457e^{-0.079t}$ ($R^2 = 0.8629$) and $c = 16.177e^{-0.088t}$ ($R^2 = 0.8469$) at 15, 25 and 35 °C,

respectively. The shortest half-life of PYR was 7.9 days when incubated at 35 °C.

Environmental conditions have important effects on the growth of microorganisms and their biodegradation functions. Commonly, the reproduction speed of bacteria was faster under higher temperatures because the growth and enzymatic activity of microorganisms in vivo is relatively high at higher temperatures. The optimum temperature for enzymatic activity typically ranges from 35 to 40 °C. Thus, in the optimal temperature range, bacterial reproduction was accelerated and PYR degradation was improved. In

Table 1 Equation of PYR biodegradation kinetics by PL5 in different pHs

	Reaction kinetics equation	Correlation coefficient (R^2)	Half-life ($t_{1/2}$)
pH 6.0	$c = 17.457e^{-0.079t}$	0.8269	8.8
pH 7.0	$c = 18.617e^{-0.075t}$	0.9308	9.2
pH 8.0	$c = 18.929e^{-0.065t}$	0.9191	10.7
pH 9.0	$c = 19.081e^{-0.037t}$	0.9451	18.7

Fig. 4 PYR concentration after degraded by PL5 under different temperatures

weakly acidic or neutral environments, bacterial populations may be significantly increased, whereas under alkaline conditions, bacterial growth is inhibited, which further affects the degradation (Chen et al. 2012). Kastner et al. found that phenanthrene and anthracene degradation by *Sphingomonas paucimobilis* was extremely sensitive to pH of soil (Kastner et al. 1998). *Pseudomonas putida* required a pH of 6.0–7.0 for optimal phenanthrene and PYR degradation (Ping et al. 2011). In comparison, strain PL5 had a better PYR degradation ability and was relatively sensitive to the pH of the environment. Thus, in order to achieve satisfactory results, we can improve the PYR degradation capabilities of strain PL5 by adjusting the nutrients and system conditions.

Biodegradation of PYR in different soils

At pH 7.0 and 30 °C, strain PL5 had different capabilities in degradation of PYR in the three different tested soils (Fig. 5). In the paddy soil, PYR degradation was fastest, with 57.5% of PYR being degraded by strain PL5 after 10 days. In comparison in fluvo-aquic soil, PYR degradation was the lowest, with only 31.2% of PYR being degraded by strain PL5 after 10 days. The results indicated that the ability of strain PL5 to degrade PYR was

Fig. 5 PYR concentration after degraded by PL5 in soils

influenced by soil properties. Soil properties, such as soil texture, organic matter (Tremolada et al. 2012), and cation exchange capacity (CEC), affect the growth and degradation of soil microorganisms. The fluvo-aquic soil had a high fine silt content, poor ventilation, prominent water and gas contradictions, and low CEC, all of which affected the growth of strain PL5. Therefore, the degradation capacity of PL5 in fluvo-aquic is the lowest. A similar pattern of PYR degradation by strain PL5 was observed in all three soils, with PYR degrading rapidly during days 0–4 and was slower from days 4 to 10.

The biodegradation of PYR by strain PL5 in paddy soil was better than that in the liquid medium, which may be due to the nutrient availability and environmental conditions in paddy soil being conducive to strain growth, thereby improving the degradation capacity. In the process of degrading high molecular weight PAHs, microorganisms can secrete certain enzymes to start the degradation process that perform typical catalytic reactions (Ping et al. 2014, 2017). According to the kinetic model (El-Mansi et al. 2007), strain PL5-mediated degradation of PYR in paddy soil exhibited first-order kinetics ($c = 17.372e^{-0.0797t}$, $R^2 = 0.9021$) in comparison with sterilized paddy soil without PL5 ($c = 19.621e^{-0.0045t}$, $R^2 = 0.5122$). The half-life of PYR in paddy soil with strain PL5 was 8.7 days, compared to 154.3 days in soil without strain PL5 such that

the PYR half-life was shortened 19-fold in the presence of strain PL5.

There are many other reports regarding the degradation of PYR in soils. The degradation rate of PYR in soil was 56% by the fungi *Basidiomycetes* sp. (Anastasi et al. 2009) less than 40% in 10 days by *Mycobacterium* PYR-1 in soil sludge (Ramirez et al. 2001). After a 90-day incubation, the percentage of remaining PYR was 51.9% in unsterilized soil without microorganisms but the degradation rates increased to 94.9 and 90.6% when inoculated with *Phanerochaete chrysosporium* (Chen and Ding 2012). In soils contaminated with 1000 mg kg⁻¹ of a three-PAH mixture, *Trichoderma asperellum* H15 was shown to degrade 74% of phenanthrene, 63% of PYR, and 81% of BaP after 14 days (Zafra et al. 2015). In comparison, strain PL5 had better ability in degradation of PYR in soil.

The degradation of PYR by PL5 in paddy soil was better than in fluvo-aquic and red soils, because there are different in characteristics of soils. The results indicated that by adjusting environmental conditions, the degradation ability of PL5 could be improved, which can be applied to environments with actual pollution. The results of this study and previous reports are mostly confined to the laboratory; however, the actual remediation of contaminated soils by microorganisms is facing many problems. Microorganisms, particularly bacteria, are extremely sensitive to ambient temperature, pH, and pollutants. In the degradation process, toxic intermediate metabolites are often produced and accumulated, and more environmental pollutants exist in the residual binding state, all of which affect microbial degradation. To solve these problems, microbial mutation breeding, construction of high-efficiency degrading microorganisms (a multi-enzyme coupling), and immobilized microbial technology should be studied. Moreover, biological bacterial fertilizers could be used for microbial remediation of pollution in the environment.

Conclusions

PYR-degrading bacterium PL5 was isolated from hydrocarbon-contaminated soil and could use PYR as the carbon source. Strain PL5 was further identified as one strain of *Pseudomonas monteilii*. The PYR degradation rate was 51.8% by the strain PL5 after 10 days with an initial concentration of 20 mg L⁻¹ PYR at 25 °C and pH 7.0. The ability of PL5 to degrade PYR at different pH values was as follows pH 6.0 > pH 7.0 > pH 8.0 > pH 9.0. The PYR-degrading ability of the strain under different temperature conditions was 35 > 25 > 15 °C. Regulation of environmental conditions can improve the degradation ability of strain PL5. In paddy soil, PYR degradation was fastest,

with 57.5% of PYR being degraded by PL5 in 10 days. However, in fluvo-aquic soil, only 31.2% of PYR was degraded by PL5 in 10 days. The half-life of PYR was shortened 19-fold in paddy soil through PL5-mediated degradation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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