Supplementary Materials for

A revisit on the enhancing effect of Tween-80 on the bioremediation of PAH-contaminated soil with a surfactant-compatible strain

Likun Zhao [a], Rui Yue [a,b], Hua Li [a], Shiyu Bao [a], Chaoqi Chen [a,*], Kaihong Chen [a], Jin Qi [a], Xuhui Mao [a,*]

[a] School of Resource and Environmental Sciences, Hubei International Scientific and Technological Cooperation Base of Sustainable Resource and Energy, Wuhan University, Wuhan 430079, China

[b] No. 208 Hydrogeological and Engineering Geological Team, Chongqing Bureau of Geological and Mineral Resource Exploration and Development, Chongqing, 400700, China

* Corresponding Authors: clab@whu.edu.cn (X. Mao); chenchaoqi@whu.edu.cn (C. Chen)

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Text S1. The preparation of MSM and double-layer agar plates.

The mineral salt medium (MSM) was prepared with KH_2PO_4 (1.7 g/L), $K_2HPO_4 \cdot 3H_2O$ (5.6 g/L), NH_4Cl (1.0 g/L), NaCl (0.5 g/L), $MgSO_4$ (100 mg/L), $MnSO_4 \cdot H_2O$ (20 mg/L), $FeSO_4 \cdot 7H_2O$ (10 mg/L), and $CaCI_2$ (15 mg/L).

The overlayer and underlayer of agar plates were prepared separately. MSM containing 1% agar and 1.5% agar was sterilized at 121°C for 20 min, respectively. The MSM containing 1.5% agar was poured onto sterilized plates (diameter of 90 mm) to form the underlayer. In addition, 0.2 mL of acetone containing 8 mg/mL of Pyr was added to MSM containing 1% agar. The mixture was set for 5-10 min to allow the volatilization of acetone and poured onto the underlayer to form the overlayer. The final concentration of Pyr in the overlayer of the agar plates was 400 mg/L.

Text S2. The procedure for the inoculation of strain DH-6.

Before inoculation, the strain DH-6 was enriched in LB broth and collected in the logarithmic phase. The cells were harvested by centrifuging at 5000 rpm for 5 min, washed three times, and resuspended using MSM solution. The cell density was adjusted to an optical density of 1.0 at a wavelength of 600 nm (OD_{600}). The suspension was then incubated at 30°C for 2 hours before being added to the slurry.

Text S3. Extraction of PAHs in liquid and solid phases.

The extraction method for PAHs in the liquid phase is as follows: briefly, 300 μL of the sample was mixed with 900 μL n-hexane in a 2 mL centrifuge tube, shaken vigorously for 10 min, and centrifuged at 12000 rpm for 10 min at 37°C. Approximately 400 μL of supernatant was filtered to the analytical vial, and measured by high-performance liquid chromatography (HPLC).

The extraction method for PAHs in the solid phase is as follows: firstly, take a 2 g

soil sample and add 10 mL n-hexane solution. Vortex mix the mixture for 15 min and perform ultrasonic extraction for 30 min. Then, centrifuge the mixture at 5000 rpm for 10 min and collect the extraction solution. Repeat the extraction process once and combine the two extraction solutions. Add a suitable quantity of anhydrous sodium sulfate and copper powder, vortex mix for 5 min, then nitrogen blow-off to a volume of 0.5 mL, and adjust the volume to 1 mL with n-hexane. Next, purify the sample using a column filled with magnesium silicate to remove impurities. Elute the column with 10 mL of elution solvent (dichloromethane: n-hexane = 1:1, v/v), collect the eluate, and then evaporate the solvent using a stream of nitrogen gas until the volume reduces to 0.5 mL. Finally, adjust the volume to 1 mL with n-hexane, and measured by HPLC.

Text S4. Conditions of the HPLC system.

Conditions of high-performance liquid chromatography (HPLC, Shimadzu) system were as follows: C18 column (4.6 ×150 mm), column temperature (35^{o}C), flow rate (1 mL/min), mobile phase (water: methanol = 10:90), injection volume (20 μL), and detection wavelength (254 nm). All established standard curves had R^2>0.999. The concentrations of PAHs were determined from peak areas at corresponding retention times.

Text S5. Testing steps for determining the contribution of biosorption

The total removal efficiency of PAHs by strain DH-6 is composed of decay, biodegradation and biosorption. Firstly, the sample collected at "t" time was divided into two parts. The one part was centrifuged at 5000 rpm for 5 minutes, and the PAHs content in the supernatant was tested as C_{t1}. The other part was sonicated for 10 minutes in an ice bath to ensure the lysis of DH-6 cells, and the PAHs content was tested as C_{t2}. Secondly, the total removal efficiency (Φ) of PAHs was calculated using the equation $\Phi=(1 - C_{t1}/C_0)$

× 100%, where C_0 is the initial concentration of PAHs. Finally, the biosorption efficiency (Φ_a) of PAHs was calculated using the equation $\Phi_a=\Phi - (1 - C_{t2}/C_0) \times 100\%$.

Text S6. Quantitative PCR analysis of functional genes.

The strain DH-6 was cultured in the negative control medium (MSM with 10 g/L Tween-80) and the experimental medium (MSM with 10 g/L Tween-80 and 100 mg/L Pyr), respectively. The sampling time of RNA extraction was designed according to degradation curves of Pyr in each of treatments. The RNA was sequenced by on Illumina sequencing platform using Next-Generation Sequencing (NGS). The rpsL was regarded as a reference gene. The fold change in gene expression was calculated by $2^{-\Delta\Delta Ct}$. The program primers of each genes are listed below.

pcaG-F: GCGGCATCAACATCCACTTG.

pcaG-R: AAACGGTAGGCGAGCTGTC.

pcaH-F: GACCTGCTGCTCAACTTCG.

pcaH-R: AGGTAGCGGTCGTTCTTGTG.

ligA-F: CGCTTCATCTTCGCTCTGG.

ligA-R: CGGCAGGTAGGTCAACACT.

flnD2-F: CTCCAGAACCGCAACAACCT.

flnD2-R: AGCATCGCAGCACCTTCAG.

phtAb-F: GTGACCAACGCGATTTCCG.

phtAb-R: GAAGCCACCGAACACATTGAC.

mobA-F: CCGCAACCAGGAACGCTAT.

mobA-R: AGCAGGGTTTCCAGCAAGG.

rpsL-F: GCAAGCGCATGGTCGACAAGA.

rpsL-R: CGCTGTGCTCTTGCAGGTTGTGA.

Text S7. Identification of intermediates of PAHs.

The degradation intermediates of PAHs were extracted using liquid-liquid extraction. Briefly, 20 mL of samples were adjusted to pH＜2. The samples were then sonicated for 10 minutes in an ice bath to ensure the lysis of DH-6 cells. Samples were mixed with 20 mL ethyl acetate. The extraction was repeated and the extracts were dewatered using sodium sulfate anhydrous. The extracts were further concentrated by nitrogen sweeping.

The extract was analyzed using a Liquid Chromatography High Resolution Mass Spectrometer (LCHRMS, a shimadzu prominence UFLC-20A system (Shimadzu Corporation, Kyoto, Japan) coupled with a quadrupole time-of-flight mass spectrometer (Ab sciex Triple QTOF5600+, AB Sciex, Redwood, CA, USA)) and a Gas Chromatography-Mass Spectrometry (GC-MS, Agilent & GC-7890A; MS-5975C, USA).

For LCHRMS, the chromatographic column was Hypersil GOLD C18 column (2.1 × 100 mm, 3 μm pore size, Thermo). The mobile phase consisted of water and acetonitrile, and the flow rate was set at 0.3 mL/min. The injection volume was 10 μL. The mass spectrometer includes an electrospray ionization (ESI) source. The Ion Source Gas1 was set at 55 psi, Ion Source Gas2 at 60 psi, and the Curtain Gas at 30 psi. The temperature of the system was maintained at 550°C. The IonSpray Voltage was set to float between -4500V and 5500V. The Declustering Potential was set at 80V, and the Collision Energy was set at 35V with a Collision Energy Spread of 15V. The analysis was performed in the m/z range of 100 to 1000. The analysis was performed in the negative ion mode using Time-of-Flight Mass Spectrometry (TOF–MS), and ten TOF–MS/MS data sets were collected. The data acquisition was carried out using the information-dependent acquisition (IDA) function.

For GC-MS, the extracts were incubated with an equal volume of 99% BSTFA + 1%

TMCS before injection. The chromatographic column used was HP-5MS (30.0m×250μm, 0.25μm). The initial temperature was set at 60ºC for 3 minutes, then ramped at a rate of 20ºC /min to 180ºC and held for 5 minutes, followed by a ramp of 10ºC/min to 300ºC and held for 5 minutes. The injector temperature was 280ºC. The transfer line temperature was 300. Helium was used as the carrier gas with a flow rate of 1.0 mL/min. Splitless injection mode was employed, and the injection volume was 1 μL. The mass spectrometry includes an electron ionization (EI) source with an electron energy of 70 eV. The ion source temperature was 230ºC and the quadrupole temperature was 150ºC. The scan mode was used with a scan range for mass ranging from 35 to 600.

Text S8. Test of seed germination and plant growth.

Briefly, 100 g of freeze-dried soil was added to each plate (diameter of 90 mm). In each plate, 30 wheat seeds (Triticum aestivum L., obtained from the Beijing Academy of Agriculture and Forestry Sciences, China.) were sowed. The plates were incubated in the dark/light cycle (14/10 h) at 25 ± 1ºC. The moisture was maintained at 60% by watering every day. In addition, the untreated soil was used as a control. All experiments were conducted in replicates. The numbers of germinated seeds on day 1, day 3, and day 5 were counted manually, respectively. On day 5, the soil was separated from plants by soaking in sterilized water for 5 min. The stem and roots were carefully separated using a sterilized scissor. The stem length and root length were measured manually. The stem and roots were oven-dried at 105ºC to constant weight before being weighed.

Text S9. Fenton oxidation of PAHs in the contaminated field soil.

The Fenton oxidation of PAHs was also examined in a slurry bioreactor. In the reactor, 0.1 kg of the contaminated field soil was mixed with 0.9 L of MSM (with 10 g/L Tween-80). Subsequently, a pre-reaction was conducted for 1 hour at 30ºC and 200 rpm

176 to enhance the solubility of PAHs in the solution. In the slurry reactor, solutions of ferrous
177 sulfate ($FeSO_4$) and hydrogen peroxide (H_2O_2) were separately added to ensure that the
178 concentrations of Fe^{2+} and H_2O_2 in the mixed solution were 20 mmol/L and 100 mmol/L,
179 respectively. The oxidation process was conducted at a temperature of 30°C, and a stirring
180 rate of 200 rpm for 24 hours. After oxidation, the pH and the concentrations of PAHs in
181 the soil were tested. The results indicated that after oxidative degradation, the pH of soil
182 was 6.49, and the removal efficiency of PAHs was 40.6%. The soil samples were freeze-
183 dried for wheat planting.

184 **Table S1.** Physicochemical properties of the spiked soil and the PAH-contaminated field soil*.

Parameters	Spiked soil	Contaminated field soil
Moisture (%)	2.30	5.20
pH	8.82 ± 0.02	7.08 ± 0.01
Conductivity (mS/m)	9.0 ± 0.1	22.8 ± 0.1
Organic matter (g/kg)	2.4 ± 0.1	10.3 ± 0.11
Total nitrogen (mg/kg)	260 ± 10	260 ± 10
Total phosphorus (mg/kg)	582 ± 3	544 ± 3
Sand particles, 0.05–2 mm (%)	32.4	25.2
Powder particles, 0.002–0.05 mm (%)	66.5	55.0
Clay particles, ≤0.002 mm (%)	1.10	15.2

185 *The contaminated field soil was sampled from an abandoned coking plant site located in the Anhui
186 Prov., China.

187 **Table S2.** Initial concentrations of PAHs and dosage of Tween-80 in liquid medium.

PAHs	Spiked concentrations of PAHs (mg/L)	Dosage of Tween-80 (g/L)
Naphthalene(Nap)	100	5
Phenanthrene (Phe)	100	5
Fluoranthene (Flu)	100	10
Pyrene (Pyr)	100	10
Benzo[a]anthracene (BaA)	100	15
Benzo[b]fluoranthene (BbF)	100	15
Benzo[b]pyrene (BaP)	10	1.5
Dibenz[a,h]anthracene (DBahA)	10	1.5

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189 **Table S3** Initial concentrations of PAHs in the contaminated field soil*.

PAHs		Concentrations (mg/kg)	
		Soil A	**Soil B**
LMW-PAHs	Naphthalene (Nap)	0.65	0.00
	Phenanthrene (Phe)	0.05	0.20
	Anthracene (Ant)	1.29	0.00
	Other LMW-PAHs	3.16	0.00
HMW-PAHs	Fluoranthene (Flu)	8.59	0.40
	Pyrene (Pyr)	8.21	0.30
	Benzo[a]anthracene (BaA)	10.35	15.30
	Benzo[b]fluoranthene (BbF)	7.06	22.60
	Benzo[a]pyrene (BaP)	2.25	7.20
	Dibenz[a,h]anthracene	3.86	0.00
	Other HMW-PAHs	12.04	1.70
	ΣPAHs	57.51	47.70

190 *Soil A was sampled from an abandoned coking plant site located in Anhui, China, and was used for
191 the lab-scale slurry bioreactor experiments. Soil B was sampled from an abandoned coking plant site
192 located in Tianjin, China, and was used for the pilot-scale slurry bioreactors experiments.

193 **Table S4.** Detected intermediates of PAHs in the bioremediation process.

Substrate	Possible metabolites	Formula	Mass	Detection Methods
Naphthalene (Nap)	Salicylic Acid	$C_7H_6O_3$	138.03	LCHRMS
Phenanthrene (Phe)	3,4-Dihydroxybenzoic Acid	$C_7H_6O_4$	154.03	GC-MS
	Salicylic Acid	$C_7H_6O_3$	138.03	LCHRMS
Fluoranthene (Flu)	Salicylic Acid	$C_7H_6O_3$	138.03	LCHRMS
Pyrene (Pyr)	Catechol	$C_6H_6O_2$	110.04	GC-MS
	Salicylic Acid	$C_7H_6O_3$	138.03	LCHRMS
	3,4-Dihydroxybenzoic Acid	$C_7H_6O_4$	154.03	LCHRMS
Benzo[a]anthracene (BaA)	Catechol	$C_6H_6O_2$	110.04	GC-MS
	Salicylic Acid	$C_7H_6O_3$	138.03	LCHRMS
	3,4-Dihydroxybenzoic Acid	$C_7H_6O_4$	154.03	LCHRMS
Benzo[a]pyrene (BaP)	Salicylic Acid	$C_7H_6O_3$	138.03	LCHRMS

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Table S5. Comparison of this study with the literatures concerning bioremediation of PAHs in liquid media with Tween-80.

Genus name	Concentration of Tween-80	Concentration of PAHs	Removal efficiency of PAHs	References
Sphingomonas sp.	393 mg/L (0.3 mM)	50 mg/L Ant	89.6% of Ant on 96 hours.	(Al Farraj et al., 2021)
Klebsiella oxytoca	13.1 mg/L	0.12 mg/L Pyr	95% of Pyr on day 18.	(Zhang and Zhu, 2012)
Pseudomonas aeruginosa	160 mg/L	100 mg/L Pyr	98.9% of Pyr on 168 hours.	(Ghosh and Mukherji, 2016)
Burkholderia cepacia	500 mg/L	100 mg/L Pyr.	50% of Pyr on 80 hours.	(Chen et al., 2013)
Phanerochaete sordida	2 g/L	50 mg/L BaP	53.3% of BaP on day 32.	(Li et al., 2023)
Pseudomonas aeruginosa	256 mg/L	50 mg/L BaP	79.7% of BaP on day 25.	(Meng et al., 2019)
Pseudomonas aeruginosa DH-6	0.5-50 g/L	100 mg/L of Nap, Phe, Flu and Pyr; 10 mg/L of BaP and DBahA, respectively.	78.5-99.3% for each PAH on day 7.	**This study**

Table S6. Comparison of this study with the literatures concerning the bioremediation of PAH-contaminated field soil with Tween-80.

Genus name	Soil type	Concentration of Tween-80	Liquid /soil ratio	Concentration of PAHs	Extraction of PAHs	Removal efficiency of PAHs	References
Klebsiella sp.	Spiked soil	25 g/kg	10:1 v/w	200 mg/kg of Phe, Fla, and Pyr, respectively.	56.4 - 82.7% for each PAHs.	89.1% of Phe, 81.3% of Fla, and 78.3% of Pyr on day 30.	(Xu et al., 2019)
Bacterial consortium	Spiked soil	150 g/kg	30:1 v/w	500 mg/kg of Phe.	93.7% of Phe	74.4% of Phe on day 7.	(Gharibzadeh et al., 2016)
Lasiodiplodia theobromae	Spiked soil	5 g/kg	10:1 v/w	50 mg/kg BaP.	--	32.1 % of BaP on day 35.	(Wang et al., 2014)
Lasiodiplodia theobromae	Field soil	5 g/kg	10:1 v/w	91.4 mg/kg of 16 US EPA PAHs.	--	73.2 % of ΣPAHs on day 30.	(Wang et al., 2014)
Mycobacterium sp.	Field soil	25-50 g/kg	10:1 v/w	258 mg/kg of 16 US EPA PAHs.	9.1% of ΣPAHs	62.4 % of ΣPAHs on day 30.	(Gong et al., 2015)
Pseudomonas aeruginosa	Field soil	25 g/kg	2:1 v/w	57.51 mg/kg of 16 US EPA PAHs.	63.6% of ΣPAHs	88.1% of ΣPAHs, 80.5 - 99.4% of HMW-PAHs on day 14.	**This study**

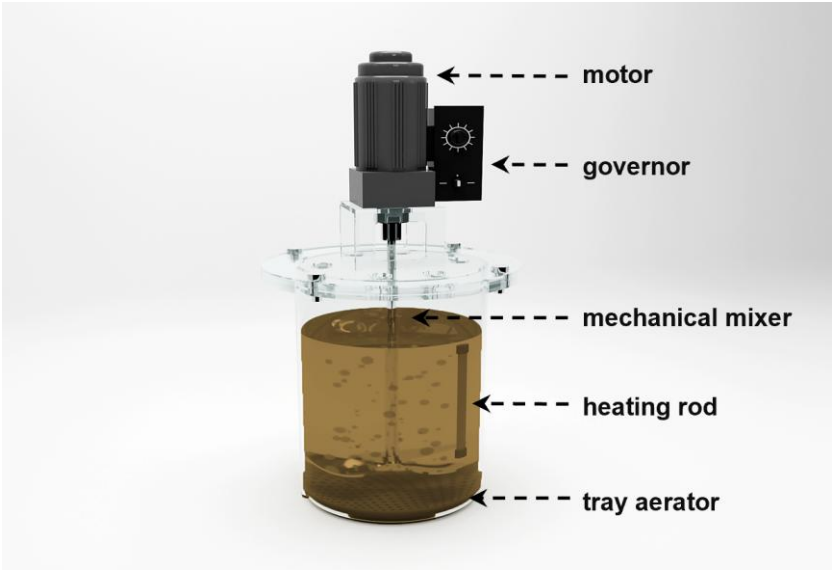


Fig. S1. The lab-scale slurry bioreactor used in this study. The bioreactor includes a mechanical mixer with impeller-type blades, a motor, a governor, a heating rod, and a tray aerator located at the bottom.

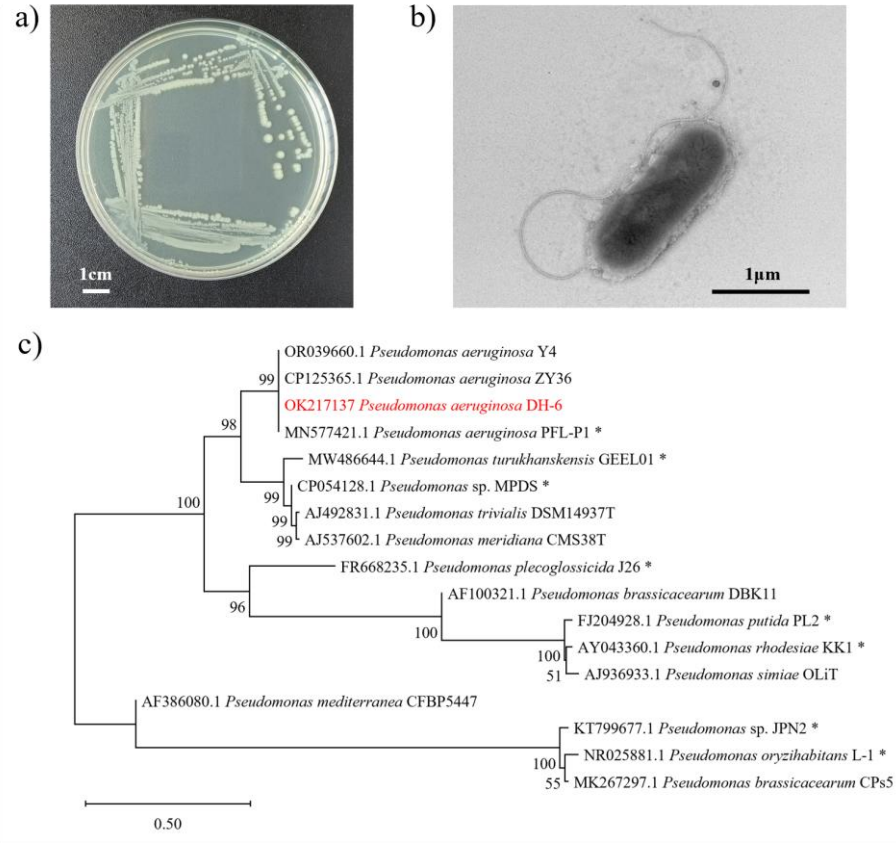
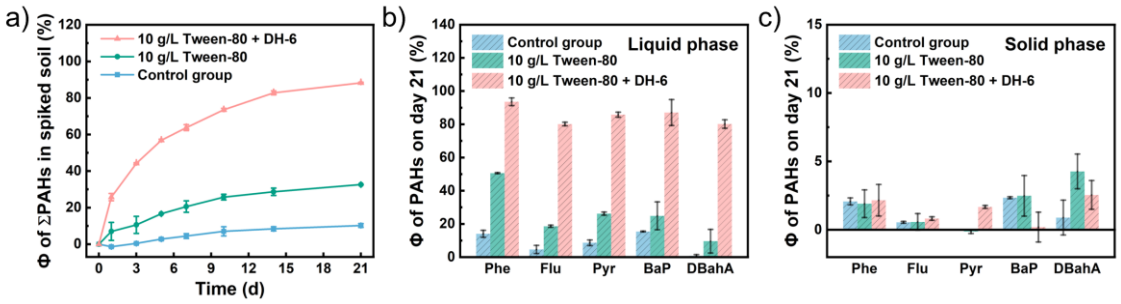


Fig. S2. (a) Appearance of the formed colonies, (b) TEM image of strain DH-6, and (c) phylogenetic relationships of strain DH-6. Pseudomonas isolates known to degrade PAHs are indicated with an asterisk (*).



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208 **Fig. S3.** Removal efficiency of (a) ΣPAHs and five spiked PAHs in (b) liquid phase and (c) solid phase
209 on day 21. The initial concentration of ΣPAHs in the spiked soil was set at 200 mg/kg, including 60
210 mg/kg of Phe, 60 mg/kg of Flu, 60 mg/kg of Pyr, 10 mg/kg of BaP, and 10 mg/kg of DBahA. The
211 water-soil mass ratio of the slurry bioreactor was maintained at 2:1, with 10 g/L Tween-80 added to
212 the liquid phase. The symbol "Φ" represents the removal efficiency.

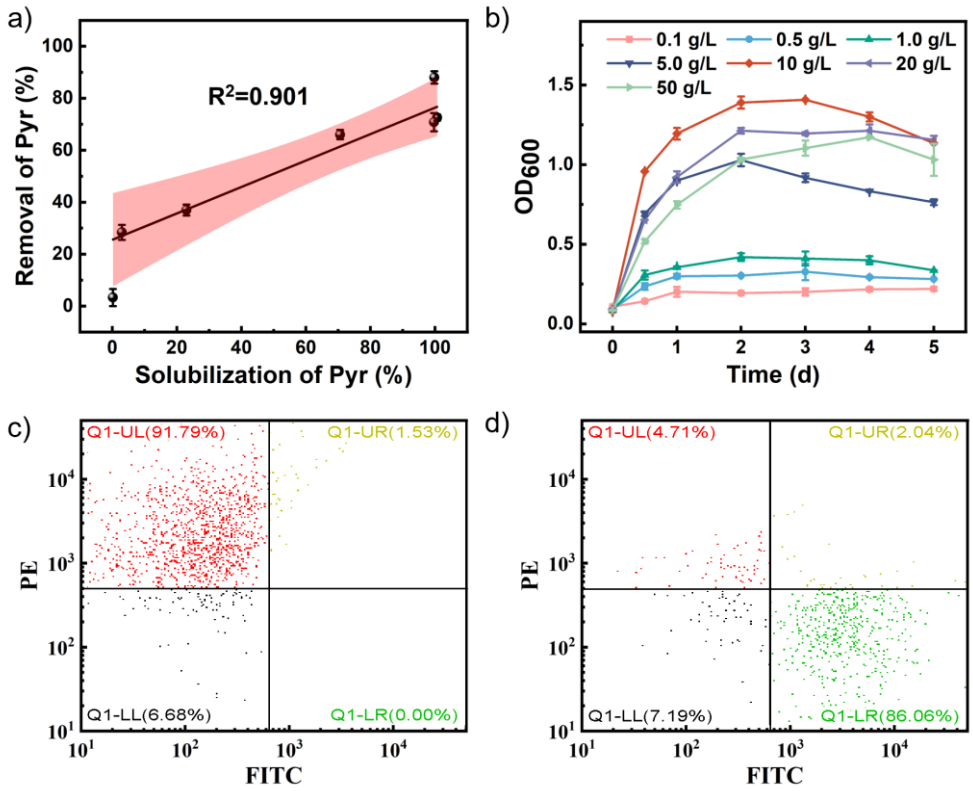


Fig. S4. (a) Linear relationship between the solubilization of Pyr and its removal efficiency. (b) OD_{600} of strain DH-6 in the MSM supplemented with 100 mg/L of Pyr and different concentrations of Tween-80. Flow cytometry (FCM) analysis of strain DH-6 in PS (c) and PT (d) during the logarithmic growth phase (36 h). PS: MSM supplemented with Pyr as the sole carbon source. PT: MSM supplemented with Pyr and 10 g/L Tween-80. STYO9/PI fluorescence dyes were used to differentiate between live and dead bacteria. Q1-LR represents live bacteria, Q1-UL represents dead bacteria, Q1-LL represents impurities, and Q1-UR represents damaged bacteria.

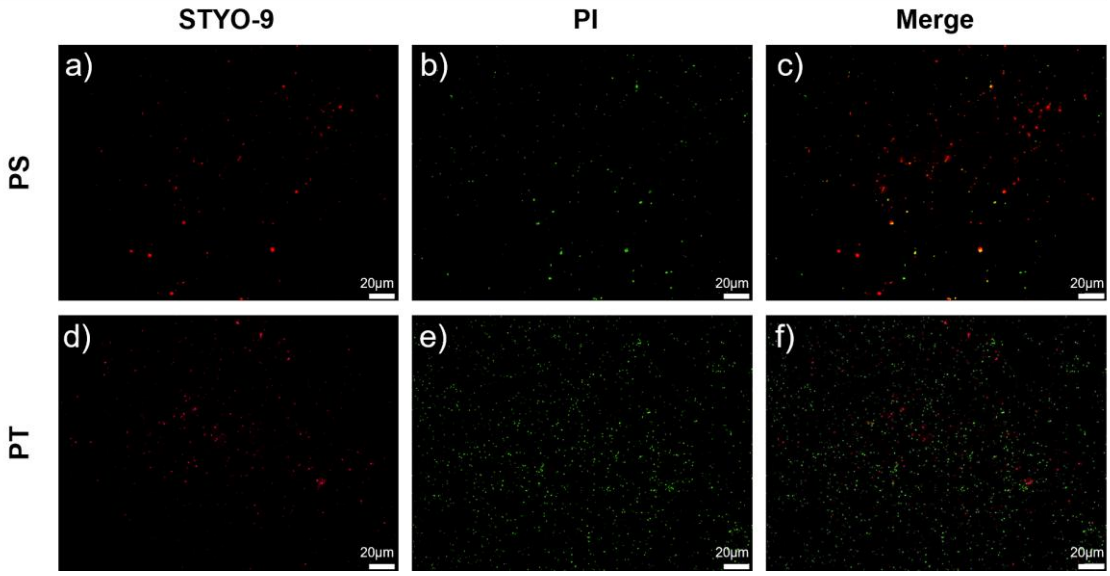
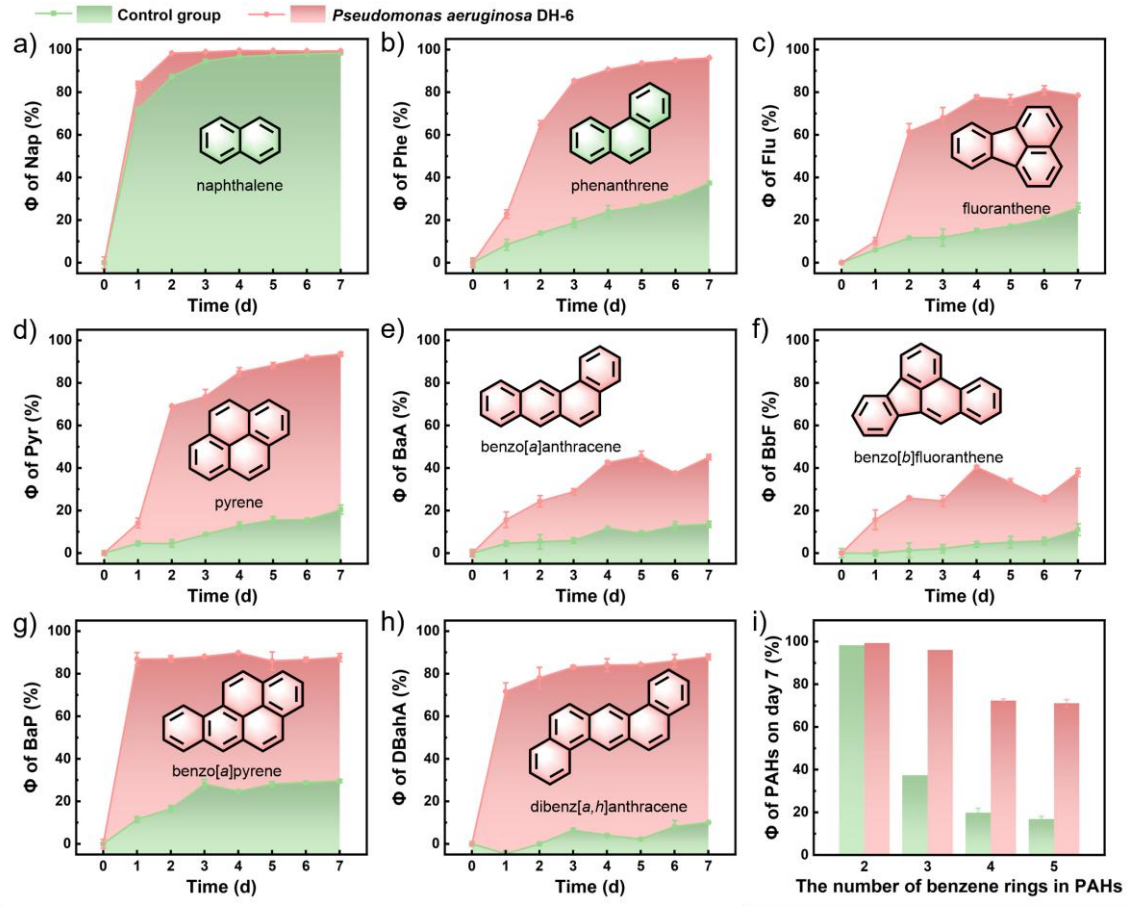


Fig. S5. Results of the microbial activity analysis on strain DH-6 in PS (a-c) and PT (d-f) during the logarithmic growth phase (36 h). PS: MSM supplemented with Pyr as the sole carbon source. PT: MSM supplemented with Pyr and 10 g/L Tween-80. Visualized using SYTO-9 to stain live biofilm cells green and PI to stain dead cells red, were examined by fluorescence microscopy.



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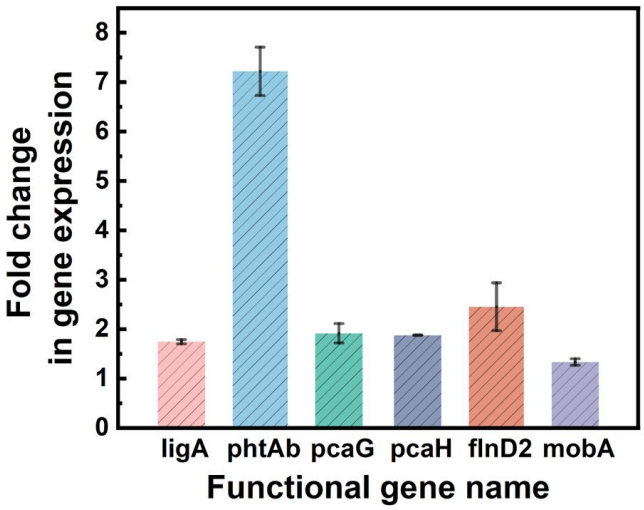
227 **Fig. S6.** Removal efficiency of (a) Nap, (b) Phe, (c) Flu, (d) Pyr, (e) BaA, (f) BbF, (g) BaP, (h) DBahA

228 in the MSM treated with DH-6, and (i) average removal efficiencies of PAHs on day 7. The symbol

229 "Φ" represents removal efficiency.



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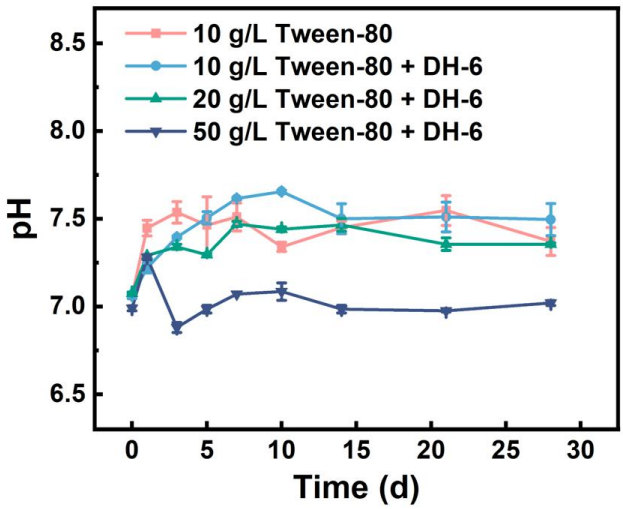
231 **Fig. S7.** QPCR of functional genes related to degrading PAHs in strain DH-6. Experience group: MSM

232 supplemented with 10 g/L Tween-80 and 100 mg/L Pyr; control group: MSM supplemented with 10

233 g/L Tween-80.



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235 **Fig. S8.** pH variations for the contaminated field soils that were subjected to different experimental

236 treatments.

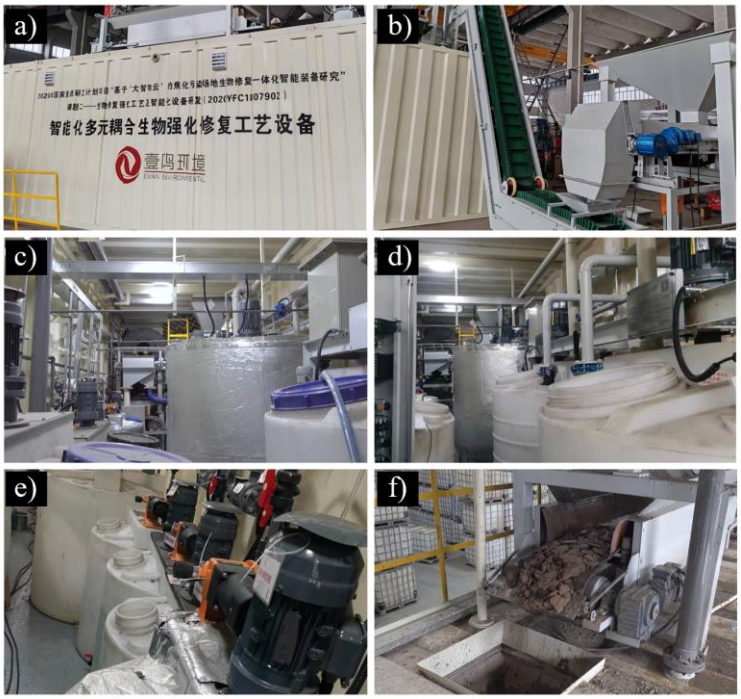

Fig. S9. The pilot-scale equipment for the experiments on contaminated field soil. (a) Container of the pilot-scale equipment. (b) In-feed conveyor. (c) Slurry bioreactor. (d) Reactor for oxidation. (e) Microbial agent barrels and chemicals barrels. (f) Out-feed conveyor.

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