Article

Construction and Degradation Performance Study of Polycyclic Aromatic Hydrocarbons (PAHs) Degrading Bacterium Consortium

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Abstract: PAHs are widely distributed in the environment and pose a serious threat to ecological security and human health. The P&A (*Pseudomonas aeruginosa* and *Alcaligenes faecalis*) bacterium consortium obtained in this study comes from oily sludge and is reused for the degradation of PAHs mixture in oily sludge. Few articles pay attention to the PAHs mixture in oily sludge and reuse the bacterium consortium for its degradation. The PAHs solution degradation efficient of P&A bacterial consortium under different environmental conditions, bioaugmentations, and exogenous stimulations were studied by ultraviolet visible spectrophotometer and gas chromatography–mass spectrometry. The result shows that, after 8 days of degradation under 35 °C, pH 7, with 5% (volume percent) of the inoculation amount, the degradation rate of NAP, PHE, and PYR solution could higher than 90%, 80%, and 70%, respectively. The additional crude oil could further improve the NAP, PHE, and PYR degradation efficiency. The minimum inhibitory concentration of Cu²⁺, Zn²⁺, and Pb²⁺ to bacterium were 2.002, 17.388, and 9.435 mM, respectively. The addition of surfactants had negative or limited positive effect on the PAHs degradation rate. Furthermore, the average degradation rates of NAP, PHE, and PYR, in oily sludge of local petroleum polluted area by P&A bacterial consortium, could all reach above 80%. Based on gas chromatography–mass spectrometry test results before and after incubation, P&A bacterial consortium also provides more opportunities for other organic compounds' degradation.

Keywords: PAHs degradation; bacterial consortium; *P. aeruginosa* and *A. faecalis*; oily sludge

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1. Introduction

PAHs, which are formed by direct connection, bending connection, or aggregation of multiple benzene rings [1–3], mainly occur during incomplete combustion [4] of fossil fuel, such as coal, oil, and gas, waste incineration sites, or the formation of crude oil [5,6], and are a class of compounds with genotoxicity, carcinogenicity, mutagenicity, and teratogenicity [7,8]. Studies have shown that PAHs and their derivatives could pose a threat to almost all organisms. PAHs have long-term health effects even at low levels, such as cataracts, kidney and liver damage, and jaundice, because they are persistent in the environment. They are highly lipophilic and readily adsorb onto particles in both water and aerosols, indicating that exposure to PAHs can occur through inhalation of atmospheric particles, ingestion of contaminated particles (e.g., soil), or dermal contact with contaminated material (e.g., soil, sediment, and water) [9–15]. In the early 1980s, the United States Environmental Protection

Agency (EPA) listed 16 PAHs as environmental priority monitoring pollutants [16,17]. The European Commission and China also identified PAHs as priority hazardous substances causing environmental and human health concerns [18].

Because of rigid aromatic structure, high partition coefficient, and high resonance energy, this type of persistent organic compounds easily adsorbs on organic substances above the soil or sediment particle. Moreover, PAHs exist in crude oil and incomplete combustion products of oil, so oil sludge contains a large number of PAHs. The low fluidity, high emulsification, and complex components (e.g., residual oils, benzene series, aromatic hydrocarbons, polychlorinated biphenyls, asphaltene, colloids, bacteria, and toxic heavy metal elements) of oil sludge also increase the difficulty of PAHs degradation. Therefore, PAHs in oily sludge has become one of the difficulties and hotspots of its harmless.

Although PAHs in oily sludge can be degraded or eliminated by volatilization, ultraviolet photolysis, or chemical oxidation [19,20], biodegradation technology [21,22] has the incomparable superiority compared to the traditional method with cost-effectiveness and non-invasion advantages. Natural terrestrial plants in nature, tall fescue, willow, reed, and sudangrass [23–27] can promote the decomposition of PAHs through the rhizo-degradation effect. In addition, various forms of bacterium that can survive in oily sludge (*Pseudomonas*, *Bacillus*, and *Acinetobacter*) and fungus (*White rot fungi*) [28–31] can be isolated and cultured and then reused for the degradation of PAHs in oily sludge, which can not only quickly adapt to the complex degradation environment but also contribute important roles in the aspect of PAHs' biodegradation.

Furthermore, many studies have noticed that the bacterial consortium promoted better organics degradation effects from individual bacteria [32]. The possible reason is that, in the process of bacterial consortium degradation, since the intermediate biotransformation product of one bacteria can be used as the basis for the catabolism and growth of others, a better degradation efficiency of mixed PAHs could be achieved by bacterial consortium [33]. Based on strong synergistic metabolism, the response to complex environment, and degradability to multiple pollutants [34] advantages, bacterial consortium technology has become the first option for PAHs and heterocyclic derivatives degradation method in recent years.

The research focused on the PAHs mixture of oily sludge, the bacteria in oily sludge were used to construct the bacterial consortium and reused for the degradation of PAHs mixture in oily sludge. In this study, 10 bacteria isolated from oily sludge were used to catabolize bicyclic NAP (naphthalene), tricyclic PHE (phenanthrene) and tetracyclic PYR (pyrene). The single variable method was used to screen and construct, optimized and improve the P&A bacterial consortium activity. During the experiment, UV-vis (ultraviolet visible) spectrophotometer and GC-MS (gas chromatography-mass spectrometry) were used to detect the residual amount of NAP, PHE, and PYR in solution. Moreover, GC-MS and qualitative analysis software were also used to identify the types of organic compounds in oily sludge. The P&A bacterial consortium obtained in this study can not only effectively degrade NAP, PHE, and PYR but also degrade Acenaphthylene, 1H-Phenylene, 4H-Cyclopenta[d,e,f]phenanthrene, Fluoranthene, Triphenylene, 9H-Cyclopenta[a]pyrene, and Benzo[k]fluoranthene. This work could greatly increase the application range of bacterial consortium and is promising in the degradation of PAHs contents' oily sludge in a petroleum-polluted area.

2. Experimental Methods and Materials

2.1. Materials

The NAP (naphthalene, analytical pure), PHE (phenanthrene 97 wt %), and PYR (pyrene 97 wt %) were purchased from local store. The crude oil (70.26% saturated hydrocarbon, 12.54% aromatic hydrocarbon, 7.38% asphaltene, and 9.82% gum) and oil sludge (oil content 5.62%, 0.2090 mg/g NAP, 3.1116 mg/g PHE, 1.00595 mg/g PYR, and organic compounds are mainly aromatic hydrocarbons) were collected from a local oil-polluted area in Shaanxi Province, China.

GC-MS (7890B-5977B, Agilent Technologies Inc., Palo Alto, CA, USA), autoclave (LDZX-30KBS, Shanghai Shen'an Medical Instrument Factory, Shanghai, China), vortex turbidimeter (Vortex-2, Shanghai Hushi Industrial Co., Ltd., Shanghai, China), ultrasonic cleaner (KQ-100DE, Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, China), bed temperature incubator (GTCS-2011, Changzhou Zhengrong Instrument Co., Ltd., Changzhou, China), biochemical incubator (SPX-250BIII, Tianjin taist Instrument Co., Ltd., Tianjin, China), UV-vis spectrophotometer (T2600, Shanghai Youke Instrument Co., Ltd., Shanghai, China), low-speed bench centrifuge (TDL-80-2B, Shanghai Anting Scientific Instrument Co., Ltd., Shanghai, China), etc.

2.2. Experimental Procedures

2.2.1. Basic Bacterium Information Test

The basic bacterium of oil sludge was tested by the streak plate method. After the individual bacteria were inoculated on Luria–Bertani (LB) liquid medium and incubated for 12 h at 35 °C, then transferred to LB solid medium and cultivation in biochemical incubator for 24 h at 35 °C, the color, transparency, cell shape, and cell morphology were observed and recorded. The bacteria were stored in a refrigerator at 4 °C. The basic information of 10 bacteria was tested in this study, the result is shown in Table 1.

Table 1. Basic information of bacteria.

NO.	Name	Characteristics of Colony				
		Colour	Transparency	Edge	Cell Morphology	GenBank Number
1-1	<i>Pseudomonas aeruginosa</i> A	White	Opaque	Neat	Bacilli	KX665604
1-2	<i>Microbacterium oxidans</i>	Light yellow	Translucent	Neat	Bacilli	KX665599
1-3	<i>Ochrobactrum intermedium</i>	Milky white	Opaque	Neat	Bacilli	KX665600
1-4	<i>Stenotrop Homonas</i>	Milky white	Opaque	Neat	Bacilli	KX665603
1-5	<i>Brevibacillus laterosporus</i>	Grayish white	Opaque	Untidy	Bacilli	KX665602
1-6	<i>Alcaligenes faecalis</i>	White	Transparent	Untidy	Corynebacterium	KX665601
2-1	<i>Pseudomonas aeruginosa</i> B	Grayish green	Translucent	Neat	Bacilli brevis	—
2-2	<i>Achromobacter</i> sp.	Yellow	Translucent	Neat	Bacilli brevis	—
2-3	<i>Cellulomonas</i>	Light yellow	Opaque	Untidy	Bacilli	—
2-4	<i>Serratia marcescens</i>	Dark red	Opaque	Untidy	Bacilli brevis	—

2.2.2. GC-MS Operating Conditions

Chromatographic column: DB-5MS (30 m × 0.25 mm × 0.25 µm), split ratio: 10:1, Temperature: 280 °C, Injection Volume: 1.0 µL, Column Flow: 1.0 mL/min (constant flow), Column Temperature: 80 °C for 2 min, Rise to 180 °C (20 °C/min) keep for 5 min, Rise to 290 °C (10 °C/min) keep for 5 min, Electron Bombardment Source: EI, Ion source Temperature: 230 °C, Ionization Energy: 70 eV, Interface Temperature: 280 °C, Quadrupole Temperature: 150 °C, Mass Scanning Range: 45–450 u, Solvent delay Time: 5 min, Scanning Mode: full scan scan/SIM (Ion Mode).

2.2.3. Quantitative Analysis of Single and Mixture PAHs

Cyclohexane (q.s) was added into the medium and shaken on the vortex turbidimeter for 5 min, followed by centrifuging for 10 min at 3000 rpm, and the organic phase was collected. Cyclohexane (q.s) was added again to the aqueous phase, repeat shaken, centrifuged, and collected three times. The extract was extracted to a sand core funnel containing 5 mm of anhydrous Na₂SO₄ to absorb water for 1 h. The organic phase was collected, and the absorbance was determined by the UV-vis spectrophotometer [35,36].

The residuals of single NAP, PHE, and PYR in the medium were calculated according to the standard curve.

Hexyl hydride (q.s) was added into the medium and shaken on the vortex turbidimeter for 5 min, followed by ultrasonic extraction below 35 °C for 30 min and centrifugation at 3000 rpm for 10 min, and the organic phase was collected. The organic phase was extracted to a sand core funnel containing 5 mm anhydrous Na₂SO₄ to absorb water for 1 h. Then, a 10 mL glass syringe was used to inject the dehydrated organic phase through a 0.22 µm membrane into the sample vial. The residual of NAP, PHE, and PYR in the medium were determined by GC-MS. The residual of mixed PAHs in the sample was calculated according to the standard curve.

2.2.4. Quantitative Analysis of Crude Oil

The medium was transferred into a 250 mL separating funnel, and petroleum ether and 1:1 H₂SO₄ were added into separating funnel to completely integrate the crude oil into the organic phase. After standing for 30 min, the aqueous phase was released from below and the organic phase was poured out from above. The absorbance was determined by UV-vis spectrophotometer, and the residual of crude oil in the medium was calculated according to the standard curve [35,36].

2.2.5. Sensitivity Analysis of P&A Bacterial Consortium to Heavy Metal Ions

The circular filter papers with a diameter of 6 mm were submerged into CuSO₄·5H₂O, (Pb(NO₃)₂), and ZnSO₄·7H₂O standard solution with concentrations of 0.1, 0.5, 1, 5, 10, and 20 g/L, respectively. After let the papers dried overnight at 37 °C and autoclaved, the drug sensitivity test paper (DSTP) was made. A total of 0.25 mL of bacterial solution with optical density 600 = 1.0 (OD₆₀₀ = 1.0) was applied evenly on LB solid medium, and six pieces of DSTP were lied tightly on it, then cultured at 35 °C for 24 h. The size of the bacteriostatic circle around the paper were recorded to detect whether there is bacteriostatic effect.

2.2.6. Resistance Analysis of P&A Bacterial Consortium to Heavy Metal Ions

Stepwise dilution method was used for MIC (minimum inhibitory concentration) test by using 11 tubes with number 0–10, then 250 mg CuSO₄·5H₂O, Pb(NO₃)₂ and ZnSO₄·7H₂O were diluted into 5 mL LB liquid medium in tube No. 0, 2.5 mL LB liquid medium was added into tubes No. 1–10, and after that, 2.5 mL of solution from tube No. 0 were injected into tube No. 1, and the rest of the tubes from 2–8 could be performed in the same manner, respectively. Then, 0.25 mL of bacterial solution with OD₆₀₀ = 1.0 was injected into autoclaved tubes No. 0–9. All test tubes were cultured in a biochemical incubator at 35 °C for 3 days. The heavy metal ion concentration of the tube without visible bacterial growth is the MIC of the bacterium.

2.2.7. PAHs Degradation Experiment

The individual bacteria on the LB solid medium was inoculated with an inoculation ring into LB liquid medium and cultured for 24 h at 35 °C. Then, 5 mL of bacterial solution was transferred into new LB liquid medium and cultured until OD₆₀₀ = 1.0. Inoculate a total of 5 mL of a bacterial solution with OD₆₀₀ = 1.0, whose viable bacteria count result is 10¹⁰ CFU/mL, into a PAHs (NAP, PHE and PYR) contained medium. The residual NAP, PHE, PYR, and crude oil in the PAHs degradation medium were calculated according to the methods in Sections 2.2.2 and 2.2.3 after 8 days of degradation in different conditions. Flow charts of heavy metal ions and PAHs degradation experiment are shown in Figure 1.

Figure 1. Flow chart of heavy metal ions and PAHs degradation experiment.

3. Results and Discussion

3.1. Construction of P&A Bacterial Consortium

3.1.1. Optimization of PAHs Degradation by Individual Bacteria

Optimization of Individual Bacteria by Single PAHs

Each of 10 bacteria was used to consume 500 mg/L NAP, 50 mg/L PHE, and 100 mg/L PYR, respectively [37,38], and the absorbances of the solutions were determined by UV-vis spectrophotometer on Days 2, 4, 6, 8, and 10. Within 10 days of consumption, the average degradation rates of NAP, PHE, and PYR were continuing to increase up to 90.24%, 34.17%, and 34.36%, respectively (Figure 2), but the increase rates on Days 8–10 were significantly slower than others, so the degradation time of subsequent experiments was determined to be 8 days.

Figure 2. Degradation rates of single PAHs by purebred strain.

It could be also seen that the degradation rates of NAP by 10 strains were all about 80% (Figure 2a) within 8 days. The top six bacterium with the best degradation effect on NAP, PHE, and PYR and their degradation rates within 8 days are shown in Table 2.

Table 2. The best degradation rates of NAP, PHE, and PYR within 8 days.

Serial NO.	NAP		PHE		PYR	
	NO.	Degradation Rates	NO.	Degradation Rates	NO.	Degradation Rates
1	1-1	87.40%	1-1	38.58%	1-5	45.83%
2	1-3	86.05%	2-1	37.15%	1-1	44.88%
3	1-2	85.36%	1-6	37.10%	1-3	37.42%
4	1-6	85.25%	2-3	34.35%	1-6	31.02%
5	1-5	84.36%	2-2	33.69%	2-1	30.29%
6	2-1	84.28%	1-5	31.99%	2-3	29.73%

Figure 2 and Table 2 show that, NAP, PHE, and PYR degradation rates reach 87.40%, 38.58%, and 44.88% when 1-1 was used, and 84.28%, 37.15%, and 30.29% when 2-1 was used. Bacteria 1-1 and 2-1 belong to *Pseudomonas*, with the most extensive degradation object range and species so far [39]. One strain of *P. anguilliseptica* isolated from an urban area was reported with both degradation of PAHs and improved degradation efficiency of bacterial consortium [40].

Similarly, based on the results in Figure 2 and Table 2, NAP, PHE, and PYR degradation rates reach 84.36%, 31.99%, and 45.83% when 1-5 was used, and 85.25%, 37.10%, and 31.02% when 1-6 was used. Bacteria 1-5 and 1-6 belong to *Bacillus* and *Alcaligenes*, respectively. Four potential candidates with higher degradation effect single substance of PAHs were selected from 10 bacteria.

Optimization of Individual Bacteria by Mixed PAHs

Since PAHs persistently exist in mixture forms in the natural [41], the bacterium comprehensive degradation capacity for all NAP, PHE, and PYR compounds should be considered. Four optimum individual bacterium (1-1, 2-1, 1-6, and 1-5) were used as degradation bacteria. The mixed 200 mg/L NAP, 20 mg/L PHE, and 40 mg/L PYR were used to test the degradation capacity for bacterium, that selected above, to find the optimum mixed PAHs degradation bacterium.

NAP degradation rates was not used, since all bacteria could degrade the 200 mg/L NAP completely (Figure 3). In the mixed PAHs system, the degradation rates shown in Figure 3 for PHE and PYR were 79.24% and 65.09%, and 89.11% and 79.14%, when the Bacteria 1-1 and 1-6, were used respectively. Bacteria 1-1 and 1-6 showed better degradation effects than 1-5 and 2-1. Since Bacteria 1-1 and 1-6 were isolated from the same oily sludge (3.1), the symbiotic relationship between 1-1 and 1-6 is possible. Based on the experiment above and basic bacterium information test results, the bacteria 1-1 and 1-6 were selected to conducting the degradation bacterium in the following studies.

3.1.2. Optimization of Proportion of Two Individual Bacterium in Bacterial Consortium

It is impossible that there are only individual bacteria in the natural degradation process of the pollution system. PAHs are often degraded by the bacterial consortium since the diversity of bacterial consortium is conducive to the degradation of PAHs. Some bacteria can provide oxygenase, other degradation enzymes, and growth factors to promote the degradation of PAHs through synergistic effect. For example, *P. putida* cannot degrade PYR without the present of *Flavobacterium* and its growth matrix PHE [42].

In order to optimize the bacterial proportion (volume percent) in degradation bacterial consortium, two degradation bacteria(1-1 and 1-6) were mixed in different ratios to degrad the mixture of PAHs. Since 200 mg/L NAP was completely degraded by bacteria in experiment section of Optimization of Individual Bacteria by Mixed PAHs, the NAP was increased to 500 mg/L in PAHs mixture in the following test, and the results were shown in Figure 4.

Figure 3. Degradation rates of mixed PAHs by purebred strain (Δ : PHE degradation rate; $\star$: PYR degradation rate).

Figure 4. Degradation rates of mixed PAHs by bacteria consortium with different ratio (Δ : PHE degradation rate; $\star$: PYR degradation rate).

NAP degradation rates were not used, since all bacterial consortium could degrade the 500 mg/L NAP completely (Figure 4). It could be observed that, when the inoculation volume ratio was 1:1, the degradation rates of PHE and PYR were higher than others (92.02% and 88.65%) (Figure 4), which was also higher than individual bacteria 1-6 (PHE 89.11% and PYR 79.14%) and 1-3 (PHE 79.24% and PYR 65.09%) (Figure 3). This also proved the previous conjecture that there was no competitive relationship between 1-1 and 1-6, and they are interactive with each other. The synergistic effect of the two bacterium can promote the degradation rates of PHE and PYR.

Through the experiment in this section, a P&A bacterial consortium composed of *P. aeruginosa* (1-1) and *A. faecalis* (1-6) was successfully constructed. The optimal degradation P&A bacterial consortium was in a volume ratio of 1:1 when $OD_{600} = 1.0$ and the best degradation term under laboratory conditions was 8 days. The two bacteria were from oily sludge, which also shows the environmental adaptability of P&A bacterial consortium.

3.2. Effect Analysis of Environmental Factors on Degradation

Although the P&A bacterial consortium had been constructed, the degradation rates of NAP, PHE, and PYR also depend on environmental factors, bacteria, and PAHs 'initial

concentrations. The effects of temperature, pH, bacteria, and PAHs' initial concentrations on the degradation of PAHs were studied by single variable method. Since 500 mg/L NAP was completely degraded by bacterium in experiment 3.2.2, the PAHs mixture contained 2000 mg/L NAP, 20 mg/L PHE, and 40 mg/L PYR in the following test, except Experiment (c). The PAHs initial concentrations in Figure 5c are shown in Table 3 as follows. The results are shown in Figure 5.

Figure 5. Effects of environmental conditions on the degradation rate of PAHs (◇: NAP degradation rate; △: PHE degradation rate; ☆: PYR degradation rate).

Table 3. The PAHs initial concentrations in Figure 5c.

NO.	1	2	3	4	5
NAP/mg/L	1000	2000	3000	4000	5000
PHE/mg/L	10	20	30	40	50
PYR/mg/L	20	40	60	80	100

The effects of environmental conditions on NAP, PHE, and PYR degradation efficiency are shown in Figure 5. It can be seen from Figure 5a that temperature is one of the most important environmental factors that is affecting the growth and survival of microorganisms; when the degradation temperature is 35 °C, the comprehensive degradation rates of NAP, PHE, and PYR are higher than others (99.51%, 92.60%, and 91.71%), and when it is higher or lower than 35 °C, a considered decrease of degradation rate could be observed. Within the appropriate temperature range, the enzymatic reaction efficiency, the solubility and mass transfer rates of PAHs are increased [43,44]. Based on the Figure 5b, the higher degradation rates of NAP, PHE, and PYR could be achieved (93.86%, 78.98%, and 77.08%) when pH is

about 7. However, the NAP, PHE, and PYR degradation rates would be decreased at other pH, proving that the enzyme activity will be reduced if pH is out of the optimum pH range of 6.5~7.5 [45].

With the increasing initial concentration (IC) of NAP, PHE, and PYR in the degradation system, the degradation rates showed a downward trend (Figure 5c). However, when the IC values of NAP, PHE, and PYR were 1000, 10, and 20 mg/L (Table 3), respectively, NAP and PHE were degraded by the P&A bacterial consortium completely, and the degradation rate of PYR was as high as 85.55%. In order to carry out the follow-up experiment and observe the experimental phenomenon, the dosages of NAP, PHE, and PYR in the follow-up experiment were determined as 2000, 20, and 40 mg/L, and the degradation rates of NAP, PHE, and PYR under this IC were 95.23%, 88.13%, and 70.72%, respectively. The lower initial concentration of PAHs made it not only difficult to provide sufficient carbon source but also difficult to induce microorganisms to produce corresponding degrading enzymes. High-concentration PAHs have toxicity to microorganisms [29], and suitable initial concentration also is the important consideration factor for further analysis.

It can be observed from Figure 5d that when the bacterial inoculum is 5% (volume percent), the degradation rates of NAP, PHE, and PYR were 96.37%, 88.54%, and 73.82%, respectively, which were higher than others. The accumulation of biomass would take a long time when the bacterial inoculum is lower than the appropriate amount, but higher bacterial inoculation will lead to a large number of cell deaths because of nutrient deficiency.

All above, the optimum comprehensive degradation factors about temperature are 35 °C, the pH is about 7, the NAP_{IC} , PHE_{IC} , and PYR_{IC} are 2000, 20, and 40 mg/L, respectively, and for bacterial inoculum, which with $\text{OD}_{600} = 1.0$ is 5 mL (volume percent: 5%), the best degradation rates for NAP, PHE, and PYR are higher than 90%, 80%, and 70%, respectively. Therefore, the subsequent experiments will be carried out on this basis.

3.3. Effect Analysis of Exogenous Substances on PAHs Degradation

3.3.1. Crude Oil

During the microbial degradation of PAHs in oily sludge, the content of residual oil in sludge would affect the degradation efficiency of PAHs. Additionally, the residual oil in randomly stacked oily sludge can enter the soil directly or indirectly, affecting the physical and chemical properties and functions of the soil. Toxic substances can inactivate plant enzymes and penetrate plant structure, resulting in the failure of plant growth [46]. Furthermore, excessive residual oil would pollute groundwater after being washed by rain. After horizontal expansion in groundwater, some residual oil can enter surface runoff with runoff sediment, resulting in excessive chemical oxygen demand (COD) and petroleum substances in surface water [47]. The test of crude oil effect on the degradation of NAP, PHE, and PYR by P&A bacterial consortium was explored.

It is shown that the crude oil content factor did not impact NAP, PHE, and PYR degradation in this experiment, and all of them were completely degraded (Figure 6). Moreover, within the P&A bacterial consortium system, the degradation rate of crude oil reached 80.85% when the addition of crude oil was 5 g/L. Compared with Section 3.2, the possible reason for the complete degradation of NAP, PHE, and PYR caused by the addition of crude oil is that there are short linear and branched alkanes with simple structure in crude oil [48]. They can not only provide a relatively high-quality carbon source for the P&A bacterial consortium, which greatly increased the bioaccumulation in the system in a short time, but also stimulate the P&A bacterial consortium to produce corresponding degradation enzymes to accelerate the degradation rates of NAP, PHE, and PYR. After the easily degradable short chain alkanes and straight chain alkanes were consumed, a large number of bacteria began to degrade the NAP, PHE, and PYR; therefore, its degradation rate was greatly increased.

Figure 6. Effect of initial oil content on PAHs degradation efficiency ($\diamond$: NAP degradation rate; Δ : PHE degradation rate; $\star$: PYR degradation rate; $\triangle$: Crude oil degradation rate).

The possible reason for the different crude oil degradation rates (Figure 6) is that, under appropriate stimulation, microorganisms can emulsify crude oil by changing the hydrophobicity of cell surface or secreting biosurfactants themselves [49,50]. This effect is limited when crude oil content is insufficient, and the crude oil is difficult to disperse in the solute, resulting in its low degradation rate. With the increase of crude oil content (to 5 g/L), this effect is gradually obvious. However, when the crude oil content increases to 10 g/L, the intermediate products produced by crude oil are toxic to the P&A bacterial consortium (the results of viable bacteria count showed that the number of viable bacteria was lower than others), which lead to the crude oil degradation rate declining.

3.3.2. Heavy Metal Ions Sensitivity Analysis

Heavy metals are common components in oily sludge. In the process of microbial treatment of oily sludge, many heavy metals become protein-precipitant and cause microbial death. Some of microorganisms exhibit a biphasic response to a number of heavy metals (Cu^{2+} , Zn^{2+}); an appropriate amount of heavy metals contribute to the growth of microorganisms, but they kill microorganisms if excessive [51]. Other metals have toxic effects (Pb^{2+}) at a low concentrations. Previous experiments ignored the disadvantage of heavy metals. Therefore, it is necessary to explore the sensitivity and resistance of P&A bacterial consortium to heavy metal particles. The bacteriostatic circles produced by the P&A bacterial consortium are shown in Figure 7, and the average diameter of the bacteriostatic circles are shown in Table 4.

Table 4. Average diameter of bacteriostatic circles produced by DSTP.

Compound Concentration (g/L)	Average Diameter of Bacteriostatic Circles (mm)								
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$			$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$			$\text{Pb}(\text{NO}_3)_2$		
	1-1	1-6	P&A	1-1	1-6	P&A	1-1	1-6	P&A
0.1	—	—	—	—	—	—	—	—	—
0.5	—	7	7	—	—	—	—	—	—
1	—	8	13	—	—	—	—	—	—
5	—	9	16	—	10	5	—	—	7
10	15	16	22	8	12	10	8	—	10
20	30	30	30	16	14	19	11	8	13

Figure 7. Bacteriostatic circles produced by different DSTP. (a) Bacteriostatic circles produced by $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ DSTP. (b) Bacteriostatic circles produced by $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ DSTP. (c) Bacteriostatic circles produced by $\text{Pb}(\text{NO}_3)_2$ DSTP.

DSTP is lied tightly on the surface of LB solid medium, which was inoculated with different bacterium. The heavy metal ions in the paper diffuse in agar, and the concentration of heavy metal ions decreases logarithmically with the extension of diffusion distance. The bacterium forms a transparent bacteriostatic circle around the paper. The size of bacteriostatic circle can reflect the sensitivity of bacteria to heavy metal ions. Additionally, it has a negative correlation with the MIC, that is, the larger the diameter of bacteriostatic circle, the higher the antibacterial activity [52]. It can be seen from Figure 7 and Table 4 that, with the increase of the concentration of heavy metal ions, the diameters of the bacteriostatic circles are also increased. When the concentrations of compounds are higher than 0.5, 5, and 5 g/L, respectively (i.e., the concentrations of Cu^{2+} , Zn^{2+} , and Pb^{2+} are higher than 2.002, 17.388, and 15.096 mM, respectively), the growth of bacterium was inhibited.

Resistance Analysis

The negative effects of compound concentrations on the growth of P&A bacterial consortium are shown in Figure 8.

The MIC of P&A bacterial consortium to different compounds were 0.5 g/L (Cu^{2+} 2.002 mM), 5 g/L (Zn^{2+} 17.388 mM), and 3.125 g/L (Pb^{2+} 9.435 mM), respectively, which are higher than the reported contents of Cu^{2+} , Zn^{2+} , and Pb^{2+} in oil sludge [53]. It indicates that P&A bacterial consortium can tolerate high concentrations of Cu^{2+} , Zn^{2+} , and Pb^{2+} , and grow normally in oily sludge.

Figure 8. Resistance of P&A bacterial consortium to different concentrations of different compounds. (a) Resistance of P&A bacterial consortium to different concentrations of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. (b) Resistance of P&A bacterial consortium to different concentrations of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. (c) Resistance of P&A bacterial consortium to different concentrations of $\text{Pb}(\text{NO}_3)_2$.

3.3.3. Surfactants

Many types of surfactants have a high solubilization effect on PAHs [54] and emulsification and dispersion on crude oil [55]. In order to investigate the effect of surfactants on PAHs degradation, four surfactants aqueous solutions (Tween-80, TritonX-100, APG-1214, and Sodium dodecyl sulfate) were added into different media under their critical micelle concentrations (CMC, 100, 50, 500, and 500 mg/L).

Due to the addition of surfactant, the degradation rates of NAP, PHE, and PYR in the Tween-80 group were decreased to 87.85%, 52.32%, and 40.95%, and those in the APG-1214 group were decreased to 86.24%, 66.07%, and 61.72%. The degradation rates of crude oil in all groups were increased (Figure 9). This indicates that surfactants preferentially

increase the solubility of crude oil; thus, the degradation rate of crude oil is increased. As shown in Figures 6 and 9, although the NAP, PHE, and PYR degradation rates in the Triton X-100 group do not decrease significantly, the crude oil degradation rate only increases by 3.85% (from 80.85% to 84.70%). It can be seen that the non-ionic surfactant (Tween-80 and APG-1214) had negative effect on the degradation of NAP, PHE, and PYR in the presence of crude oil, and the negative effects of non-ionic surfactant (Triton X-100) and anionic surfactant (SDS) are not obvious. However, their positive effects are also very limited, which means that no additional surfactants are needed.

Figure 9. Effects of surfactants on the degradation efficiency of PAHs (◇: NAP degradation rate; △: PHE degradation rate; ☆: PYR degradation rate; ▽: Crude oil degradation rate).

3.4. Degradation of PAHs in Oily Sludge by P&A Bacterial Consortium

The PAHs degradation study in this paper above were conducted under ideal conditions. However, there are many uncontrollable factors in the degradation process of PAHs in actual oily sludge, leading to the differences in degradation effect between them. Therefore, the oily sludge from oil polluted area in Shaanxi Province, China, was used to test the practical application possibility of P&A bacterial consortium in the test below (Figures 10 and 11).

The P&A bacterial consortium was used to degrade oily sludge that did not only contain NAP, PHE, and PYR (0.2090 mg/g NAP, 3.1116 mg/g PHE, and 1.00595 mg/g PYR) but also other types of PAHs compounds. After 8 days, more than 80% of NAP, PHE, and PYR was degraded in triplicate parallel groups experiments (Figure 10). At the same time, it was found from chromatogram analysis of oily sludge that the P&A bacterial consortium had the ability to degrade Acenaphthylene, 1H-Phenylene, 4H-Cyclopenta[d,e,f]phenanthrene, Fluoranthene, Triphenylene, 9H-Cyclopenta[a]pyrene, and Benzo[k]fluoranthene in oily sludge (Figure 11).

Figure 10. Degradation of PAHs in oily sludge by PA bacterial consortium.

Figure 11. Chromatogram analysis of oily sludge. (a) Chromatographic analysis of oily sludge before degradation; (b) chromatographic analysis of degraded oily sludge; 1. Naphthalene; 2. Acenaphthylene; 3. 1H-Phenylene; 4. Phenylene; 5. 4H-Cyclopenta[d,e,f]phenanthrene; 6. Fluoranthene; 7. Pyrene; 8. Triphenylene; 9. 9H-Cyclopenta[a]pyrene; 10. Benzo[k]fluoranthene).

4. Conclusions

An optimal PAHs degradation bacterial consortia (*P. aeruginosa* and *A. faecalis*) and their volume ratio (1:1) were screened out by this study from 10 bacterium in local oily sludge. The PAHs solution degradation efficient of P&A bacterial consortium under different environmental conditions, bioaugmentations, and exogenous stimulations were studied by UV-vis spectrophotometer and GC-MS. The result shows that, after 8 days degradation under 35 °C, pH 7, with 5% (volume percent) of the inoculation amount, the degradation

rates of 2000 mg/L NAP, 20 mg/L PHE, and 40 mg/L PYR were higher than 90%, 80%, and 70%, respectively. In the report of Sponza et al. and Ventaka et al., the highest biodegradation rates of PHE and PYR were only 74% and 60% [56,57], although A. Arun et al. used *C. versicolor* and *F. palustris*, which could degraded 93.7% of PYR, but its degradation term was as long as 28 d [58]. In contrast, the P&A bacterial consortium has more advantages in degradation efficiency and degradation term. Additionally, the experimental results also show that the additional crude oil further improved the degradation efficiency of P&A bacterial consortium. The MIC values of Cu^{2+} , Zn^{2+} , and Pb^{2+} to bacterium were 2.002, 17.388, and 9.435 mM, respectively. The addition of surfactants had a negative or limited positive effect on the PAHs degradation rate. Furthermore, the average degradation rates of NAP, PHE, and PYR in oily sludge by P&A bacterial consortium also reached above 80%. Based on GC-MS test result before and after incubation, PAHs degradation ability of P&A bacterial consortium is not only limited to NAP, PHE, and PYR, but also includes others such as Acenaphthylene, 1H-Phenylene, 4H-Cyclopenta[d,e,f]phenanthrene, Fluoranthene, Triphenylene, 9H-Cyclopenta[a]pyrene, and Benzo[k]fluoranthene. However, there are few reports on the degradation of PAHs mixture in oily sludge by bacterial consortium.

The P&A bacterial consortium has outstanding performance on PAHs degradation activity in oil sludge and high concentration heavy metal ion environment. Based on this advantage, the P&A bacterial consortium can be prepared into immobilized microorganisms to improve the microbial cell density in unit volume medium and further improve its anti-toxic ability. At the same time, the carrier material can act as a loosening agent. It also can wrap the nutrients by microorganisms to play a stable role in complex environment, which means that the P&A bacterial consortium has prospects in the application in oily sewage, oily sludge, and soil treatment.

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