applied sciences

MDPI

Article

Comparison of Petroleum Hydrocarbons Degradation by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

Zhaoyang You [1,2], Haiyang Xu [1], Shujuan Zhang [1,*], Hyunook Kim [3,*], Pen-Chi Chiang [2], Wenfei Yun [1], Li Zhang [1,2] and Miao He [1]

1 College of Urban Construction, Nanjing Tech University, Nanjing, Jiangsu 210039, China; youzhaoyang@njtech.edu.cn (Z.Y.); hexhy@njtech.edu.cn (H.X.); 2899431306@njtech.edu.cn (W.Y.); njzl@njtech.edu.cn (L.Z.); hm0204@njtech.edu.cn (M.H.)

2 Graduate Institute of Environmental Engineering, National Taiwan University, 71 Chou-Shan Road, Taipei City 10673, Taiwan; pcchiang@ntu.edu.tw

3 Department of Environmental Engineering, University of Seoul, Seoul 02504, Korea

* Correspondence: zhangshujuan@njtech.edu.cn (S.Z.); h_kim@uos.ac.kr (H.K.)

Received: 23 November 2018; Accepted: 4 December 2018; Published: 9 December 2018

Featured Application: Bioremediation is an economical and effective option to remove petroleum hydrocarbons (PHs) from soil and water ecosystems. In-depth understanding of the performance of different microorganisms is helpful in constructing bacterial communities to effectively remediate polluted ecosystems. Our study investigated the contribution of biotic and abiotic factors on variation in the PHs degradation efficacy of *Klebsiella pneumoniae* (Kp) and *Pseudomonas aeruginosa* (Pa). Our work advances the current understanding of characteristics of highly effective strains in PHs biodegradation and their use in construction of highly effective bacterial communities.

Abstract: The aim of this work was to develop bacterial communities to effectively degrade petroleum hydrocarbons (PHs). We investigated the biotic and abiotic contributors to differences in PHs degradation efficacy between two bacterial strains, *Klebsiella pneumoniae* (Kp) and *Pseudomonas aeruginosa* (Pa), screened out from the activated sludge of a petroleum refinery. We characterized the temporal variations in degradation efficacy for diesel and its five major constituents as a sole carbon source and identified more constituents they degraded. The growth characteristics, surface tension, hydrophobicity and emulsifiability of these two strains were measured. We further estimated the relationships between their degradation efficacy and all the biotic and abiotic factors. Results showed that the Pa strain had higher diesel degradation efficacy (58% on Day 14) and utilized more diesel constituents (86%) compared to Kp. Additionally, the growth of the Pa strain in diesel medium was faster than that of the Kp strain. The Pa strain had a lower surface tension and higher hydrophobicity and emulsifiability than Kp, while the surfactant produced by Pa was identified as rhamnolipids. Degradation of PHs was positively related to bacterial growth, hydrophobicity and emulsification but negatively related to surface tension. Overall, differences in degrading capacity for diesel constituents, relative growth rate, and biosurfactant production contributed to the variation in the PHs degradation efficacy of these two bacterial strains.

Keywords: biodegradation; bacteria; diesel constituents; biosurfactant; degradation efficacy; growth rate

1. Introduction

Petroleum hydrocarbons (PHs) are among the most abundant pollutants in soil and water environments [1]. It is reported that about eight million tons of petroleum are released into the

water environment every year [2]. Leakage of PHs commonly occurs during exploration, production, refining, storage and transportation [3]. For example, a rig explosion resulted in the escape of four million barrels of petroleum into the Gulf of Mexico in 2010 [4]. The European Environment Agency reported that about 34% of contaminated sites in Europe were related to mineral oil (including common petroleum substances such as polycyclic aromatic hydrocarbons (PAHs) and volatile aromatic hydrocarbons) in 2006 and this value has since increased to 53% [5]. Besides surface water, PHs leakage also contaminates groundwater. According to the United States Environmental Protection Agency, in 2000, 25% of underground storage tanks for petroleum had leakage which directly resulted in serious groundwater pollution in the United States [6,7]. Toxic and persistent constituents of PHs such as olefinic and paraffinic hydrocarbons, monoaromatic hydrocarbons (e.g., benzene, toluene, and xylene) pose a great threat to human beings [1,8–10]. Hence, developing an economical and effective way to remove PHs from water ecosystems is a major goal to maintain the health of ecosystems and human beings.

There are several methods to mitigate the risks of PHs contamination of the health of ecosystems and effects on human health, including bioremediation [11–14] and a variety of physico-chemical treatments, e.g., separation tank treatment [15], coagulant addition [16,17], and membrane separation [18]. Although physico-chemical treatments are effective in degrading these pollutants, they are limited by the high operational costs and generation of secondary toxic compounds [19]. Compared to these methods, bioremediation has received immense attention because it costs less in chemicals and energy and is environment-friendly. In this process, these PHs, serving as the energy and carbon source for microorganisms, are transformed or degraded into non-hazardous (such as microbial biomass and/or CO_2) or less-hazardous chemicals (such as glycerinum) [20,21]. As implementation of bioremediation is heavily dependent on microorganisms' performance, an in-depth understanding of factors that affect their performance is required.

Many microorganisms are able to degrade PHs, such as protozoa, fungi and bacteria. Specifically, grazing protozoa were reported to be able to carry out naphthalene mineralization up to four times faster than the control in flasks with sediment slurry and seawater [22]. Fungi were also reported to degrade PHs, which is mediated by the cytochrome P-450 system and/or soluble extracellular enzymes, including lignin peroxidase, manganese peroxidase and laccases [23]. Additionally, filamentous fungi, basidiomycetes, white rot fungi and deuteromycetes have also shown the ability to reduce the abundance of PAHs by effectively degrading a broad range of the PHs that mainly compose diesel fuels [24,25].

Although many microorganisms can degrade PHs pollutants, bacteria play a dominant role [26,27]. Of various strains obtained from enrichment cultivation of oil-contaminated soils from an oil field in China, 22 strains could utilize diesel as a sole source of carbon and energy and 11 strains could degrade the total PHs of diesel by more than 70% in seven days [28]. Another study found that when two bacterial strains (*Mycobacterium hyalinum* and *Cladosporium carrianii*) were applied at the same time, a significant synergistic effect was observed, and almost complete (99%) degradation of diesel in was achieved within five days [29]. Compared to bacteria, there are fewer kinds of fungi and protozoa that can degrade PHs, and their average degradation efficacy is also lower [30,31]. Therefore, a comprehensive understanding of how bacteria perform in degrading PHs is of great benefit in the construction of effective bioremediation systems.

The biodegradation process for PHs is influenced by many factors, such as the constituent content of diesel fuels [32]. Diesel is a complex mixture of PHs with carbon numbers of C_{10}–C_{22}. For $0^{\#}$ diesel, among the 70 compounds detected, 8% of mass fraction was aromatic hydrocarbons, 90% was saturated alkanes, and 2% was alcohol, acid and olefins [33]. For aromatic hydrocarbons, half were monocyclic aromatic hydrocarbons (e.g., substituted benzene) while the other half were PAHs (e.g., naphthalene, anthracene, and phenanthrene). For saturated alkanes, 69% were C_{13}–C_{22} straight alkanes, 15% were isomers and 6% were naphthenic hydrocarbons [34]. Importantly, the extent of their biodegradation varied [30], depending on the carbon number [35], branch number [36] as well as

the biotoxicity of aromatic hydrocarbons [37]. The order of biodegradation difficulty was: olefins < alkanes < PAHs < branched alkanes < naphthenic hydrocarbons [27,38].

This biodegradation process is also manipulated by the production of biosurfactant [39,40], a type of metabolite of PHs produced by microorganisms [41,42]. For example, the strain DHT-GL (*Pseudomonas aeruginosa*) was able to produce biosurfactant identified as glycolipid on yeast peptone glucose medium and reduce its surface tension from 55 to 30 dN/cm after 96 h growth. In addition, this biosurfactant not only increased emulsification of the oil but also increased the adhesion of PHs to the cell surface of other bacteria [43]. These surfactants were reported to reduce the surface tension of liquid [44], emulsify solubilization and increase the surface hydrophobicity of the bacterial cells [45,46]. The specific biosurfactant function seems to be connected with environmental conditions and corresponding regulatory systems. The range of rhamnolipid functions covers solubilization, modification of surface properties, stimulation of bacterial motility, formation and disruption of biofilms, virulence and anti-microbial activity [42]. These effects increased the probability of contact between the cell and PHs and the adhesion of hydrocarbon, leading to improvement of the hydrophobic substrate [47]. Many microorganisms produce biosurfactants to increase the bioavailability of poorly available substrates [24]. Therefore, bacteria producing biosurfactants are hypothesized to be more effective in degrading PHs [48,49].

In addition to the constituent content of PHs and biosurfactants, growth characteristics of microorganisms might also contribute to bacterial performance in degradation of PHs [50]. One of the possible reasons is that PHs degradation by bacteria is mainly dependent on their strong intracellular enzymes, such as monooxygenase and hydroxylase [51,52]. Synthesis and secretion of these enzymes are impacted by the growth rate and metabolism of the cells [53]. It has been reported that fast-growing bacteria have a higher degradation efficacy than their slow-growing counterparts, and there is a positive correlation between them [50,54–56].

Two PH-degrading bacterial strains were screened in our laboratory, and we found that they have a high diesel degradability both as a single strain and as a community. Meanwhile, one of them was able to produce biosurfactant while the other was not (Supplementary Materials; Figures S1–S3). Using them to biodegrade diesel, we aimed to explore both biotic and abiotic factors that contributed to the performance of the two strains to degrade PHs. We hypothesized that the strain with higher efficacy for diesel degradation would be able to produce biosurfactant and utilize higher levels of diesel constituents, with a higher growth rate.

2. Materials and Methods

2.1. Microorganisms

Klebsiella pneumoniae (Kp) and *Pseudomonas aeruginosa* (Pa) were screened out from the activated sludge collected from a petroleum refinery in Nanjing, China. These two bacterial strains were identified with 16S rDNA sequencing (Sangon Biotech, Shanghai, China) and the establishment of a phylogenetic tree indicated that the Kp strain was *Klebsiella pneumoniae* (AR0139) and Pa was *Pseudomonas aeruginosa* (DSM50071) [13]. The optimum growth conditions of the Kp strain were pH of 7.5, temperature of 30 °C, and 2% salinity, while those of Pa were pH of 7.5, temperature of 35 °C, and 1% salinity [57].

Before we selected Kp and Pa, four bacterial strains (i.e., KX-1, Kp, KX-4, and Pa) were screened and constructed in equal proportions. The results showed that the biodegradation efficacy of the combination of Kp and Pa was the highest (Figure S1) and individually, these two strains could degrade diesel well (Figure S2). In addition, one of them could produce (Figure S3). Due to the aforementioned reasons therefore, we used Kp and Pa in our study.

2.2. Chemicals and Culture Mediums

Luria-Bertani medium (LB) was made by adding 10-g peptone, 5-g yeast extract and 10-g NaCl into 1-L deionized (DI) water. Then the medium pH was adjusted to pH 7.4. The medium was used

for activation, enrichment and preservation of the strains before degradation experiments. Mineral salt medium (MSM) was made by adding 1.0-g K_2HPO_4, 0.5-g KH_2PO_4, 5.0-g NaCl, 2.0-g $NaNO_3$, 1.0-g $MgSO_47H_2O$, 0.1-g KCl, 0.01-g $FeSO_47H_2O$, and 0.1-g $CaCl_2$ into 1 L DI water. The medium pH was then adjusted to 7.2 ± 0.2. The medium was used for studying degradation of different PH substrates by strains. The diesel medium was prepared by adding 15 mL diesel to 985 mL MSM. Finally, the specific diesel constituent medium was made by adding 15 mL diesel constituent to 985 mL MSM.

All the mediums were autoclaved at 121 °C for 20 min before adding diesel or its constituent. All the organic solvents used in this study were HPLC grade, and inorganic chemicals were analytical grade or higher.

2.3. Petroleum Hydrocarbons for Degradation

In this study, a total of 14 different diesel components were monitored as test materials (Figure S4). The mass fraction of these selected components was about 55% of the total 0# diesel (Figure S4a). The percentages of each of these components are shown in Figure S4b. The component with the highest content was chain alkanes which accounted for more than 90% of the total mass fraction.

2.4. Surface Tension Test

The surface tension of the diesel broths after 3, 4, and 5 days of cultivation was determined with a surface tension meter (Sigma 702ET, Sweden Hundred Wood Technology, Shanghai, China). Culture broths were centrifuged at 10,000 rpm for 10 min and filtered to harvest cell-free supernatant, which was used for surface tension determination. The strains reducing surface tension to 45 mN m^{-1} or lower were considered as effective biosurfactant producers [58].

2.5. Bacterial Biomass and PHs Degradation Efficacy Test

The diesel broths cultivated for 2, 4, 6, 8, 10, 12, and 14 days were collected and used for analysis of the biomass and degradation efficacy. Bacterial biomass was determined by the optical density (OD) value measured at a wavelength of 600 nm by UV spectrophotometer (Metash UV-5500, Shanghai Metash Instruments Co. LTD, Shanghai, China) [28]. Meanwhile, the dilution-plate method was used to estimate the number of living bacteria (culturing 48 h at 30 °C).

The degradation efficacy of the strains for diesel or its constituents was determined by ultraviolet spectrophotometry [59], as shown in Equation (1). The culture broths were centrifuged, then poured into a dipper to which 5 mL H_2SO_4 (50%), 1 g NaCl and 15 mL light petroleum were added. The organic phase was fully oscillated and statically stratified. Therefore, the organic phase was treated with sodium sulfate anhydrous and filtered into a 50 mL volumetric flask. We repeated the above steps twice to lower water content and to collect as much remaining diesel, or its constituents, as possible. Then, the solution was raised to a final volume of 50 mL and the absorbance of this solution was recorded at 256 nm with a 10 mm optical path quartz colorimeter. Fitting by the least squares method of linear regression, a relationship curve between absorbance and diesel concentration (mg/L) was obtained. The equation of the standard curve for diesel concentration is Y = 0.0022X + 0.0012 (R^2 = 0.9999).

$$\text{Degradation efficacy (\%)} = (1 - \text{Residual diesel concentration}/\text{Initial diesel concentration}) \times 100\%. \quad (1)$$

2.6. Hydrophobicity and Emulsification Test

The bacterial adhesion to hydrocarbons was used to test the hydrophobicity of the strains. Bacterial cells were harvested from culture broth and centrifuged at 8000× g for 20 min at 4 °C. Pellets were washed three times with DI water. The cells were then resuspended in a saline solution containing 0.9% NaCl to test their OD at 600 nm (0.5). These suspended cells (3 mL) were mixed with 0.3 mL normal sixteen alkane (C_{16}) in a screw-top test tube and then vortexed for 3 min. After this step, the C_{16} and aqueous phases were separated by stewing for 30 min. This aqueous phase was

carefully transferred into a cuvette with a Pasteur pipette and its turbidity was measured with UV spectrophotometer at 600 nm. Hydrophobicity was expressed as the percentage of adherence to C_{16}, as follows in Equation (2):

$$\text{Hydrophobicity (\%)} = 100\% \times (1 - \text{OD of the aqueous phase/OD of the cell suspension}). \quad (2)$$

A value less than 10% suggests cells should be considered hydrophilic, while a value greater than 50% suggests the cells are hydrophobic [60].

After the above centrifugation, supernatant (3 mL) was mixed with C_{16} (0.03 mL). This mixture was vortexed for 1 min and stewed for 24 h to exclude the water phase. The turbidity of the remaining solvent phase was measured with a UV spectrophotometer at 600 nm. The degree of emulsification was represented by OD_{600} [61].

2.7. GC-MS Analysis

During the degradation process (cultivating for 2, 5, 8, 11 and 14 days), the diesel broths of the Kp and Pa strains were analyzed using GC-MS (Agilent GC7890A, Santa Clara, CA, USA; the chromatographic column: HP-5MS (30 m × 0.25 mm × 0.25 μm), Agilent, Santa Clara, CA, USA). For the GC analysis, the medium was first centrifuged (8000× *g* for 20 min at 4 °C) and the supernatant (3 mL) of the centrifuged medium was extracted with hexane. The liquid phase was filtered with a 0.45 μm filter. The operating temperatures of the injector and detector were 250 and 200 °C, respectively. The oven temperature program was as follows: 100 °C held for 0.5 min and raised to 290 °C at 8 °C min^{-1}, with an isothermal period of 20 min at the end. The ionization energy was 70 eV and the inlet velocity was 1 mL min^{-1}. The limit of detection was determined by calculating 3 × the signal-to-noise ratio (S/N).

2.8. FTIR and HPLC-MS Analyses

Structural characterization of the bacterial secretions was done using FTIR and HPLC-MS analyses. The metabolite extraction from the culture broth was carried out as described by Mariaamalraj et al. [62]. Extraction was performed by following the standardized solvent extraction method with ethyl acetate and the organic phase was subsequently purified by silica-gel column chromatography. Primary characterization of the biosurfactant was carried out using the anthrone test, the saponification test, and the rhamnose test [62] (Figure S9).

The column-purified secretions were analyzed in a NICOLET 6700 FTIR-Spectrophotometer (Thermo-Fisher, Waltham, MA, USA) using the ATR (attenuated total reflectance) mode in a range of 500–4000 cm^{-1} to detect functional groups and bond types.

HPLC-MS was also used to confirm the presence of metabolites formed during biodegradation. The liquid medium and biomass extracts from the 72 h exposures were analyzed and compared with individual metabolite standards. An HPLC coupled to a triple quadrupole mass detector series 6410 with an electrospray ionization source in the negative mode (Agilent Technologies, Santa Clara, CA, USA) was used.

2.9. Statistics

Data were tested for normality with the Shapiro-Wilk test and the homogeneity of variances was confirmed with Levene's test using Origin Pro 2017C. The effects of time, strains and their interactions were analyzed with two-way analysis of variance (ANOVA). When an effect was significant in the ANOVA, it was tested by one-way ANOVA followed by Fisher's LSD post hoc test. In addition, Spearman correlation coefficients were calculated between different indicators (colony floc unit (CFU), OD_{600}, hydrophobicity, emulsification and surface tension value) and the degradation efficacy using R [63]. For all analyses, a significance level of $p < 0.05$ was applied. Data are expressed as the mean of three replicates with standard deviation.

3. Results

3.1. Biodegradability of Diesel and Its Main Constituents

The Pa and Kp strains showed a significant difference in their PH degradation efficacy, such that the degradation efficacy of the Pa strain was 15% higher than that of the Kp strain (Figure 1a). The degradation efficacy of these two strains increased with cultivation time. Maximum values of 58% were observed after 10 days cultivation for the Pa strain, and of 45% for the Kp strain, although little change was observed until Day 14. There was a significant difference between the two strains in degrading the diesel constituents phenanthrene, C_{16}, C_{20} and C_{24} alkanes (Figure 1b–f). Specifically, the Pa strain showed a higher degradation efficacy than the Kp strain throughout the cultivation time. This difference was greater for phenanthrene, C_{20} and C_{24}, than for C_{16}. For another diesel constituent –C_{12}, the Kp strain had a significantly better degrading capability than the Pa strain (Figure 1c); the Kp strain could degrade 90% of C_{12}-containing organics within six days cultivation, while the Pa strain could remove less than 15% of the compounds throughout the culture period.

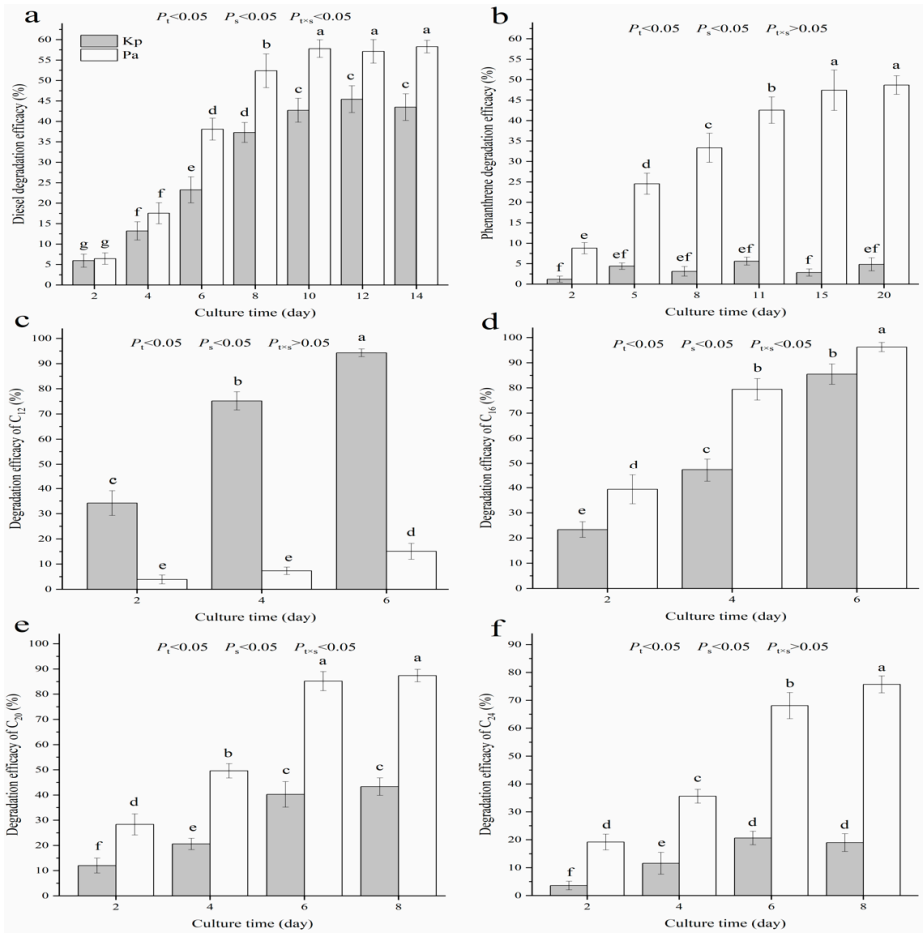

Figure 1. Degradation efficacy of Kp and Pa strains for: (**a**) diesel, (**b**) phenanthrene, (**c**) n-dodecane (C_{12}), (**d**) n-hexadecane (C_{16}), (**e**) n-eicosane (C_{20}), and (**f**) n-tetracosane (C_{24}). The corresponding hydrocarbon degradation efficacy values represent the mean ± SD of three independent replicates (n = 3). In each data sample, P_t, P_s, and $P_{t\times s}$ represent the P value of time, strain and interaction between the strains, respectively. Different letters above the columns represent significant differences according to Fisher's HSD test ($p < 0.05$).

In summary, degradation efficacy increased with culture time and the Pa strain performed much better than the Kp strain in reducing phenanthrene and C_{16}, C_{20} and C_{24} alkanes, while Kp was superior in degrading C_{12} alkanes.

3.2. Biodegrading Range of Diesel Constituents

The Pa strain had a wider degradation range than the Kp strain (Figure 2). The GC-MS spectrums showed that the main carbon number of diesel constituents was distributed among C_{10}–C_{23} (Figure 2a); strain Kp mainly degraded C_{10} to C_{14} (Figure 2b), while strain Pa mainly degraded C_{15} to C_{24} (Figure 2c).

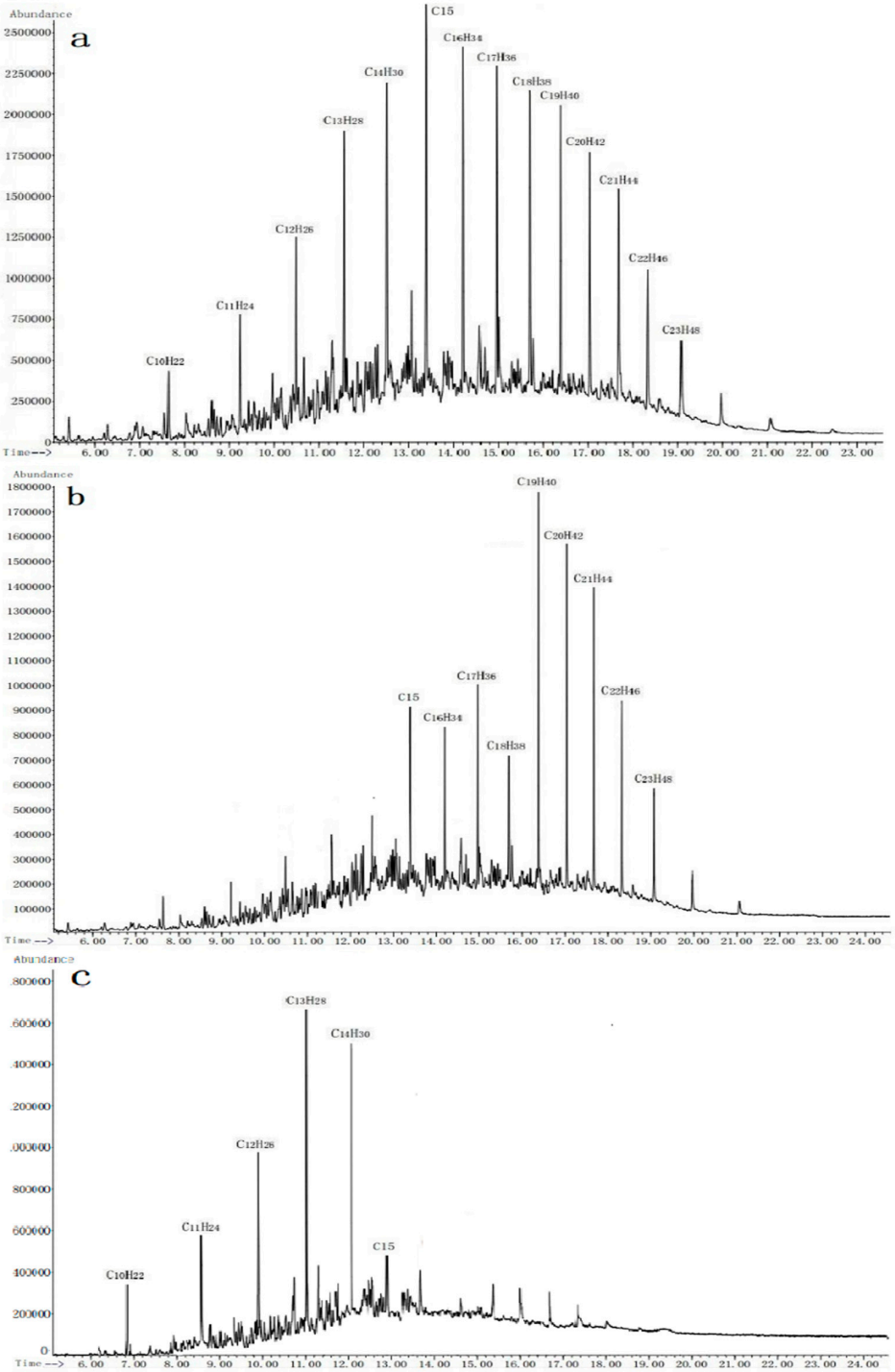

Figure 2. GC-MS spectra of diesel constituents in the absence and presence of Kp and Pa. (**a**) original 1.5% diesel sample; (**b**) culture medium after 8-day degradation by Kp; and (**c**) culture medium after 8-day degradation by Pa.

3.3. Growth Characteristics of Kp and Pa in Media with Diesel or Its Constituents as Sole Carbon Source

We first analyzed the diesel-constituent ranges that the two strains were able to utilize as a sole carbon source. We found that the Pa strain was able to grow (including good-growth and medium-growth) on 86% of diesel constituents on a mass basis, compared to 68% for the Kp strain (Figure 3a,b). Additionally, the Pa strain grew better than the Kp strain on eight diesel constituents (accounting for 75%), while the Kp strain grew better on only seven diesel constituents (accounting for 48%). In terms of alkanes, the Pa strain preferred long-chain constituents (Figure 3b) while the Kp strain preferred short-chain constituents (Figure 3a).

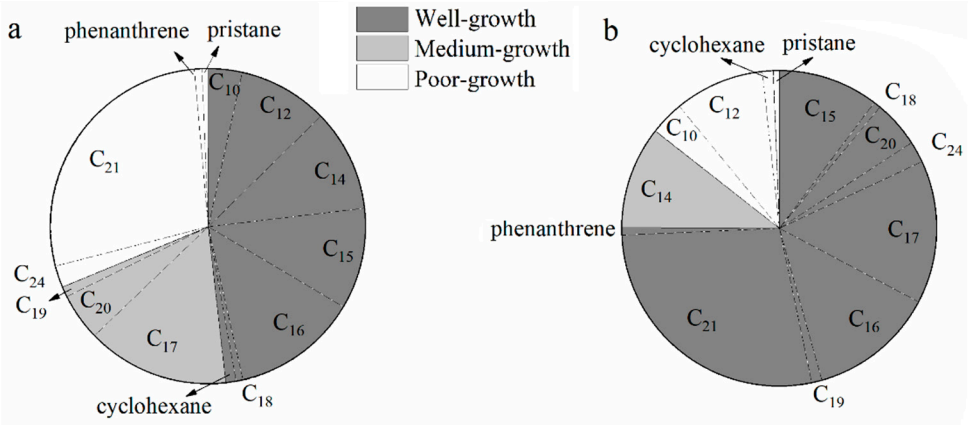

Figure 3. Growth characteristics of Kp (**a**) and Pa (**b**) cultivated with diesel constituents as a sole carbon source. Dark grey, light grey and white areas indicate good-growth (OD600 $\geq$ 1), medium-growth (0.4 < OD600 < 1) and poor-growth (OD600 $\leq$ 0.4), respectively.

In the culture medium with C_{21} as a sole carbon source, the Pa strain was able to grow while the Kp strain could not. In addition, the Kp strain grew poorly in C_{20}- and C_{24}-containing medium, while the Pa strain grew well in C_{20}–C_{24}- as well as in C_{16}-containing medium. In diesel medium (Figure 4a), the biomass of the Pa strain was significantly higher than that of the Kp strain in each period. The relative growth rates of the Kp strain and Pa were 0.4 d^{-1} and 0.6 d^{-1}, respectively (Figure 4b). In the decline period, the declining rate of the Pa strain (-0.03 d^{-1}) was lower than that of the Kp strain (-0.05 d^{-1}). In cyclohexane medium, the Pa strain showed a decline with a relative growth rate of -0.26 d^{-1} (Figure 4c,d). In contrast, the Kp strain showed "S" type growth with a relative growth rate of 0.8 d^{-1}. The biomass production of the Kp strain was higher than that of Pa throughout the culture time.

In summary, the Pa strain grew faster than Kp in diesel medium; however, in cyclohexane medium, Pa was not able to grow while Kp grew well.

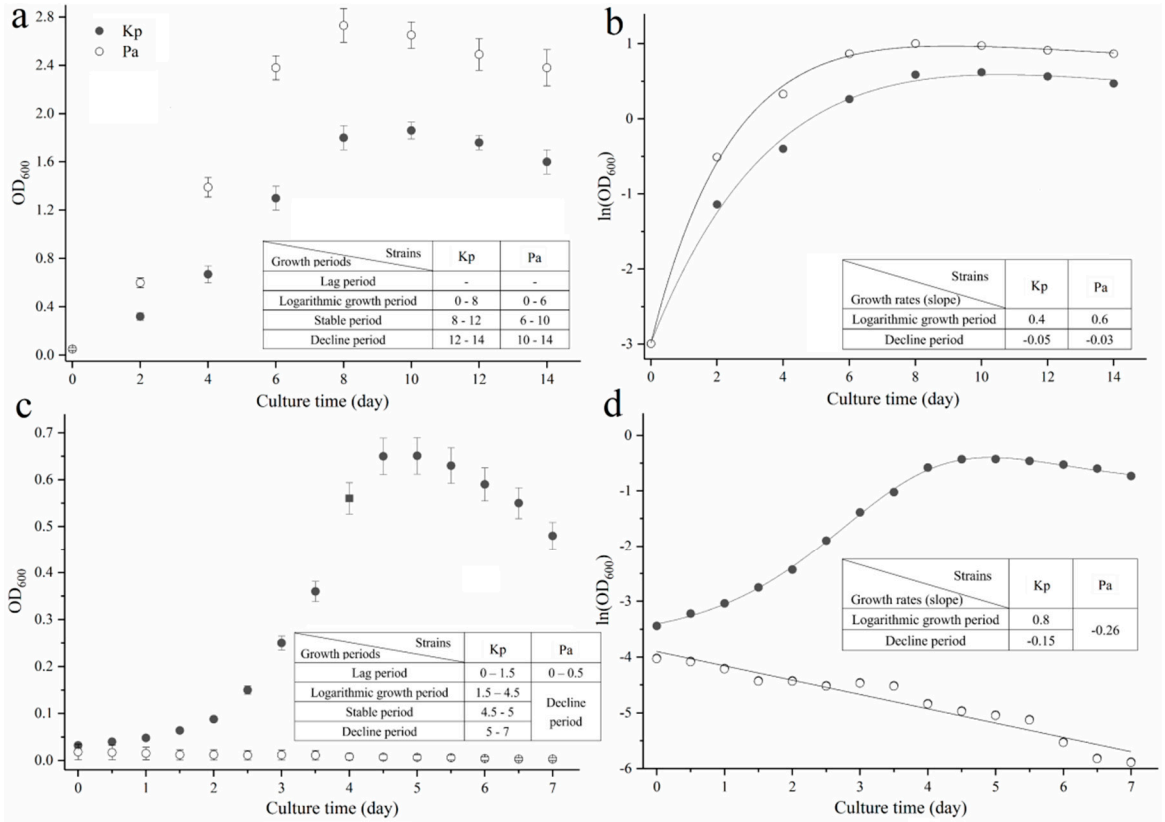

Figure 4. Growth curves of strains Kp and Pa in diesel medium (**a**,**b**) and cyclohexane medium (**c**,**d**). Data in (**a**,**c**) are the original values while those in (**b**) and (**d**) are the corresponding logarithmic values. Relative growth rates were estimated with the slopes during the logarithmic growth period based on the Baranyi-Roberts Growth Model [64].

3.4. Surface Tension, Hydrophobicity and Emulsifiability

The difference between the surface tensions of the two strains was significant. The surface tension of the Pa strain was below 32 mN/m while that of the Kp strain was above 60 mN/m throughout the culture time (Figure 5a). As shown in Figure 5b, the difference in hydrophobicity between the Kp strain and Pa was significant. The initial hydrophobicity of the Pa strain (15%) was higher than that of the Kp strain (9%). This difference in the hydrophobicity increased as the culture time elapsed; however, a decrease was noted on Day 14.

The difference in emulsification between the two strains was also significant (Figure 5c). The emulsifiability of the Kp strain with a maximum value of 0.1, was much lower than that of the Pa strain (0.3–0.6). All the above estimators indicated that the Pa strain might be producing biosurfactant.

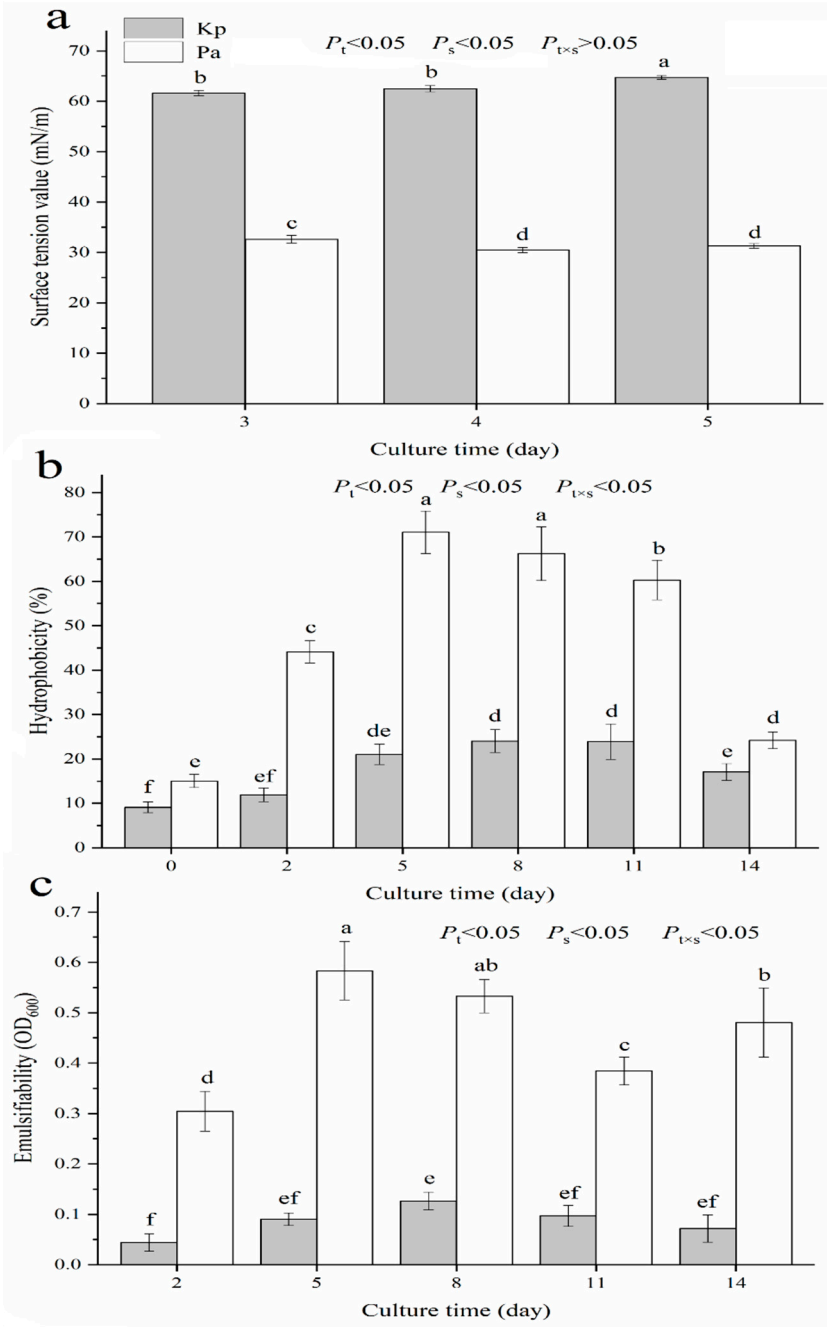

Figure 5. Differences in surface tension (**a**), hydrophobicity (**b**), and emulsifiability (**c**) between the two strains, Kp and Pa.

3.5. Group Characterization of Bacterial Secretions

In order to confirm if the Pa strain produced biosurfactant, we identified its secretions with FTIR and HPLC-MS. FTIR displayed the absorption band of –OH stretch at 3406 cm^{-1}. Those at 2925 and 2860 cm^{-1} were caused by the C–H expansion vibration. The carbonyl and carboxyl expansion vibration at 1737 and 1645 cm^{-1} indicated the existence of lipids, and peaks at 1457 and 1385 cm^{-1} suggested the bending of C–H (–CH_3 and –CH_2). The absorption peak at 1173 cm^{-1} was the stretching vibration peak of C–O (Figure S5). The above estimators suggested that the secretions of the Pa strain were composed of glycolipids.

These compounds had their peaks at 11.01 and 27.35 min, indicating that their molecular weights were 527 and 334, respectively. This indicated that these two compounds were single rhamnolipid

Rha-C12:1-C10 and Rha-C10 [65] (Figures 6 and 7a,b). The molecular weights of the compounds observed at 13.56 and 16 min were 553 and 555, respectively, indicating that these two compounds were double rhamnolipid Rha-Rha-C10:1 and Rha-Rha-C10, respectively (Figures 6 and 7c,d). The peak at 27.35 min represented Rha-C10 (namely 2-*O*-α-L-rhamnyl-β-hydroxydecyl acid; molecular weight: 334), one of the most common structures in rhamnolipids (Figure 6). Among these homologues, the mono-rhamnolipid structure Rha-C12:1-C10 with the RT of 11.01 min had the highest content.

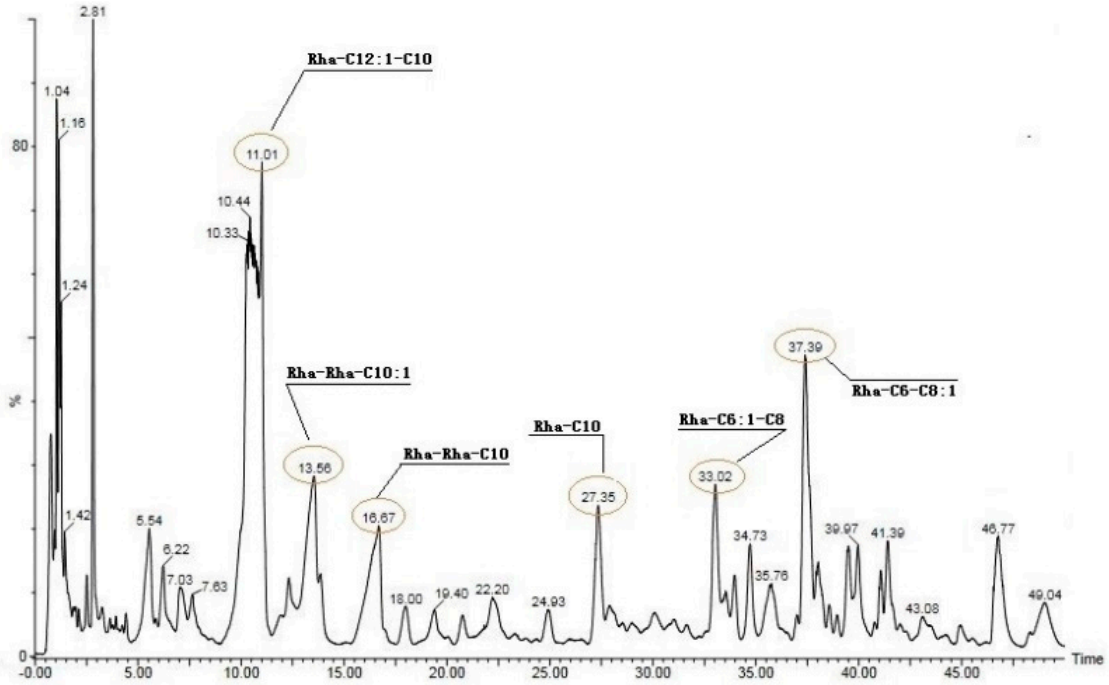

Figure 6. HPLC-MS analysis of Pa secretion.

These results suggested that the Pa strain might produce biosurfactant, which was identified as rhamnolipids with the highest content of mono-rhamnolipid structure. This biosurfactant enhances the surface hydrophobicity of the strain and improved the emulsification of the solution. Meanwhile, there was no evidence showing that the Kp strain would produce any biosurfactant.

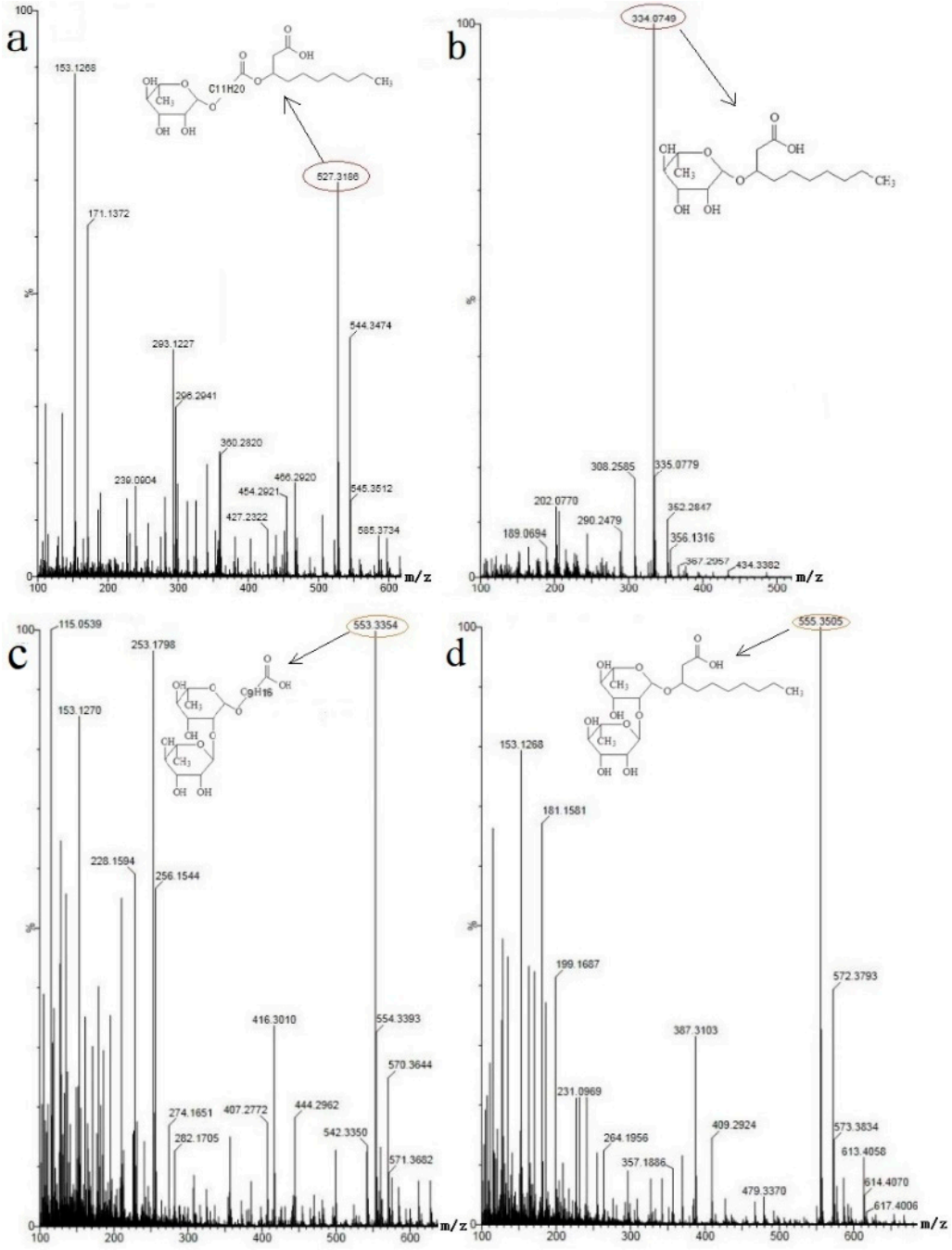

Figure 7. Mass spectrometry of bacterial secretions.

3.6. Potential Factors Related to Degradation Efficacy

To show whether degradation efficacy of the strains was related to the aforementioned biotic and abiotic factors, we constructed a matrix of Spearman correlation coefficients (Figure S6). This matrix showed that all these factors were positively correlated with the degradation efficacy of each strain.

Irrespective of the estimator (CFU or OD_{600}) used to indicate biomass, degradation efficacy was positively correlated with biomass and there was no clear difference in the distribution of data point clouds between these two strains (Figure S7).

We found degradation efficacy was positively correlated with hydrophobicity and emulsifiability but negatively with surface tension values. We also found the data point clouds of the Pa strain were separate from those of the Kp strain (Figure S8).

4. Discussion

We have presented the variation in performance of two bacterial strains, Kp and Pa in the degradation of PHs [1]. We found that the Pa strain was able to reduce diesel (a common form of PHs) by 58%, while the Kp strain degraded 45% of diesel within 14 days. This difference in degradation efficacy for given PHs was even greater in previous studies where one bacterial strain (*Burkholderia fungorum* T3A13001) could degrade pyrene by 59–62% while the other strain (*Caulobacter* sp. T2A12002) only did so by 21–24% over an 18-day incubation. This difference was also reported for two bacterial strains (*Pseudomonas* (22.39–38.32%) and *Rhodococcus* sp. (87.35–99.89%)) in the degradation of diesel and its component *n*-alkane [66]. Additionally, this difference was found for extreme halophilic bacterial strains: one strain could degrade up to 47% of crude oil while another degraded 13% [67].

From two petroleum-contaminated soils, ten strains were screened out and were able to produce biosurfactant and degrade oil. These strains could produce glycolipid biosurfactants, which ranged from 1.93 to 3.7 g L^{-1}. The results showed that four isolates could effectively degrade crude oil in saline mineral broth, with the highest removal efficacy of 39%. Gas chromatography analysis revealed that both lighter and heavier PHs were degraded by microbial cells. It was also demonstrated that branched alkanes as well as linear alkanes were degraded by the isolates. Based on 16S rDNA sequencing, all four isolates were identified as *Pseudomonas aeruginosa* [68]. A hydrocarbon-degrading, biosurfactant-producing, and plant-growth-promoting endophytic bacterium, *Pseudomonas aeruginosa* L10, was isolated from the roots of a reed, Phragmites australis, in the Yellow River Delta, Shandong, China. *P. aeruginosa* L10 effectively degraded C_{10}–C_{26} n-alkanes from diesel, as well as common PAHs such as naphthalene, phenanthrene, and pyrene. In addition, *P. aeruginosa* L10 could produce biosurfactant. Moreover, they identified genes related to PHs degradation, such as putative genes encoding monooxygenase, dioxygenase, alcohol dehydrogenase, and aldehyde dehydrogenase. Genome annotation revealed that *P. aeruginosa* L10 contained a gene cluster involving in the biosynthesis of rhamnolipids, rhlABRI, which could be responsible for the observed biosurfactant activity [69].

There are also many applications of bacterial communities. An in vitro experiment was performed by taking petrol pump soils and diesel in flasks with micronutrient and macronutrient supplements. The bioreactor was inoculated with bacterial communities containing *Moraxella saccharolytica*, *Alteromonas putrefaciens*, *Klebsiella pneumoniae* subsp. aerogenes and *Pseudomonas fragi* along with soil and diesel. The ability of the bacterial inoculum to degrade diesel was analyzed through GC-MS. Smaller chain compounds were obtained after the experimental period of 30 days. The diesel degradation rate was better with the bacterial consortium than with individual bacteria. The bacterial consortium can be a better choice for faster and complete remediation of soils contaminated by hydrocarbons [70].

Together with these previous studies, our study provided strong evidence that PHs degradation capacity is species-specific and a comprehensive understanding of the contributing factors to this species-specific degradation capacity is of importance to enhance microbial remediation of a PHs polluted environment.

Different species have different capacities to utilize given diesel constituents as a sole source of carbon and energy. For example, the *Dietzia* strain DQ12-45-1b was reported to utilize a wide range of *n*-alkanes (C_6–C_{40}) and aromatic compounds of crude oil as a sole carbon source while other strains only use some of these compounds ($\leq C_{25}$) [71]. Similarly, Habib et al. [66] reported that the bacterial strain ADL15 tended to degrade shorter *n*-alkanes (*n*-C_{10}, *n*-C_{11}, *n*-C_{12}) more effectively than the middle-chain *n*-alkanes, while the other bacterial strain, ADL36, could degrade most of the recognizable *n*-alkanes significantly. Therefore, we hypothesized that the difference in diesel degradation between the two strains was partly due to the fact that they had different capacities to utilize diesel constituents. As expected, the Pa strain performed much better than the Kp strain in reducing diesel constituents (especially those with high content), such as C_{14}, C_{17} and C_{21} alkanes (with their contents varying

from 5.77% to 15.2%), but not C_{12} which only accounts for 5.06%. These results suggested that specific bacterial strains or communities are required to utilize given PHs pollutants.

We further assessed the growth characteristics of these two strains in mediums where diesel or its constituents were provided as a sole carbon and energy source. This was because the degradation of PHs by bacteria depends mainly on their strong intracellular enzymes [51,52] and the synthesis and secretion of biosurfactants which are affected by the bacterial growth rate [53]. Therefore, we expected that there would be a positive correlation between PHs degradation efficacy and bacterial growth rate [50,54–56]. As expected, compared with the Kp strain, Pa grew faster in diesel medium and was able to utilize more constituents (on both a number-basis and content-basis). This indicated that the Pa strain had a better capacity to utilize diesel as a carbon source. We also confirmed that degradation efficacy was positively correlated with bacterial growth as indicated by either OD_{600} or CFU. Although the Pa strain did not grow, Kp grew very well in cyclohexane medium. Pa still performed better in degrading diesel which was because cyclohexane accounted for only 0.6% of diesel on a content-basis. Our result suggested that high growth rate, especially in constituents with high content, should be attended to when selecting strains for PHs biodegradation.

The addition of surfactants could significantly promote PHs degradation of oily sludge [72], which might be due to the reduced surface tension [73], and/or enhanced hydrophobicity [46], and/or emulsifiability [74]. Importantly, some microorganisms have been reported to produce biosurfactants. For example, Qiao and Shao [75] identified that a novel biosurfactant (proline lipid) was produced by a marine oil degrading bacterium, *Alcanivorax dieselolei* B-5. In our study, Pa had a lower surface tension value and higher hydrophobicity and emulsifiability than Kp. These results motivated us to investigate whether biosurfactant production contributed to the better performance of Pa in degrading PHs. As expected, we found Pa could excrete glycolipid and we further identified this glycolipid to be rhamnolipid [65]. This compound was found to serve as a surfactant which enhances the solubility of PHs and renders them more accessible for biodegradation [76,77]

We also found that degradation efficacy was positively correlated with hydrophobicity and emulsifiability. In fact, rhamnolipids produced by Pa are well known to interact with toxic xenobiotics—closing them in micelles and making them less toxic to bacteria. The reduction in the toxicity of phenols can be explained by a combination of toxin accumulation in biosurfactant micelles and hydrophobic interactions of phenols with rhamnolipid-based dissolved organic carbon. These results provide evidence that, next to the effect of the micelle formation, hydrophobic interactions with rhamnolipid-based dissolved organic carbon affect the bioavailability of phenols [78]. Besides which, the surfactant-mediated treatment increases hydrocarbon solubilization and potentially facilitates biodegradation. This property of rhamnolipids is of interest to those using biosurfactants for microbial treatment of hydrocarbon-rich wastewaters co-contaminated with toxic compounds [79]. Moreover, more studies have been conducted about rhamnolipids' influence on cell hydrophobicity and hydrocarbons degradation—for both single species, and communities. Both surfactants altered cell surface hydrophobicity of the consortia in a similar manner, i.e., increased for the hydrophilic and decreased for the hydrophobic cultures. Additionally, the surfactants exhibited a similar influence on diesel fuel biodegradation; enhanced biodegradation was observed for initially slow-degrading cultures and the opposite for fast degraders. This indicates that in surfactant-mediated biodegradation, the effectiveness of surfactants depends on the specification of microorganisms and not on the type of surfactant [46,80]. Nevertheless, production of biosurfactant does not mean that other bacteria will not degrade biosurfactant as a preferred carbon source. A study showed that the biodegradation susceptibility order can be established as follows: biodiesel > rhamnolipids > diesel oil. The complete degradation time for the abovementioned carbon sources was estimated to be 5, 10 and 28 days, accordingly. Similar results were observed for nitrate-reducing experiments, although the degradation processes were carried out at a much slower rate [81,82]. Therefore, we confirmed that biosurfactant production by Pa contributed to the higher PHs degradation by the strain.

5. Conclusions

The degradation efficacy of Pa for diesel (a common form of PHs) was 15% higher than that of Kp. In addition, Pa also performed much better in degrading the main constituents of diesel: phenanthrene and C_{16}, C_{20} and C_{24} alkanes (except C_{12}). Specifically, Pa degraded 86% of diesel constituents on a mass basis, compared to Kp (68%). In addition, although Pa grew faster in diesel medium than Kp, in cyclohexane medium, the growth of Kp was higher. Pa was able to produce biosurfactant which was identified as rhamnolipids with the highest content of mono-rhamnolipid structure. This biosurfactant enhanced the surface hydrophobicity of the Pa strain and improved the emulsification of the solution.

This study confirmed that specific bacterial strains or communities would be required to degrade specific PHs pollutants. In particular, strains producing biosurfactant might play an important role in PHs biodegradation, indicating that it might be of great significance to increase the proportion of surfactant strains to improve biodegradation efficiency.

When these two strains are applied for bioremediation of PHs, the potential leak of these species into the environment should be considered and prevented since they are pathogenic. Especially, inoculation of these species in situ is not recommended unless there is robust evidence showing that they are non-invasive or not able to lead to any disease.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/8/12/2551/s1.

Author Contributions: Z.Y. and S.Z. proposed the main idea and conceptualization. H.X. performed experiments, results analysis, and scientific discussions and wrote the paper. L.Z., W.Y. and M.H. also contributed to some of the experiments. Z.Y., S.Z., H.K. and P.-C.C. helped to revise the clarity of the work, as well as write and organize the paper. Finally, H.K. assisted in the language correction and submission of the article.

Funding: This work was financially supported by the Natural Science Foundation of the Jiangsu Province in China (No. BK20160689). H. Kim was financially supported by the Korea Institute of Energy Technology Evaluation and Planning (No. 2017010000780).

References

1. Nakazawa, M.M.; Gavazza, S.; Kato, M.T.; Florencio, L. Evaluation of rhamnolipid addition on the natural attenuation of estuarine sediments contaminated with diesel oil. *Environ. Sci. Pollut. R.* **2017**, *24*, 25522–25533. [CrossRef]
2. Shpiner, R.; Liu, G.; Stuckey, D. Treatment of oilfield produced water by waste stabilization ponds: Biodegradation of petroleum-derived materials. *Bioresour. Technol.* **2009**, *100*, 6229–6235. [CrossRef] [PubMed]
3. Shen, T.; Pi, Y.; Bao, M.; Xu, N.; Li, Y.; Lu, J. Biodegradation of different petroleum hydrocarbons by free and immobilized microbial consortia. *Environ. Sci. Proc. Impact.* **2015**, *17*, 2022–2033. [CrossRef] [PubMed]
4. Atlas, R.M.; Hazen, T.C. Oil Biodegradation and Bioremediation: A Tale of the Two Worst Spills in US History. *Environ. Sci. Technol.* **2011**, *45*, 6709–6715. [CrossRef] [PubMed]
5. Pinedo, J.; Ibanez, R.; Lijzen, J.P.A.; Irabien, A. Assessment of soil pollution based on total petroleum hydrocarbons and individual oil substances. *J. Environ. Manag.* **2013**, *130*, 72–79. [CrossRef] [PubMed]
6. Wu, J.W.; Zheng, X.L.; Li, L.L.; Sun, J. Adsorption of aqueous oil on sands and its studies of effective factors. *Environ. Sci.* **2006**, *27*, 2019.
7. Wang, Z.Q.; Wu, Q.; Zou, Z.G.; Chen, H.; Yang, X.C.; Zhao, J.C. Study on the groundwater petroleum contaminant remediation by air sparging. *Environ. Sci.* **2007**, *28*, 754.
8. Ugochukwu, U.C.; Ochonogor, A. Groundwater contamination by polycyclic aromatic hydrocarbon due to diesel spill from a telecom base station in a Nigerian City: Assessment of human health risk exposure. *Environ. Monit. Assess.* **2018**, *190*, 249. [CrossRef]
9. Cheng, Q.; Ge, W.; Chai, C.; Wu, J.; Ma, D.; Zeng, L.; Zhu, X.; Chen, Q.; Li, J. Polycyclic Aromatic Hydrocarbons in Soil Around Coal-Fired Power Plants in Shandong, China. *Pol. J. Environ. Stud.* **2019**, *28*, 53–65. [CrossRef]

10. Wojtera-Kwiczor, J.; Zukowska, W.; Graj, W.; Malecka, A.; Piechalak, A.; Ciszewska, L.; Chrzanowski, L.; Lisiecki, P.; Komorowicz, I.; Baralkiewicz, D.; et al. Rhizoremediation of diesel-contaminated soil with two rapeseed varieties and petroleum degraders reveals different responses of the plant defense mechanisms. *Int. J. Phytoremediat.* **2014**, *16*, 770–789. [CrossRef]
11. Polyak, Y.M.; Bakina, L.G.; Chugunova, M.V.; Mayachkina, N.V.; Gerasimov, A.O.; Bure, V.M. Effect of remediation strategies on biological activity of oil-contaminated soil—A field study. *Int. Biodeterior. Biodegrad.* **2018**, *126*, 57–68. [CrossRef]
12. Safdari, M.S.; Kariminia, H.R.; Nejad, Z.G.; Fletcher, T.H. Study Potential of Indigenous Pseudomonas aeruginosa and Bacillus subtilis in Bioremediation of Diesel-Contaminated Water. *Water Air Soil Poll.* **2017**, *228*, 1–7. [CrossRef]
13. Vazquez, S.; Monien, P.; Minetti, R.P.; Jurgens, J.; Curtosi, A.; Primitz, J.V.; Frickenhaus, S.; Abele, D.; Mac Cormack, W.; Helmke, E. Bacterial communities and chemical parameters in soils and coastal sediments in response to diesel spills at Carlini Station, Antarctica. *Sci. Total. Environ.* **2017**, *605*, 26–37. [CrossRef] [PubMed]
14. Wang, Y.; Nie, M.Q.; Wan, Y.; Tian, X.T.; Nie, H.Y.; Zi, J.; Ma, X. Functional characterization of two alkane hydroxylases in a versatile Pseudomonas aeruginosa strain NY3. *Ann. Microbiol.* **2017**, *67*, 459–468. [CrossRef]
15. Rashidi, H.R.; Sulaiman, N.M.N.; Hashim, N.A.; Hassan, C.R.C.; Emami, S.D. Simulated textile (batik) wastewater pre-treatment through application of a baffle separation tank. *Desalin. Water. Treat.* **2016**, *57*, 151–160. [CrossRef]
16. Sang, Y.M.; Gu, Q.B.; Sun, T.C.; Li, F.S. Color and organic compounds removal from secondary effluent of landfill leachate with a novel inorganic polymer coagulant. *Water. Sci. Technol.* **2008**, *58*, 1423–1432. [CrossRef] [PubMed]
17. De Oliveira, T.V.C.; Cardoso, S.L.; Vieira, R.B.; Vieira, P.A.; Cardoso, V.L. Biodegradation of effluent contaminated with diesel oil and gasoline using chitosan as a natural coagulant in a continuous process. *Braz. J. Chem. Eng.* **2016**, *4*, 863. [CrossRef]
18. Mailler, R.; Gasperi, J.; Rocher, V.; Gilbert-Pawlik, S.; Geara-Matta, D.; Moilleron, R.; Chebbo, G. Biofiltration vs conventional activated sludge plants: what about priority and emerging pollutants removal? *Environ. Sci. Pollut. Res.* **2014**, *21*, 5379–5390. [CrossRef]
19. Gill, R.T.; Harbottle, M.J.; Smith, J.W.; Thornton, S.F. Electrokinetic-enhanced bioremediation of organic contaminants: A review of processes and environmental applications. *Chemosphere* **2014**, *107*, 31–42. [CrossRef]
20. Kadri, T.; Rouissi, T.; Kaur Brar, S.; Cledon, M.; Sarma, S.; Verma, M. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by fungal enzymes: A review. *J. Environ. Sci. China* **2017**, *51*, 52–74. [CrossRef]
21. Kachieng'a, L.; Momba, M.N.B. Kinetics of petroleum oil biodegradation by a consortium of three protozoan isolates (Aspidisca sp., Trachelophyllum sp. and Peranema sp.). *Biotechnol. Rep. (Amst.)* **2017**, *15*, 125–131. [CrossRef] [PubMed]
22. Tso, S.F.; Taghon, G.L. Protozoan grazing increases mineralization of naphthalene in marine sediment. *Microb. Ecol.* **2006**, *51*, 460–469. [CrossRef] [PubMed]
23. Agrawal, N.; Shahi, S.K. Degradation of polycyclic aromatic hydrocarbon (pyrene) using novel fungal strain Coriolopsis byrsina strain APC5. *Int. Biodeter. Biodegr.* **2017**, *122*, 69–81. [CrossRef]
24. Moubasher, A.H.; Abdel-Sater, M.A.; Soliman, Z.S.M. Yeasts and filamentous fungi associated with some dairy products in Egypt. *J. Mycol. Med.* **2018**, *28*, 76–86. [CrossRef] [PubMed]
25. Acevedo-Sandoval, O.; Gutierrez-Alcantara, E.J.; Perez-Balan, R.; Rodriguez-Vazquez, G.; Zamorategui-Molina, A.; Tirado-Torres, D. Degradation Of Polycyclic Aromatic Hydrocarbons Using Bacterial Isolate From the Contaminated Soil And White Rot Fungus Pleurotus Ostreatus. *Appl. Ecol. Environ. Res.* **2018**, *16*, 3815–3829. [CrossRef]
26. Shaeyan, M.; Tirandaz, H.; Ghanbarpour, S.; Seyedipour, N.; Shavandi, M.; Dastgheib, S.M.M. Bioremediation of a drilling waste-contaminated soil; biotreatability assessment and microcosm optimization for developing a field-scale remediation process. *Iran. J. Biotechnol.* **2018**, *16*, 193–199. [CrossRef]
27. Al-Hawash, A.B.; Li, S.; Alhujaily, A.; Zhang, X.; Ma, F.; Dragh, M.A.; Abbood, H.A. Principles of microbial degradation of petroleum hydrocarbons in the environment. *Egypt. J. Aquat. Res.* **2018**, *44*, 71–76. [CrossRef]

28. Zhang, Z.Z.; Gai, L.X.; Hou, Z.W.; Yang, C.Y.; Ma, C.Q.; Wang, Z.G.; Sun, B.P.; He, X.F.; Tang, H.Z.; Xu, P. Characterization and biotechnological potential of petroleum-degrading bacteria isolated from oil-contaminated soils. *Bioresour. Technol.* **2010**, *101*, 8452–8456. [CrossRef]
29. You-Qing, L.; Hong-Fang, L.; Zhen-Le, T.; Li-Hua, Z.; Ying-Hui, W.; He-Qing, T. Diesel pollution biodegradation: Synergetic effect of Mycobacterium and filamentous fungi. *Biomed. Environ. Sci.* **2008**, *21*, 181–187.
30. Xue, J.; Yu, Y.; Bai, Y.; Wang, L.; Wu, Y. Marine Oil-Degrading Microorganisms and Biodegradation Process of Petroleum Hydrocarbon in Marine Environments: A Review. *Curr. Microbiol.* **2015**, *71*, 220–228. [CrossRef]
31. Wei, G.U.O.; Mengchang, H.E.; Zhifeng, Y. A Review of Studies on the Degradation of Petroleum Hydrocarbon in Soils and Sediments by Microorganism. *Bull. Mineral. Petrol. Geochem.* **2007**, *26*, 276–283.
32. Montagnolli, R.N.; Lopes, P.R.; Bidoia, E.D. Assessing Bacillus subtilis biosurfactant effects on the biodegradation of petroleum products. *Environ. Monit. Assess.* **2015**, *187*, 1–17. [CrossRef] [PubMed]
33. Yu, M.X.; Li, Z.; Ji, Q.N.; Wang, S.W.; Su, D.G.; Lin, Y.S. Effect of thermal oxidation of activated carbon surface on its adsorption towards dibenzothiophene. *Chem. Eng. J.* **2009**, *148*, 242–247. [CrossRef]
34. Adam, G.; Duncan, H.J. Effect of diesel fuel on growth of selected plant species. *Environ. Geochem. Health* **1999**, *21*, 353–357. [CrossRef]
35. Sugiura, K.; Ishihara, M.; Toshitsugu Shimauchi, A.; Harayama, S. Physicochemical Properties and Biodegradability of Crude Oil. *Environ. Sci. Technol.* **1997**, *31*, 45–51. [CrossRef]
36. Takei, D.; Washio, K.; Morikawa, M. Identification of alkane hydroxylase genes in Rhodococcus sp. strain TMP2 that degrades a branched alkane. *Biotechnol. Lett.* **2008**, *30*, 1447. [CrossRef] [PubMed]
37. Zhang, H.; Tang, J.; Wang, L.; Liu, J.; Gurav, R.G.; Sun, K. A novel bioremediation strategy for petroleum hydrocarbon pollutants using salt tolerant Corynebacterium variabile HRJ4 and biochar. *J. Environ. Sci. China* **2016**, *47*, 7–13. [CrossRef] [PubMed]
38. Ulrici, W. *Contaminant Soil Areas, Different Countries and Contaminant Monitoring of Contaminants*, Biotechnology: Second, Completely Revised Edition; Wiley Online Library: Hoboken, NJ, USA, 2008; pp. 11–12.
39. Ron, E.Z.; Rosenberg, E. Biosurfactants and oil bioremediation. *Curr. Opin. Biotechnol.* **2002**, *13*, 249–252. [CrossRef]
40. Morán, A.C.; Olivera, N.; Commendatore, M.; Esteves, J.L.; Siñeriz, F. Enhancement of hydrocarbon wastebiodegradation by addition of a biosurfactantfrom Bacillus subtilis O9. *Biodegradation* **2000**, *11*, 65–71. [CrossRef]
41. Tsomides, H.J.; Hughes, J.B.; Thomas, J.M.; Ward, C.H. Effect of surfactant addition on phenanthrene biodegradation in sediments. *Environ. Toxicol. Chem.* **1995**, *14*, 953–959. [CrossRef]
42. Chrzanowski, Ł.; Czaczyk, K. Why do microorganisms produce rhamnolipids? *World J. Microbiol. Biotechnol.* **2012**, *28*, 401–419. [CrossRef] [PubMed]
43. Kumar, M.; Leon, V.; Materano, A.S.; Ilzins, O.A. Enhancement of oil degradation by co-culture of hydrocarbon degrading and biosurfactant producing bacteria. *Pol. J. Microbiol.* **2006**, *55*, 139. [PubMed]
44. Lu, J.R.; Zhao, X.B.; Yaseen, M. Biomimetic amphiphiles: Biosurfactants. *Curr. Opin. Colloid In.* **2007**, *12*, 60–67. [CrossRef]
45. Lindman, B.; Wennerström, H. Miceles. Amphiphile aggregation in aqueous solution. *Top. Curr. Chem.* **1980**, *87*, 1–83. [PubMed]
46. Owsianiak, M.; Szulc, A.; Chrzanowski, Ł.; Cyplik, P.; Bogacki, M.; Olejnikschmidt, A.K.; Heipieper, H.J. Biodegradation and surfactant-mediated biodegradation of diesel fuel by 218 microbial consortia are not correlated to cell surface hydrophobicity. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 545–553. [CrossRef] [PubMed]
47. Tian, W.; Yao, J.; Liu, R.; Zhu, M.; Wang, F.; Wu, X.; Liu, H. Effect of natural and synthetic surfactants on crude oil biodegradation by indigenous strains. *Ecotoxicol. Environ. Saf.* **2016**, *129*, 171. [CrossRef] [PubMed]
48. Muthukamalam, S.; Sivagangavathi, S.; Dhrishya, D.; Sudha, S.R. Characterization of dioxygenases and biosurfactants produced by crude oil degrading soil bacteria. *Braz. J. Microbiol.* **2017**, *48*, 637–647. [CrossRef] [PubMed]
49. Reiling, H.E.; Thanei-Wyss, U.; Guerra-Santos, L.H.; Hirt, R.; Käppeli, O.; Fiechter, A. Pilot plant production of rhamnolipid biosurfactant by Pseudomonas aeruginosa. *Appl. Environ. Microbiol.* **1986**, *51*, 985–989.
50. Xu, J.; Deng, H.; Huang, T.; Song, S. Enhanced biodegradation of crude oil in contaminated soil by inoculation of hydrocarbon-degraders. *Desalin. Water Treat.* **2014**, *52*, 5126–5135. [CrossRef]

51. Van Hamme, J.D.; Singh, A.; Ward, O.P. Recent advances in petroleum microbiology. *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 503–549. [CrossRef]
52. Pi, Y.; Meng, L.; Bao, M.; Sun, P.; Lu, J. Degradation of crude oil and relationship with bacteria and enzymatic activities in laboratory testing. *Int. Biodeterior. Biodegrad.* **2016**, *106*, 106–116. [CrossRef]
53. Fuchs, G.; Boll, M.; Heider, J. Microbial degradation of aromatic compounds—From one strategy to four. *Nat. Rev. Microbiol.* **2011**, *9*, 803–816. [CrossRef] [PubMed]
54. Kronenberg, M.; Trably, E.; Bernet, N.; Patureau, D. Biodegradation of polycyclic aromatic hydrocarbons: Using microbial bioelectrochemical systems to overcome an impasse. *Environ. Pollut.* **2017**, *231*, 509–523. [CrossRef] [PubMed]
55. Ostendorf, D.W.; Schoenberg, T.H.; Hinlein, E.S.; Long, S.C. Monod kinetics for aerobic biodegradation of petroleum hydrocarbons in unsaturated soil microcosms. *Environ. Sci. Technol.* **2007**, *41*, 2343–2349. [CrossRef] [PubMed]
56. Wang, Z.-Y.; Xu, Y.; Wang, H.-Y.; Zhao, J.; Gao, D.-M.; Li, F.-M.; Xing, B. Biodegradation of Crude Oil in Contaminated Soils by Free and Immobilized Microorganisms. *Pedosphere* **2012**, *22*, 717–725. [CrossRef]
57. Xu, X.; You, Z.; Zhang, S.; Tang, Y.; Li, Z.; Xu, H. Synergistic mechanisms of degradation of diesel oil by bacterial consortium. *Environ. Sci. Technol.* **2018**, *41*, 47–55.
58. Patowary, K.; Saikia, R.R.; Kalita, M.C.; Deka, S. Degradation of polyaromatic hydrocarbons employing biosurfactant-producing Bacillus pumilus KS2. *Ann. Microbiol.* **2015**, *65*, 225–234. [CrossRef]
59. Wang, L.; Tang, Y.; Wang, S.; Liu, R.L.; Liu, M.Z.; Zhang, Y.; Liang, F.L.; Feng, L. Isolation and characterization of a novel thermophilic Bacillus strain degrading long-chain n-alkanes. *Extremophiles* **2006**, *10*, 347–356. [CrossRef] [PubMed]
60. Bouchez-Naitali, M.; Rakatozafy, H.; Marchal, R.; Leveau, J.Y.; Vandecasteele, J.P. Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. *J. Appl. Microbiol.* **1999**, *86*, 421–428. [CrossRef] [PubMed]
61. Tzintzun-Camacho, O.; Loera, O.; Ramirez-Saad, H.C.; Gutierrez-Rojas, M. Comparison of mechanisms of hexadecane uptake among pure and mixed cultures derived from a bacterial consortium. *Int. Biodeterior. Biodegrad.* **2012**, *70*, 1–7. [CrossRef]
62. Mariaamalraj, S.K.; Pasumarthi, R.; Achary, A.; Mutnuri, S. Effect of rhamnolipid on biodegradation of hydrocarbons in non-aqueous-phase liquid (NAPL). *Bioremed. J.* **2016**, *20*, 183–193. [CrossRef]
63. Coreteam, R. R: A language and environment for statistical computing. *Computing* **2015**, *1*, 12–21.
64. Perni, S.; Andrew, P.W.; Shama, G. Estimating the maximum growth rate from microbial growth curves: definition is everything. *Food Microbiol.* **2005**, *22*, 491–495. [CrossRef]
65. Wei, S. Electrospray ionization mass spectrometric detection of rhamnolipids and their acid precursors in Pseudomonas sp. BS-03 cultures. *Prog. Biotechnol.* **2005**, *25*, 83–87.
66. Habib, S.; Ahmad, S.A.; Johari, W.L.W.; Abd Shukor, M.Y.; Alias, S.A.; Khalil, K.A.; Yasid, N.A. Evaluation of conventional and response surface level optimisation of n-dodecane (n-C12) mineralisation by psychrotolerant strains isolated from pristine soil at Southern Victoria Island, Antarctica. *Microb. Cell Fact.* **2018**, *17*, 44–65. [CrossRef]
67. Almailem, D.M.; Sorkhoh, N.A.; Alawadhi, H.; Eliyas, M.; Radwan, S.S. Biodegradation of crude oil and pure hydrocarbons by extreme halophilic archaea from hypersaline coasts of the Arabian Gulf. *Extremophiles* **2010**, *14*, 321–328. [CrossRef]
68. Ebadi, A.; Olamaee, M.; Sima, N.A.K.; Nasrabadi, R.G.; Hashemi, M. Isolation and Characterization of Biosurfactant Producing and Crude Oil Degrading Bacteria from Oil Contaminated Soils. *Iran. J. Sci. Technol. A* **2018**, *42*, 1149–1156. [CrossRef]
69. Wu, T.; Xu, J.; Xie, W.; Yao, Z.; Yang, H.; Sun, C.; Li, X. Pseudomonas aeruginosa L10: A Hydrocarbon-Degrading, Biosurfactant-Producing, and Plant-Growth-Promoting Endophytic Bacterium Isolated From a Reed (Phragmites australis). *Front. Microbiol.* **2018**, *9*, 1087–1099. [CrossRef]
70. Sharma, A.; Rehman, M.B. Laboratory scale bioremediation of diesel hydrocarbon in soil by indigenous bacterial consortium. *Indian J. Exp. Biol.* **2009**, *47*, 766–769.
71. Wang, X.B.; Chi, C.Q.; Nie, Y.; Tang, Y.Q.; Tan, Y.; Wu, G.; Wu, X.L. Degradation of petroleum hydrocarbons (C6-C40) and crude oil by a novel Dietzia strain. *Bioresour. Technol.* **2011**, *102*, 7755–7761. [CrossRef]

72. Gojgic-Cvijovic, G.D.; Milic, J.S.; Solevic, T.M.; Beskoski, V.P.; Ilic, M.V.; Djokic, L.S.; Narancic, T.M.; Vrvic, M.M. Biodegradation of petroleum sludge and petroleum polluted soil by a bacterial consortium: A laboratory study. *Biodegradation* **2012**, *23*, 1–14. [CrossRef] [PubMed]
73. Lingyi, M. Screening, Purification and Analysis of a Strain Producted Glycolipid Surfactant. Ph.D. Thesis, Northeast Normal University, Changchun, China, 2015.
74. Reddy, P.G.; Singh, H.D.; Pathak, M.G.; Bhagat, S.D.; Baruah, J.N. Isolation And Functional-Characterization Of Hydrocarbon Emulsifying And Solubilizing Factors Produced by a Pseudomonas Species. *Biotechnol Bioeng.* **1983**, *25*, 387–401. [CrossRef] [PubMed]
75. Qiao, N.; Shao, Z. Isolation and characterization of a novel biosurfactant produced by hydrocarbon-degrading bacterium Alcanivorax dieselolei B-5. *J. Appl. Microbiol.* **2010**, *108*, 1207–1216. [CrossRef]
76. Mani, P.; Dineshkumar, G.; Jayaseelan, T.; Deepalakshmi, K.; Kumar, C.G.; Balan, S.S. Antimicrobial activities of a promising glycolipid biosurfactant from a novel marine Staphylococcussaprophyticus SBPS 15. *3Biotech* **2016**, *6*, 163–172.
77. Batista, S.B.; Mounteer, A.H.; Amorim, F.R.; Tótola, M.R. Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. *Bioresour. Technol.* **2006**, *97*, 868–875. [CrossRef] [PubMed]
78. Chrzanowski, L.; Wick, L.Y.; Meulenkamp, R.; Kaestner, M.; Heipieper, H.J. Rhamnolipid biosurfactants decrease the toxicity of chlorinated phenols to Pseudomonas putida DOT-T1E. *Lett. Appl. Microbiol.* **2010**, *48*, 756–762.
79. Chrzanowski, L.; Owsianiak, M.; Szulc, A.; Marecik, R.; Piotrowska-Cyplik, A.; Olejnik-Schmidt, A.K.; Staniewski, J.; Lisiecki, P.; Ciesielczyk, F.; Jesionowski, T. Interactions between rhamnolipid biosurfactants and toxic chlorinated phenols enhance biodegradation of a model hydrocarbon-rich effluent. *Int. Biodeterior. Biodegrad.* **2011**, *65*, 605–611. [CrossRef]
80. Kaczorek, E.; Chrzanowski, L.; Pijanowska, A.; Olszanowski, A. Yeast and bacteria cell hydrophobicity and hydrocarbon biodegradation in the presence of natural surfactants: Rhamnolipides and saponins. *Bioresour. Technol.* **2008**, *99*, 4285–4291. [CrossRef] [PubMed]
81. Łukasz, C.; Dziadas, M.; Łukasz, Ł.; Cyplik, P.; Białas, W.; Szulc, A.; Lisiecki, P.; Jeleń, H. Biodegradation of rhamnolipids in liquid cultures: Effect of biosurfactant dissipation on diesel fuel/B20 blend biodegradation efficiency and bacterial community composition. *Bioresour. Technol.* **2012**, *111*, 328–335.
82. Sydow, M.; Owsianiak, M.; Szczepaniak, Z.; Framski, G.; Smets, B.F.; Łukasz, Ł.; Lisiecki, P.; Szulc, A.; Cyplik, P.; Łukasz, C. Evaluating robustness of a diesel-degrading bacterial consortium isolated from contaminated soil. *New Biotechnol.* **2016**, *33*, 852–859. [CrossRef]