Article

Effects of Adding Laccase to Bacterial Consortia Degrading Heavy Oil

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Abstract: High-efficiency bioremediation technology for heavy oil pollution has been a popular research topic in recent years. Laccase is very promising for the remediation of heavy oil pollution because it can not only convert bio-refractory hydrocarbons into less toxic or completely harmless compounds, but also accelerate the biodegradation efficiency of heavy oil. However, there are few reports on the use of laccase to enhance the biodegradation of heavy oil. In this study, we investigated the effect of laccase on the bacterial consortia degradation of heavy oil. The degradation efficiencies of bacterial consortia and the laccase-bacterial consortia were $60.6 \pm 0.1\%$ and $68.2 \pm 0.6\%$, respectively, and the corresponding heavy oil degradation rate constants were 0.112 day^{-1} and 0.198 day^{-1} , respectively. The addition of laccase increased the heavy oil biodegradation efficiency ($p < 0.05$) and biodegradation rate of the bacterial consortia. Moreover, gas chromatography–mass spectrometry analysis showed that the biodegradation efficiencies of the laccase-bacterial consortia for saturated hydrocarbons and aromatic hydrocarbons were $82.5 \pm 0.7\%$ and $76.2 \pm 0.9\%$, respectively, which were $16.0 \pm 0.3\%$ and $13.0 \pm 1.8\%$ higher than those of the bacterial consortia, respectively. In addition, the degradation rate constants of the laccase-bacterial consortia for saturated hydrocarbons and aromatic hydrocarbons were 0.267 day^{-1} and 0.226 day^{-1} , respectively, which were 1.07 and 1.15 times higher than those of the bacterial consortia, respectively. The degradation of C15 to C35 n-alkanes and 2 to 5-ring polycyclic aromatic hydrocarbons by laccase-bacterial consortia was higher than individual bacterial consortia. It is further seen that the addition of laccase significantly improved the biodegradation of long-chain n-alkanes of C22–C35 ($p < 0.05$). Overall, this study shows that the combination of laccase and bacterial consortia is an effective remediation technology for heavy oil pollution. Adding laccase can significantly improve the heavy oil biodegradation efficiency and biodegradation rate of the bacterial consortia.

Keywords: heavy oil pollution; bioremediation; laccase; bacterial consortia; degradation

Citation: Dai, X.; Lv, J.; Wei, W.; Guo, S. Effects of Adding Laccase to Bacterial Consortia Degrading Heavy Oil. *Processes* **2021**, *9*, 2025.

<https://doi.org/10.3390/pr9112025>

Academic Editor: Alexey V. Vakhin

Received: 16 September 2021

Accepted: 10 November 2021

Published: 12 November 2021

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1. Introduction

The use of heavy oil is rapidly growing to meet escalating energy demands; consequently, the pollution resulting from accidents involving heavy oil are also increasing annually [1,2]. Compared with conventional crude oil, heavy oil has a much higher density and viscosity, and it contains high-molecular-weight hydrocarbons (especially highly polycyclic aromatic hydrocarbons), heterocyclic compounds, and heavy metals [3,4]. Moreover, heavy oil pollution causes severe and lasting harm to both the ecological environment and human health. Therefore, there is an urgent need for efficient remediation technologies for heavy oil pollution [5–7].

Microbial remediation technology based on the addition of efficient bacteria is promising for the treatment of heavy oil pollution owing to its low cost and prevention of secondary pollution [8]. However, it is difficult for high-efficiency bacteria to adapt to actual

heavy oil pollution environments and survive several of the biological and abiotic restrictions, such as physical and chemical requirements, as well as indigenous microbial competition [9,10]. This results in the slow start-up of the bioremediation process. In addition, the high content of refractory organic compounds, such as long-chain aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) in heavy oil, also brings about new challenges to the microbial remediation of heavy oil pollution [4,11]. The biodegradability of hydrocarbons in heavy oil generally decreases in the following order: n-alkanes, branched alkanes, branched alkenes, low molecular weight n-alkyl aromatics, monoaromatics, cycloalkanes, polycyclic aromatic hydrocarbons (PAHs), and asphaltene [4]. It has been reported that enzymes can quickly start the biodegradation of toxic and refractory pollutants. Laccase, oxidases, reductases, depolymerases, peroxidases, dioxygenases, and dehydrogenases are utilized for the degradation of different toxic environmental pollutants (PAHs, dyes, heavy metals, plastics, pesticides, etc.) [12–14]. The advantages of enzymatic degradation of toxic environmental pollutants include highly flexible operational conditions; ease of control; a rapid, cost-effective, and highly specific process; no need of nutrient supply; reduced mass transfer limitation; resistance to protozoa predation and toxic pollutants, etc. [13]. Therefore, it can be applied to both in situ bioremediation and ex situ bioremediation.

Several recent studies have demonstrated the potential of laccase to degrade hydrocarbons [15–18]. Laccase (EC 1.10.3.2), a polyphenol oxidase, is a well-known biocatalyst for oxidizing various PHAs, phenols, and aromatic amines to form quinones or oligomers, as well as to reduce molecular oxygen to produce water [15]. Wu et al. [19] used laccase to treat aged PAH-contaminated soils. Their results showed that laccase could degrade 15 priority PAHs (specified by the U.S. Environmental Protection Agency) to varying degrees (11% to 86%) after 24 h. Bautista et al. [20] used immobilized laccase to degrade 82% naphthalene, 73% phenanthrene, and 55% anthracene in PAHs. Kucharzyk et al. [14] demonstrated that the co-immobilized preparation of laccase and manganese peroxidase can effectively improve the degradation activity of petroleum-degrading microorganisms in contaminated sites and significantly promote the biodegradation of petroleum hydrocarbons, especially PAHs. Laccase has high activity in degrading hydrocarbons; however, there are few reports on the degradation of heavy oil by microorganisms combined with laccase. Liu et al. [21] demonstrated that bacteria–fungi (secreting laccase) joint remediation can effectively degrade petroleum hydrocarbons contained in soil, and the higher the laccase production and activity, the more stable the growth situation of white-rot fungi, yielding better TPH degradation rates and remediation results. This motivated us to utilize bench-scale experimental systems to investigate the effectiveness of laccase in heavy oil biodegradation processes.

This study investigated the effect of adding laccase on the bacterial consortia degradation of heavy oil. The heavy oil degradation efficiency and kinetics of the bacterial consortia and laccase-bacterial consortia were studied. Changes in saturated hydrocarbons and aromatic hydrocarbons during heavy oil biodegradation were characterized by gas chromatography–mass spectrometry (GC–MS).

2. Materials and Methods

2.1. The Heavy Oil Composition

The experimental oil was Venezuelan extra-heavy crude oil with diluents, containing 35.33% saturated hydrocarbons, 34.11% aromatic hydrocarbons, 19.87% asphaltenes, and 12.27% resins. Its API degree was 16. It was sourced from the PetroChina Liaohe Petrochemical Company, located in Liaoning Province, China.

2.2. Heavy Oil–Degrading Bacterial Consortia

The heavy oil-degrading bacterial consortia were constructed by our research group, and were composed of *Brevibacillus* sp. DL-1, *Bacillus* sp. DL-13, and *Acinetobacter schindleri* DL-34, which all are biosurfactant producing bacteria and heavy oil degrading bacteria.

They can degrade 60.75% heavy oil in 8 days. The bacterial strain was separately incubated in a rotatory shaker (160 rpm) under aerobic conditions at 30 °C for 24 h. Cells in the Luria-Bertani (LB) medium were harvested by centrifugation at 5000 rpm for 10 min and washed three times, and the OD₆₀₀ was adjusted to 0.8 using distilled saline solution. The composition and heavy oil degradation characteristics of the bacterial consortia were reported in a previous study [22]. The bacterial consortia were constructed by optimizing the degradation efficiency of heavy oil.

2.3. Crude Laccase Solution

The crude laccase solution was prepared using genetic engineering technology. The laccase-encoding gene was amplified from the total DNA of *Bacillus subtilis* subsp. *Subtilis* str. 168 using specific primers. The amplified laccase-encoding gene was then gel-purified (iNtRON) and ligated into the pMD19-T vector (TAKARA). The recombinant plasmid pMD19-T carrying the laccase-encoding gene was transferred to *E. coli* DH5 α via electroporation, and the genetically engineered bacteria containing the laccase-encoding gene were obtained via screening with blue and white spots. Plasmid DNA was extracted from selected clones using a plasmid DNA extraction kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The laccase-encoding gene was amplified from the plasmid DNA via polymerase chain reaction with specific primers and then sequenced (BGI Biotech Co., Ltd., Beijing, China).

After the laccase-encoding gene sequence was corroborated in the recombinant pMD19-T vector, the vector containing the laccase-encoding gene was digested using *EcoR* I and *Bam*H I and then ligated into the corresponding sites of the pET28a (+) vector, which was initially digested using the same restriction enzymes. The ligated mixture was then transformed into the protein expression strain *E. coli* BL21 (DE3). Laccase was prepared via the fermentation of the genetically engineered *E. coli* BL21 (DE3) for laccase overexpression, and the fermentation broth was centrifuged to collect cells at a low temperature. Subsequently, the cells were subjected to sonication for 10 min on ice in pulses of 5 s on and 10 s off at 50% amplitude. Finally, the cell lysate was centrifuged at 4 °C and 12,000 $\times$ g for 30 min to remove cell debris. The supernatant was used as the crude laccase solution.

2.4. Culture Medium

The LB medium contained 10 g/L peptone, 10 g/L beef extract, and 10 g/L NaCl. The medium was adjusted to pH 7.0 and sterilized by autoclaving at 121 °C for 30 min before use. Solid LB medium was prepared by adding 20 g of agar to 1 L of LB.

The mineral salt medium (MSM) contained 5 g/L (NH₄)₂SO₄, 3 g/L KH₂PO₄, 2 g/L Na₂HPO₄·2H₂O, 0.7 g/L MgSO₄·7H₂O, and 1 mL of trace element solution. The medium was adjusted to pH 7.0 and sterilized by autoclaving at 121 °C for 30 min before use. Solid MSM medium was prepared by adding 20 g of agar to 1 L of MSM.

The trace element solution contained 50 mg/L FeCl₃·6H₂O, 10 mg/L ZnSO₄·7H₂O, 2 mg/L CaCl₂, 0.5 mg/L MnCl₂·4H₂O, and 0.5 mg/L CuSO₄. The medium was filtered and sterilized with a 0.22 μ m filter membrane and stored for later use.

The heavy oil medium contained 0.01 g heavy oil and 30 mL MSM. The culture medium was sterilized using high-temperature and high-pressure steam at 121 °C for 30 min and stored for later use.

2.5. Heavy Oil Biodegradation

The bacterial consortia were inoculated in 30 mL of the MSM medium with 0.01 g of heavy crude oil. The vials were incubated in a rotatory shaker (160 rpm) under aerobic conditions at 30 °C for 14 d. All experiments were performed in triplicate. The residual heavy oil in each vial was extracted using 40 mL of CCl₄ at time intervals of 0, 2, 4, 6, 8, and 10 d.

The crude laccase enzyme solution and bacterial consortia were inoculated in 30 mL of the MSM medium with 0.01 g of heavy crude oil. The vials were incubated in a rotatory shaker (160 rpm) under aerobic conditions at 30 °C for 14 d. The residual heavy oil in each vial was extracted using 40 mL CCl₄ at time intervals of 0, 2, 4, 6, 8, and 10 d.

2.6. Heavy Oil Degradation Efficiency and Degradation Kinetics

The heavy oil in the extract was measured using an infrared oil content analyzer (OIL-460, Beijing ChinaInvent Instrument Tech. Ltd., Beijing, China) according to the national standard method in China [23]. The residual heavy oil in each vial was extracted using 40 mL of CCl₄ for quantification and GC–MS analysis, and the extraction process was repeated three times. The heavy oil degradation efficiency was calculated using Equation (1):

$$\text{Heavy oil degradation efficiency (\%)} = \frac{C_0 - C_t}{C_0} \times 100\% \quad (1)$$

where C_0 is the initial heavy oil concentration and C_t is the heavy oil concentration after t days of incubation.

The microbial degradation of heavy oil is typically expressed using first-order kinetics [24,25], as given in Equation (2):

$$\ln C_t = -kt + b \quad (2)$$

where C_t is the heavy oil concentration after t days of incubation, k is the biodegradation rate constant (day^{−1}), t is the time (day), and b is the fitting constant.

2.7. Separation and GC–MS Analysis of the Saturated and Aromatic Hydrocarbons of Heavy Oil

The composition of heavy oil is complex. According to its chemical structure, there are four main components of heavy oil: saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes [26]. The residual heavy oil was separated using column chromatography. The detailed method is as follows: first, 0.7 g of heavy oil was dried using nitrogen, redissolved in petroleum ether (30 °C to 60 °C), and then left to stand and precipitate. The petroleum ether solution was filtered to produce colorless liquid using absorbent cotton, which was then dried with nitrogen, concentrated to 5 mL, and separated via column chromatography. The adsorbent of the stationary phase was active silica gel/active alumina with a mass ratio of 3:2. The former was extracted with chloroform, boiled with distilled water, and activated in an electric drying oven; the latter was activated and dried in a muffle furnace. The alkanes and aromatics were eluted with petroleum ether (30 °C to 60 °C) and petroleum ether (30 °C to 60 °C)/dichloromethane (1:2, v/v) and analyzed using GC–MS with $n\text{-C}_{24}\text{D}_{50}$ and DBT-D₈ as standards, respectively. The detailed operation parameters can be found in a previous study by Cai et al. [27].

3. Results

3.1. Crude Laccase Solution Preparation for Heavy Oil Degradation

The use of crude laccase has major advantages over the use of purified laccase in terms of industrial scale operations. This is because it reduces the downstream purification processes, and the synergistic action of the mixture provides a more efficient oxidation than the purified enzyme [28,29]. Therefore, the crude laccase solution was used for heavy oil microbial degradation in this study. This solution was prepared via the overexpression of laccase in genetically engineered *E. coli* BL21, and its activity was 9.5 ± 0.7 U/mL, with ABTS as the substrate. The activity of laccase was significantly increased to 1.5 times higher than that of the wild strain (6.3 ± 0.3 U/mL). This result is consistent with the results of previous studies [30,31]. It has been widely demonstrated that the laccase heterologously expressed in *E. coli* BL21 has higher activity and yield than that found in wild strains.

3.2. Heavy Oil Degradation Efficiency and Kinetics of the Laccase-Bacterial Consortia

Figure 1 shows that after 10 d, the heavy oil degradation efficiency of the bacterial consortia was $60.6 \pm 0.1\%$, and the heavy oil concentration decreased from 223.6 ± 2.8 mg/L to 88.1 ± 0.4 mg/L. Considering the laccase-bacterial consortia, the heavy oil degradation efficiency was $68.2 \pm 0.6\%$, and the heavy oil concentration decreased from 223.6 ± 2.8 mg/L to 71.1 ± 0.9 mg/L. The heavy oil degradation efficiency of the laccase-bacterial consortia was $7.6 \pm 0.6\%$ higher than that of the bacterial consortia. Laccase has no obvious degradation effect on heavy oil. This trend demonstrates that the laccase cannot be used for heavy oil degradation alone, but can catalyze the further degradation of heavy oil degradation products by bacteria consortia. Furthermore, laccase enhanced the heavy oil biodegradation rate (Figure 2). The kinetic constants of the heavy oil degradation of the bacterial consortia and the laccase-bacterial consortia were 0.112 day^{-1} and 0.198 day^{-1} , respectively; the laccase action increased the degradation rate by 1.9 times. These results show that laccase can improve the heavy oil biodegradation efficiency and biodegradation rate by directly participating in the initial oxygenation of PAHs or other petroleum hydrocarbons [32,33].

Figure 1. Effect of laccase on heavy oil degradation of bacterial consortia.

3.3. Biodegradation of Saturated Hydrocarbons of Heavy Oil

The GC–MS results showed that the laccase-bacterial consortia had a significant removal effect on the saturated hydrocarbons of the heavy oil. On day 6 of the degradation process by the laccase-bacterial consortia, the degradation efficiency of the saturated hydrocarbons increased rapidly to a maximum of $82.4 \pm 0.7\%$. However, for the bacterial consortia degradation, the degradation efficiency of the saturated hydrocarbons reached the maximum of $66.3 \pm 0.4\%$ on day 8. The laccase-bacterial consortia increased the degradation range and degradation rate of the saturated hydrocarbons. The degradation rate constant of the laccase-bacterial consortia for saturated hydrocarbons was 0.267 day^{-1} (Figure 3b), which was significantly higher than that of the bacterial consortia for saturated hydrocarbons (0.129 day^{-1}).

Figure 2. Heavy oil degradation kinetics of the (a) bacterial consortia and (b) laccase-bacterial consortia.

Figure 3. Degradation efficiencies and degradation kinetics of the (a) bacterial consortia and (b) laccase-bacterial consortia for saturated hydrocarbons.

Furthermore, n-alkanes are the most readily degradable components in heavy oil [26] that significantly influence heavy oil degradation efficiency. GC-MS analysis showed

that the chain length of n-alkanes in the experimental heavy oil was mainly between C15 and C35, with C19 and C29 as the main alkanes. The n-alkane abundance in the heavy oil significantly decreased due to the degradation process (Table 1), whereby the degradation efficiency of the laccase-bacterial consortia for C15 to C35 was $80.9 \pm 9.2\%$ to 100% ; moreover, that for C15 to C17 was greater than 94.0% . Compared with the bacterial consortia, the degradation efficiency of C15 to C35 was significantly increased, especially for C22 to C35, the degradation efficiency increased from approximately 60% to approximately 80% . Based on the above experimental results, it can be inferred that n-alkanes can be used as small molecular mediators to participate in the laccase degradation of polycyclic aromatic hydrocarbons [18,34] and long-chain n-alkanes can also be used as co-metabolic substrates to participate in PAH degradation [35].

Table 1. Degradation efficiencies of the bacterial consortia and laccase-bacterial consortia for C15 to C35 n-alkanes.

n-Alkanes	Bacterial Consortia Degradation			Laccase-Bacterial Consortia Degradation		
	Initial Abundance	Residual Abundance	Degradation Efficiency (%)	Initial Abundance	Residual Abundance	Degradation Efficiency (%)
C15	1.4	ND	97.6 ± 0.9	2.0 ± 0.4	ND	100
C16	6.0 ± 0.1	0.1	98.0 ± 0.6	4.6 ± 0.7	0.1	98.3 ± 0.3
C17	10.4 ± 0.6	0.8	92.4 ± 0.4	7.4 ± 0.9	0.4 ± 0.1	94.0 ± 0.2
C18	14.6 ± 0.3	2.7 ± 0.1	81.7 ± 0.5	8.4 ± 0.7	0.9 ± 0.1	89.0 ± 1.5
C19	19.8 ± 0.5	4.8 ± 0.5	75.9 ± 2.1	9.1 ± 1.6	1.3 ± 0.4	84.4 ± 6.5
C20	26.0 ± 0.9	7.3 ± 0.5	72.0 ± 3.0	10.1 ± 1.2	1.7 ± 0.3	83.3 ± 4.8
C21	30.5 ± 1.6	9.1 ± 0.6	70.2 ± 3.0	10.3 ± 1.6	1.8 ± 0.4	83.0 ± 1.4
C22	32.9 ± 0.7	10.4 ± 0.9	68.4 ± 3.1	10.4 ± 2.0	1.8 ± 0.3	82.0 ± 3.4
C23	37.1 ± 1.0	12.2 ± 1.0	67.1 ± 2.5	10.9 ± 1.4	1.9 ± 0.2	81.6 ± 4.4
C24	33.5 ± 1.4	11.7 ± 0.9	64.9 ± 4.0	9.9 ± 1.2	1.7 ± 0.4	81.6 ± 6.8
C25	38.5 ± 1.0	13.9 ± 1.0	63.9 ± 2.4	11.1 ± 1.0	1.9 ± 0.2	82.4 ± 3.5
C26	30.0 ± 1.1	10.6 ± 0.9	64.3 ± 4.3	9.0 ± 0.7	1.6 ± 0.4	82.6 ± 4.9
C27	28.0 ± 0.8	9.9 ± 0.7	64.6 ± 3.3	8.3 ± 0.8	1.4 ± 0.2	82.7 ± 1.7
C28	21.4 ± 0.6	7.8 ± 0.7	63.5 ± 2.3	6.6 ± 0.5	1.1 ± 0.2	83.0 ± 2.4
C29	19.4 ± 1.0	7.3 ± 0.7	62.2 ± 5.3	5.9 ± 0.8	1.1 ± 0.2	81.9 ± 3.7
C30	13.8 ± 0.8	5.4 ± 0.5	60.9 ± 6.3	4.4 ± 0.9	0.8 ± 0.2	80.9 ± 9.2
C31	12.6 ± 0.8	4.8 ± 0.5	61.5 ± 4.4	4.1 ± 0.6	0.7 ± 0.1	81.9 ± 6.2
C32	7.8 ± 0.7	2.9 ± 0.4	63.2 ± 2.3	2.6 ± 0.4	0.5 ± 0.3	83.0 ± 7.1
C33	5.9 ± 0.6	2.3 ± 0.3	61.1 ± 3.3	2.2 ± 10.1	0.4 ± 0.1	82.0 ± 6.3
C34	5.0 ± 0.4	1.9 ± 0.4	62.9 ± 4.4	2.1 ± 0.4	0.4 ± 0.1	82.6 ± 4.8
C35	3.2 ± 0.5	1.1 ± 0.2	65.3 ± 1.0	1.6 ± 0.4	0.3 ± 0.1	82.9 ± 3.7

ND means “not detected”.

3.4. Biodegradation of the Aromatic Hydrocarbons of Heavy Oil

Aromatic hydrocarbons, especially PAHs, in heavy oil are the key components limiting heavy oil degradation [26]. On day 6 of degradation by the laccase-bacterial consortia, the degradation efficiency of aromatic hydrocarbons increased rapidly to a maximum of $76.2 \pm 0.9\%$ (Figure 4b), which was 13.06% higher than that of the bacterial consortia. The laccase increased the degradation range and degradation rate of aromatic hydrocarbons by the bacterial consortia, which is consistent with the findings of previous studies [32,36]. The kinetic constant of the laccase-bacterial consortia for aromatic hydrocarbons was 0.226 day^{-1} , which was 2.15 times that of the bacterial consortia for saturated hydrocarbons

(0.105 day⁻¹) and was higher than that of the bacterial consortia for heavy oil (0.198 day⁻¹). These results showed that aromatic hydrocarbons were not the rate-limiting step of heavy oil degradation by laccase bacteria. It can be inferred that refractory resin and asphaltene are the key components affecting the degradation rate of heavy oil [37,38].

Figure 4. Degradation efficiencies and degradation kinetics of aromatic hydrocarbons for the (a) bacterial consortia and (b) laccase-bacterial consortia.

Polycyclic aromatic hydrocarbons are important components of aromatic hydrocarbons, and they significantly influence the efficiency of heavy oil degradation. The GC–MS analysis showed that the abundance of PAHs in the heavy oil significantly decreased due to the degradation process (Table 2). The degradation efficiencies of the laccase-bacterial consortia for 2-, 3-, 4-, and 5-ring PAHs were 100%, $65.4 \pm 1.6\%$ to $95.5 \pm 0.2\%$, $52.4 \pm 8.5\%$ to $61.9 \pm 0.3\%$, and $49.9 \pm 3.4\%$, respectively. Considering the bacterial consortia degradation, the degradation efficiencies for the 2-, 3-, 4-, and 5-ring PAHs were $72.7 \pm 3.6\%$ to $96.6 \pm 4.8\%$, $54.3 \pm 5.5\%$ to $87.5 \pm 1.2\%$, $50.2 \pm 2.0\%$ to $57.3 \pm 6.9\%$, and $40.8 \pm 1.0\%$, respectively. The laccase-bacterial consortia significantly improved the PAH degradation compared to the bacterial consortia. These results corroborate those of previous studies [36,39], suggesting that laccase can catalyze the initial oxygenation of PAHs in heavy oil, and the oxidation products are more conducive to microbial biodegradation.

Table 2. Degradation efficiencies of the bacterial consortia and laccase-bacterial consortia for PAHs.

PAHs	Bacterial Consortia Degradation			Laccase-Bacterial Consortia Degradation		
	Initial Abundance	Residual Abundance	Degradation Efficiency (%)	Initial Abundance	Residual Abundance	Degradation Efficiency (%)
Nap	0.1 ± 0.03	ND	96.6 ± 4.8	ND	ND	ND
C1-Nap	0.80 ± 0.1	0.2 ± 0.1	77.2 ± 7.0	ND	ND	ND
C2-Nap	1.11 ± 0.2	0.3 ± 0.1	76.2 ± 7.2	0.14	ND	100
C3-Nap	4.0 ± 0.4	1.0 ± 0.2	74.0 ± 5.2	1.7 ± 0.04	ND	100
C4-Nap	3.6 ± 0.5	1.0 ± 0.2	72.7 ± 3.6	2.1 ± 0.1	ND	100
C5-Nap	0.44 ± 0.1	0.1 ± 0.01	74.8 ± 2.9	1.2 ± 0.2	ND	100
Phe	2.66 ± 0.3	0.7 ± 0.1	74.9 ± 3.6	1.8 ± 0.1	0.1 ± 0.002	95.5 ± 0.2
C1-Phe	6.6 ± 0.6	2.8 ± 0.6	57.1 ± 9.0	5.4 ± 0.2	1.1 ± 0.1	78.6 ± 2.6
C2-Phe	7.66 ± 0.6	3.5 ± 0.5	54.3 ± 5.5	6.4 ± 0.3	1.7 ± 0.1	74.0 ± 1.1
C3-Phe	4.4 ± 0.5	2.0 ± 0.3	54.6 ± 3.3	3.8 ± 0.2	1.3 ± 0.04	65.4 ± 1.6
Flu	0.2 ± 0.04	0.03 ± 0.02	86.2 ± 6.5	0.1 ± 0.011	ND	100
C1-Flu	1.19 ± 0.25	0.2 ± 0.04	87.5 ± 1.2	0.7 ± 0.1	ND	100
C2-Flu	2.1 ± 0.4	0.6 ± 0.1	70.1 ± 1.9	1.6 ± 0.1	0.1 ± 0.007	94.8 ± 0.4
B[b]F	0.3 ± 0.1	0.1 ± 0.03	50.2 ± 2.0	0.2 ± 0.02	0.1 ± 0.01	61.9 ± 0.3
Chr	0.5 ± 0.2	0.2 ± 0.1	54.9 ± 3.0	0.6 ± 0.04	0.3 ± 0.02	55.6 ± 7.7
C1-Chr	1.2 ± 0.2	0.5 ± 0.1	55.9 ± 3.8	1.2 ± 0.1	0.5 ± 0.02	56.3 ± 3.3
C2-Chr	1.2 ± 0.3	0.6 ± 0.1	53.7 ± 5.9	1.8 ± 0.2	0.8 ± 0.1	55.4 ± 1.0
Pyr	0.4 ± 0.1	0.2 ± 0.1	57.3 ± 6.9	0.4 ± 0.01	0.2 ± 0.03	57.3 ± 3.4
C1-Pyr	1.0 ± 0.2	0.5 ± 0.1	51.1 ± 1.3	0.9 ± 0.1	0.4 ± 0.01	52.4 ± 8.5
B[e]P	0.3 ± 0.1	0.2 ± 0.04	40.8 ± 1.0	0.3 ± 0.02	0.2 ± 0.01	49.9 ± 3.4

ND means “not detected”.

4. Discussion

The inoculation of specific bacterial consortia with multiple catabolic genes is a reasonable and feasible strategy for accelerating the efficiency of heavy oil removal from polluted environments [40–43]. However, the presence of PAHs with high toxicity and large molecular weight limits the start-up and biodegradation of the added bacterial consortia [13,14]. Therefore, we presume that the rapid degradation of PAHs (the main toxic components of heavy oil) into non-toxic or low toxic substances can quickly start heavy oil biodegradation. Enzymes will be appropriate agents for PAHs rapid degradation as they require neither nutrition from their environment nor necessity for the prevention of predators and toxic substances [18]. Enzymes have been used to biodegrade a broad variety of contaminants, including heavy metals, toxins, dyes, polycyclic aromatic hydrocarbons, and plastics, in place of harsh chemicals [18]. They efficiently degrade the high level of pollutants by utilizing them as substrates.

Laccase can catalyze the initial oxygenation reaction of PAHs degradation; it can convert or transform the PAH compounds into their corresponding quinone forms [44]. However, laccase could not be involved in further oxidation of quinones due to lacking oxidase enzymes. Therefore, its oxidation products need to be further degraded by bacteria consortia [44,45]. In this paper, we investigated the effect of laccase addition on bacterial consortia degradation of heavy oil. The results showed that the laccase can improve degradation efficiency and degradation rate of bacterial degradation of heavy oil by quickly starting the degradation of PAHs in heavy oil. Our research provides an important research idea for the degradation of highly toxic and refractory organic pollutants.

5. Conclusions

In this study, we investigated the effect of laccase on the bacterial consortia degradation of heavy oil. The degradation efficiency of heavy oil was $68.2 \pm 0.6\%$, with the degradation rate constant of 0.198 day^{-1} . The biodegradation efficiency and biodegradation rate of heavy oil was significantly higher ($p < 0.05$) than individual bacterial consortia. The laccase-bacterial consortia could significantly remove saturated hydrocarbons and aromatic hydrocarbons in heavy oil, particularly C15 to C35 n-alkanes and 2 to 5-ring PAHs. The degradation efficiencies for saturated hydrocarbons and aromatic hydrocarbons were $82.4 \pm 0.7\%$ and $76.2 \pm 0.9\%$, respectively, with degradation rate constants of 0.267 day^{-1} and 0.226 day^{-1} , respectively. It was also found that the addition of laccase significantly promoted the biodegradation of long-chain n-alkanes of C22–C35 ($p < 0.05$). These results show that the addition of laccase can significantly improve the biodegradation efficiency and biodegradation rate of heavy oil. The combined remediation technology of laccase and bacterial consortia has great application potential in both in-situ and ex-situ bioremediation of heavy oil pollution.

Author Contributions: X.D.: conceptualization, investigation, data curation, writing—original draft, writing—review and editing, visualization, supervision; J.L.: methodology, resources; W.W.: investigation, funding acquisition; S.G.: writing—review and editing, funding acquisition, supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key Research and Development Program of China (grant number 2018YFC1801200 and 2018YFC1801301) and Special Project for Reform and Development of Beijing Public Welfare Research Institutes of Beijing Academy of Science and Technology (grant number 2021G-0011).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We thank Yunyang Wan for the for the experimental materials.

Conflicts of Interest: The authors declare no conflict of interest.

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