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Original Research

Isolation of Petroleum Degraders and Petroleum-Degradation Characteristics of Crude Enzymes from *Providencia rettgeri* L1

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Abstract

A hydrocarbon-degrading bacterium *Providencia rettgeri* L1 was isolated from petroleum in Shengli oil field, China. The petroleum-degradation characteristics were investigated. The degradation rate of petroleum by the strain was 45.9% in petroleum-mineral medium (5 g/L) in 14 d measured by ultraviolet-visible spectrophotometer. The intracellular crude enzymes of *Providencia rettgeri* L1 were prepared and supplemented with formate dehydrogenase to construct an enzyme cocktail. The petroleum degradation rate of the cocktail reached 49.5% in 8 h, which was much faster than the strain. The subfraction saturates and aromatics in petroleum were analyzed by gas chromatography and gas chromatography-mass spectrometry, respectively. After degradation for 8 h, the degradation rate of saturates C_{10}-C_{40} was 50.4%, while the values for short, mid and long-chain saturates were 52.6%, 49.4% and 47.7%, respectively. Biodegradation assessment markers-pristane and phytane, were degraded by 49.3% and 44.9%, respectively. The degradation rate of aromatics was 29.5%. For 2, 3, 4 and 5-ring PAHs, the corresponding values were 47.8%, 24.2%, 29.5% and 19.8%, respectively. Source tracing experiment showed that *Providencia rettgeri* L1 was more dominant in enriched bacterial suspensions than in petroleum environment, indicating its advantage of rapid proliferation in culture media. This study can provide some insights into fast degradation of petroleum.

Keywords: *Providencia rettgeri*, petroleum, degradation, crude enzymes, high-throughput sequencing

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Introduction

The rapid development of the petroleum industry has improved people's life quality since the early 1990s. However, it has also caused substantial damage to the environment [1, 2]. Petroleum contaminants contain various organic compounds such as persistent organic pollutants (POPs) and polycyclic aromatic hydrocarbons (PAHs), both of which are notorious for their toxicity and recalcitrance nature. Soil petroleum contamination has been a major concern since it brings a threat to agriculture and the food chain, and thus to human health. Bioremediation techniques were often adopted in petroleum-contaminated soils as they are cost-effective and environment-friendly, of which bioaugmentation is one of the most popular techniques [3, 4].

Bioaugmentation provided active exogenous degrading microbes and available nutrients to promote petroleum degradation. Current researches about bioaugmentation focus on screening strains or microbial consortium with high-degrading efficiency. Many degraders were isolated, mixed consortiums were constructed, corresponding agents were prepared and then applied into the contaminated sites [5-7]. However, it took a long time for degrading microbes to degrade the petroleum contaminants. The transmembrane transport of contaminants limited their degradation rate. Intracellular enzymes played a key role in degradation of petroleum by microbes [8, 9]. The use of degrading enzymes rather than microbes would accelerate the degrading rate and shorten the remediation period, which is suitable for urgent remediation of petroleum contamination. Attempts have been made to explore the availability of enzyme remediation. Qian and Chen [10] observed enhanced oxidation of benzo[a]pyrene by crude enzyme extracts produced during interspecific fungal interaction of *Trametes versicolor* and *Phanerochaete chrysosporium*. Kucharzyk et al. [11] reported that concentrations of crude oil in sediment decreased following the treatment with ligninolytic enzymes. Ji et al. [12] constructed an enzyme cocktail containing NADH regeneration system for efficient bioremediation of oil sludge contamination. When treating the sludge with 10% oil (w/w), oil degradation rate achieved 35.6% after 12 h. Zhuo and Fan [13] comprehensively reviewed the application of enzymatic systems of white rot fungi in the removal of organic pollutants such as PAHs, pharmaceutically active compounds, endocrine disruptor compounds, pesticides, synthetic dyes and other environmental pollutants. Some promising results have been achieved.

Despite these elegant studies, researches on petroleum degradation by enzymes are still limited, and much additional work is required for the application availability of degrading enzymes. The aims of this study were to (1) isolate and identify petroleum-degrading strains; (2) study the petroleum-degrading characteristics of crude enzymes obtained from the isolated strain; (3) understand the relative abundance of the isolated strain in the bacterial community in petroleum. This study will shed light on preparation and application of petroleum-degrading enzymes derived from bacteria.

Materials and Methods

Petroleum

Petroleum (residual oil) was collected from Shengli Oilfield (37°28'N, 118°29'E), which is the second largest oil field in China. The petroleum was separated into four parts using SARA fractionation method [14], which included saturates (13.6%), aromatics (33.7%), resins (25.0%) and asphaltenes (27.7 %).

Isolation and Identification of Petroleum Degraders

The petroleum-degrading strains were cultured and isolated from the petroleum using the culture-dependent method. Petroleum was added into 30 ml of mineral medium as the sole carbon source to reach the concentration of 5 g/L. The mineral medium contained NH_4NO_3 1.0 g/L, K_2HPO_4 0.5 g/L, $MgSO_4$ 0.02 g/L, NaCl 1.0 g/L, $CaCl_2$ 0.02 mg/L and trace element solution 5 mL ($FeCl_3 \cdot 6H_2O$ 50 mg/L, $CuSO_4$ 0.5 mg/L, $MnCl_4 \cdot 4H_2O$ 0.5 mg/L and $ZnSO_4 \cdot 7H_2O$ 10 mg/L with pH 7.0-7.2). The flask was incubated at 30ºC, 160 rpm for 10 d. Then, 1 ml of aliquot was transferred to a fresh petroleum-mineral medium to perform another 10-d cultivation. Total 5 sequential rounds were conducted. The 5th enriched bacterial suspension was used to isolate the strains through serial dilution and streak plate techniques.

Identification of isolated strains was based on their colonial and cellular morphology and molecular taxonomy study [15]. The 16S rRNA gene was amplified with the universal primers 7F (5'-CAGAGTTTGATCCTGGCT-3') and 1540R (5'-AGGAGGTGATCCAGCCGCA-3'). The sequences obtained by PCR were searched on NCBI Nucleotide BLAST. The isolated strain with the best petroleum-degrading performance was sent to Majorbio Bio-Pharm Technology Company (Shanghai, China) for complete genome sequencing.

Petroleum-Degrading Capacity of the Strains

The isolated strains were activated in 30 ml of Luria-Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.0-7.2) at 30ºC, 160 rpm for 24 h. Then 10 % (v/v) of the inoculum was transferred to 30 ml of petroleum-mineral medium. The flasks were placed on a rotary shaker for 14 d to perform petroleum degradation. The petroleum-mineral medium without the inoculants was set as negative control. The experiment was conducted in triplicates.

The residual petroleum was extracted with 1:1 (v:v) dichloromethane via shaking at 25ºC, 150 rpm for 30 min and ultrasonic extraction for 10 min followed by liquid-liquid extraction for 3 times. The organic phase was collected, dehydrated by anhydrous sodium sulfate, and concentrated to 5 mL. The petroleum concentration was determined using ultraviolet-visible (UV-Vis) spectrophotometer at 230 nm [16]. The degradation rate was calculated based on the absorbance of petroleum before and after degradation.

Petroleum-Degrading Capacity of Crude Enzymes from the Strain

Preparation of Crude Enzymes

The isolated strain with the best petroleum-degrading performance was selected to prepare crude enzymes. The strain was activated in LB medium and then 10 % (v/v) of the inoculum was transferred to the induction medium (NH_4NO_3 1.0 g/L, K_2HPO_4 0.5 g/L, KH_2PO_4 1.0 g/L, $MgSO_4$ 0.2 g/L, NaCl 1.0 g/L, $CaCl_2$ 0.02 g/L, $FeCl_3$ 0.002 g/L, yeast extract 5 g/L, peptone 10 g/L, petroleum 5 g/L, pH 7.0-7.2). The flasks were shaken for 16 h at 30ºC, 160 rpm and the cells were harvested via centrifugation (4ºC, 8000 rpm, 10 min). The precipitates were transferred to the 50-ml tubes followed by addition of phosphate buffer solution (PBS, 0.01 M, pH 7.5) with the weight/volume ratio of 1 g : 15 ml. The suspension was sonicated at 320 W for 17 min (work/break, 2 s/2 s) in an ice bath. The suspension was then centrifuged at 4ºC, 8000 rpm for 10 min and the supernatant was collected as the intracellular crude enzymes.

Formate dehydrogenase (FDH) was prepared using the method described by Ji et al. (2019) [12]. The FDH gene of *Candida boidinii* was cloned and expressed in the strain *E. coli* BL21. Briefly, E.coli BL21-FDH was activated in LB medium and cultured in ZYP medium [12] (peptone 10 g/L, yeast extract 5 g/L, $Na_2HPO_4{\cdot}12H_2O$ 9 g/L, KH_2PO_4 6.8 g/L, $(NH_4)_2SO_4$ 3.3 g/L, $CaCl_2$ 0.02 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, glucose 0.5 g/L, lactose 4.81 g/L, glycerol 0.74% (v/v), ampicillin 100 mg/L, pH 7.0-7.2). The cultures were collected and centrifuged. The precipitates were suspended in PBS and sonicated. The suspension was centrifuged and the supernatant was obtained as FDH.

Degradation of Petroleum by Crude Enzymes

The total 10 ml of reaction system contained the crude enzymes obtained from the petroleum-degrading strain (1.5 ml), FDH (2.5 ml) and PBS (6 ml). The concentrations of petroleum and sodium formate in the reaction system were 5 g/L and 147 mmol/L, respectively. The residual petroleum was analyzed after reaction at 30ºC for 1~8 h. The reaction without enzymes (replaced by PBS) was the control. Experiment was conducted in triplicates.

The residual petroleum was determined using UV-Vis method described above. Then it was separated using SARA fractionation method [13]. The subfraction saturates and aromatics were subjected to further analysis with gas chromatography (GC, 7890A with FID detector, Agilent Technologies) and gas chromatography-mass spectrometry (GC-MS, 7890A-5975MSD, Agilent Technologies), respectively.

Saturates were dissolved in *n*-hexane and determined by GC to quantify hydrocarbons C_{10}-C_{40} [17]. The analytical conditions were as follows: injection volume, 1 µL; capillary column, 30 m × 0.32 mm × 0.25 µm (Agilent HP-5); nitrogen flow rate, 1.5 mL/min; air flow rate, 300 mL/min; hydrogen flow rate, 30 mL/min; makeup gas flow rate, 28 mL/min. The oven temperature started at 40ºC, held for 2 min, increased to 320ºC at 10ºC/min and held for 20 min. The inlet temperature was 300ºC and the detector temperature was 325ºC.

Aromatics dissolved in dichloromethane were injected into GC-MS for determination of 16 PAHs [18]. The analytical conditions were as follows: injection volume, 1 µL; capillary column, 30 m × 0.25 mm × 0.25 µm (Agilent HP-5 MS); helium flow rate, 1 mL/min; injector temperature, 280ºC; oven temperature, 60ºC, held for 1 min, increased to 150ºC at 20ºC/min, held for 8 min, increased to 270ºC at 5ºC/min, held for 3.36 min, increased to 290ºC at 10ºC/min, held for 10 min; ion source temperature, 230ºC; quadrupole temperature, 150ºC; ionization mode, electronic impact (EI, 70 eV). The solvent delay was 5 min. Selected ion monitoring (SIM) mode was used.

Source Tracing of the Isolated Strain

The original petroleum and the 5th enriched bacterial suspension were sent to Sangon Biotech (Shanghai, China) for high-throughput sequencing analysis to determine the relative abundance of the isolated strain in bacterial community before and after enrichment. Total bacterial genomic DNA was extracted from the samples and the V3-V4 region of 16S rRNA genes was amplified using the primer 341F (5'-CCCTACACGACGCTCTTCCGATCT GCCTACG GGNGGCWGCAG) and 805R (GACTGGAGTTCCTTG GCACCCGAGAATTCCAGACTACHVGGGTATC TAATCC). Amplicons were sequenced by using the Illumina Miseq 2×300 bp platform. Operational taxonomic units (OTUs) of the qualified sequences were defined according to a 97% similarity. The identification of bacterial taxa was based on NCBI 16S databases.

Results and Discussion

Petroleum-Degrading Capacity of Isolated Strains

Four bacterial strains (L1, L7, L14 and L19) were isolated from petroleum. These strains showed good

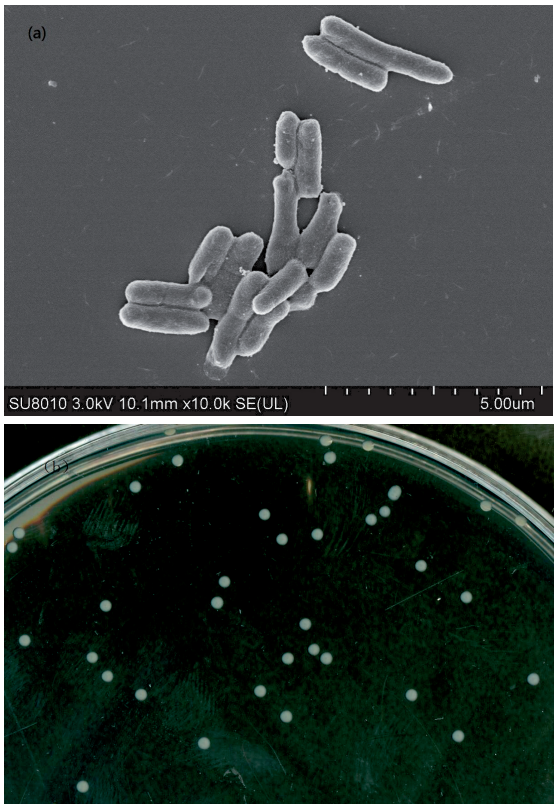

Fig. 1. Cell morphology of *Providencia rettgeri* L1 observed by scanning electron microscope a) and colony photo on LB agar medium b).

petroleum-degrading capacity with the degradation rate of 45.9%, 25.7%, 34.4% and 33.1%, respectively. Strain L1 was the best degrader among the 4 strains and was selected for further study.

Strain L1 was a gram-negative bacterium with cells 0.8-1.5 μm long and 0.4-0.6 μm wide measured by scanning electron microscope (HITACHI SUB8011, Japan) (Fig. 1a). The single colony of strain L1 on LB agar plates was round, semitransparent and white to pale yellow with regular margin (Fig. 1b). The colony surface was moist, smooth and slightly raised. The result of 16S rRNA sequence of strain L1 was submitted to NCBI Genbank (1355 bp, Accession No. MK646006.1). There was 99.0% homology to *Providencia rettgeri* 16S rRNA. A phylogenic tree was constructed based on its 16S rDNA sequence (Fig. 2). According to the morphological characteristics and 16S rRNA sequence analysis, strain L1 was identified as *Providencia rettgeri*, which was also confirmed by the result of its complete genome sequence (Genbank: CP087584.1). Strain L1 was designated as *Providencia rettgeri* L1 in the following text.

Many bacterial strains have been involved in petroleum degradation in previous studies. For example, *Alcaligens*, *Acinetobacter*, *Alcanivorax*, *Bacillus*, *Cycloclasticus*, *Enterobacter*, *Flavobacterium*, *Oleispira*, *Pseudomonas*, *Thallassolituus*, *Marinobacter*, *Rhodococcus* and *Sphingomonas* were good petroleum degraders that could utilize petroleum as carbon source [19, 20]. *Providencia* was less reported. Yuan et al. [21] compared genomes of 91 isolates of the genus *Providencia* and found that *Providencia rettgeri* and *Providencia stuartii* harbored more genes related to material transport and energy metabolism, implying a stronger ability to adapt to diverse environments. The strong adaptability is the key to pollutant degradation potential of microbes. *Providencia* has been reported to degrade organic pollutants and more

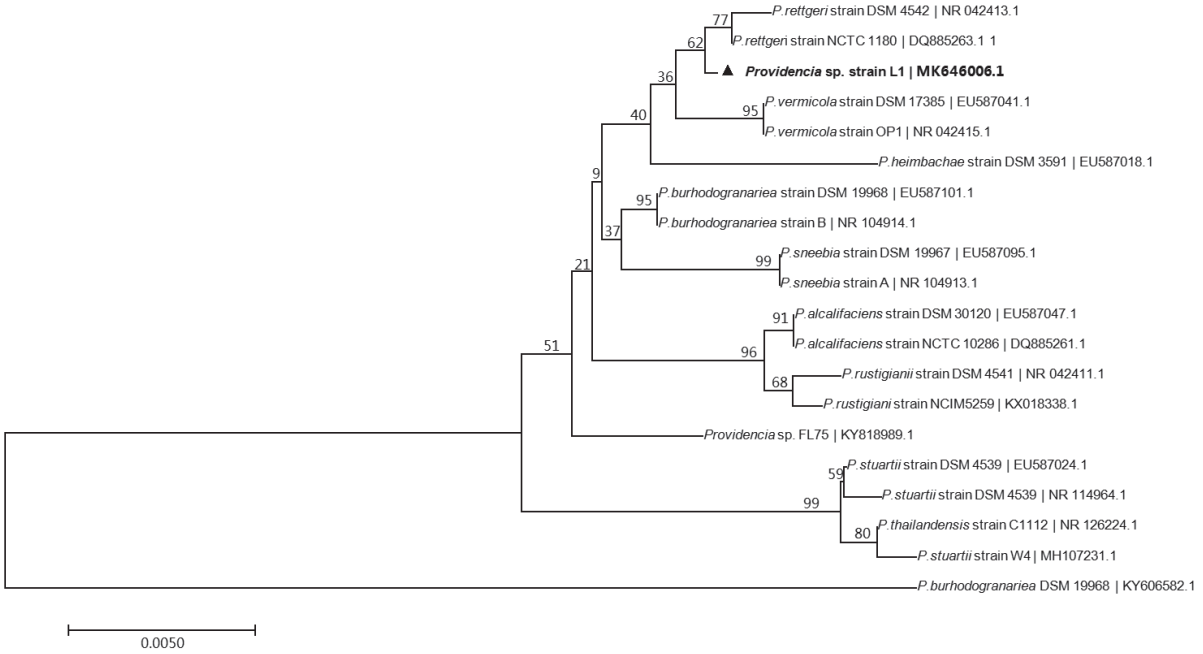

Fig. 2. Phylogenetic tree based on 16S rDNA sequence of the isolated strain *Providencia rettgeri* L1 (marked with ▲).

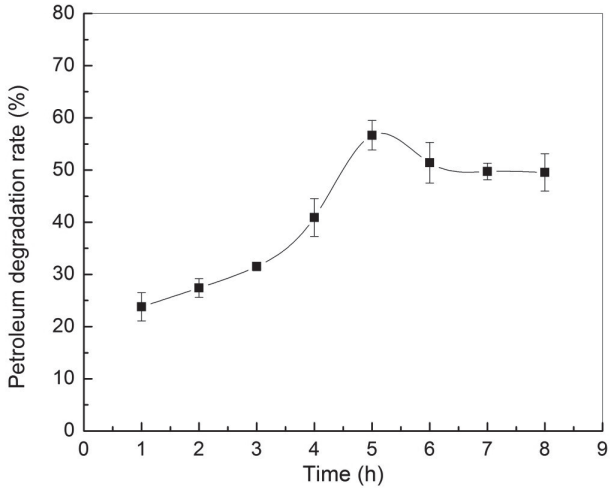

Fig. 3. Petroleum degradation rates by crude enzymes from *Providencia rettgeri* L1.

studies were on pesticides than on petroleum. Rani et al. [22] discovered that *Providencia stuartii* strain MS09 utilized chlorpyrifos to grow in LB broth containing different concentrations of chlorpyrifos ranging from 50 to 700 mg/L, revealing the role of *Providencia stuartii* on pesticide biodegradation. Zhang et al. [23] observed that biodegradation of dicarboximide fungicides in soil were significantly enhanced by two bacterial cocultures of *Providencia stuartii* JD and *Brevundimonas naejangsanensis* J3. Ayangbenro [24] isolated *Providencia stuartii* from petroleum contaminated soil and indicated that it was capable of degrading natural bitumen. However, there was still very little evidence of petroleum degradation by *Providencia*. In this study, *Providencia rettgeri* L1 isolated from petroleum showed good degradation ability for petroleum hydrocarbons and supplied the direct evidence. This also suggests that *Providencia rettgeri* L1 has the potential to be used in bioremediation of contaminated water and soil and it requires further study in the future.

Petroleum-Degrading Capacity of Crude Enzymes from *Providencia rettgeri* L1

The crude enzyme solution of *Providencia rettgeri* L1 was prepared and supplemented with FDH to degrade petroleum. As shown in Fig. 3, the degradation rate was 23.8% at 1 h, increased to 56.7% at 5 h and remained at 49.5% till 8 h. There was a slight decrease in the degradation rate of petroleum from 5 h to 8 h. It was speculated that the generation of metabolites that possibly interfered ultraviolet absorption of petroleum could be the reason. The differences in the degradation rate between at 5 h and 8 h were not significant (p>0.05). The residual petroleum at the two time points was selected for analysis of its saturates and aromatics.

Petroleum is a complex mixture of numerous individual components, in which saturates and aromatics are the major parts degraded by microbes [1]. Therefore, the two subfractions were focused in this study. The degradation rates of saturates in petroleum by crude enzymes from *Providencia rettgeri* L1 were displayed in Fig. 4. The degradation rate of saturates was 31.2% at 5 h, increasing to 50.4% at 8 h. C_{10}-C_{12} and C_{40} were not detected in the samples. The degradation rates of C_{13}-C_{39} at 8 h were higher than at 5 h, implying that longer reaction time resulted in higher degradation. The degradation rate for short-chain saturates (C_{13}-C_{20}), mid-chain saturates (C_{21}-C_{30})

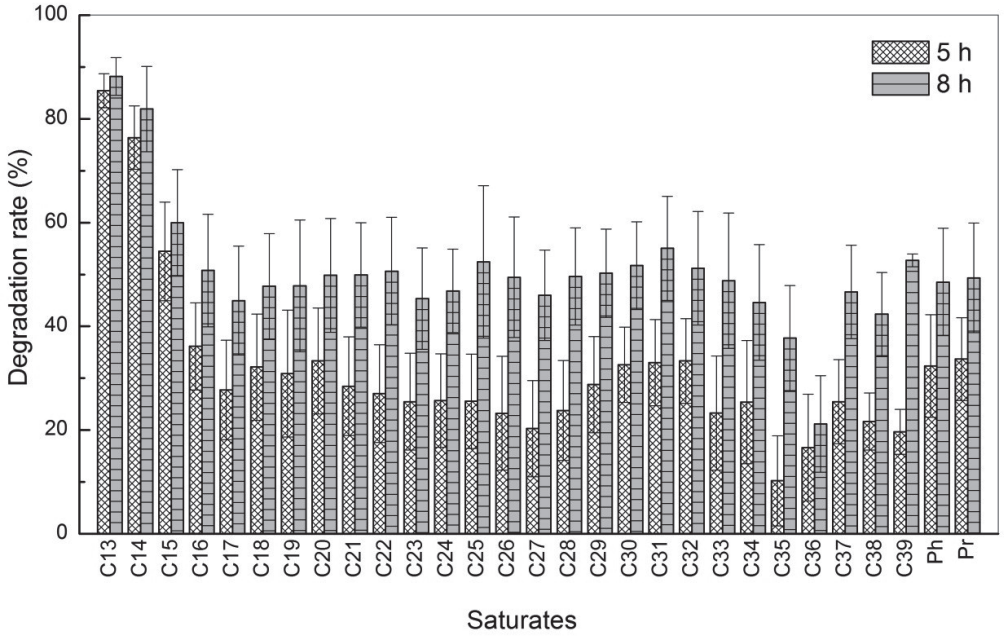

Fig. 4. Degradation rates of saturates (C_{10}-C_{40}) by crude enzymes from *Providencia rettgeri* L1.

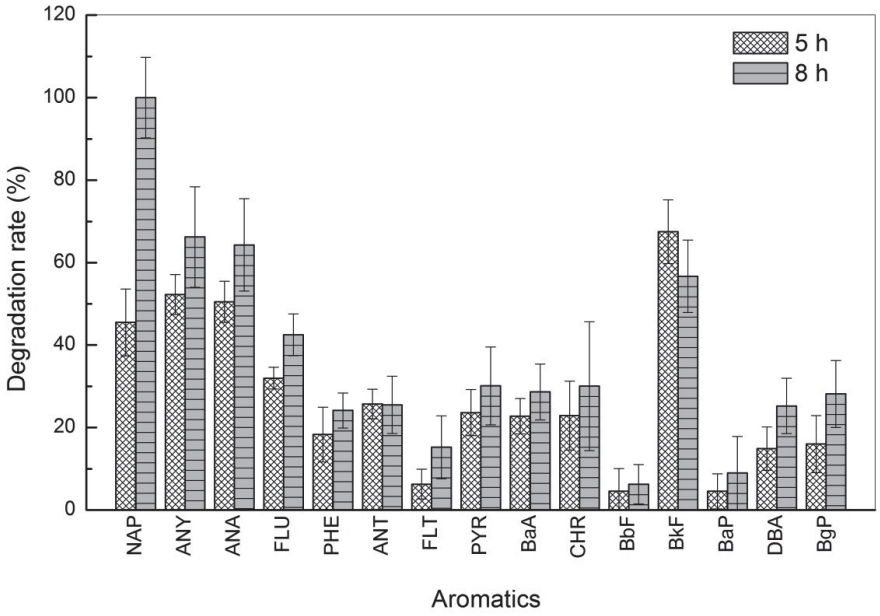

Fig. 5. Degradation rates of aromatics by crude enzymes from *Providencia rettgeri* L1.

and long-chain saturates (C_{31}-C_{40}) at 5 h was 38.5%, 26.7% and 26.4%, respectively. The values increased to 52.6%, 49.4% and 47.7% at 8 h, respectively. The degradation rates of short-chain saturates (C_{13}-C_{19}) were higher than mid-chain and long-chain saturates (C_{21}-C_{30}, C_{31}-C_{39}) at both time points. The degradation rates of single saturate ranged from 21.2% to 88.2% at 8 h. The biodegradation assessment markers, pristane

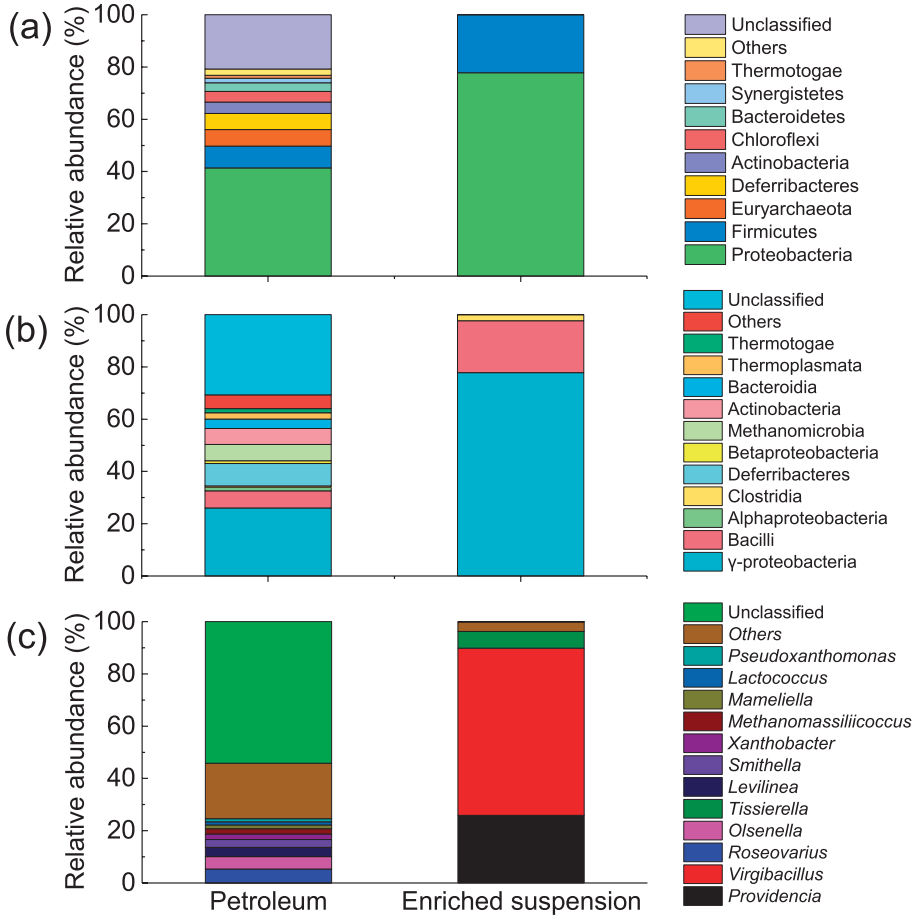

Fig. 6. Relative abundances of dominant species at a) phylum, b) class and c) genus level in bacterial community.

and phytane, showed significant degradation rates of 49.3% and 44.9%, respectively.

Aromatics in petroleum are known to possess mutagenic, teratogenic, or carcinogenic properties [25]. The degradation rates of aromatics were shown in Fig. 5. The rate was 22.7% at 5 h and 29.5% at 8 h. Indeno (1,2,3-cd) pyrene was not detected in samples. The degradation rate of PAHs varied with the number of rings, which was 47.8% for 2-ring PAHs, 24.2% for 3-ring PAHs, 29.5% for 4-ring PAHs and 19.8% for 5-ring PAHs, respectively. PAHs with fewer rings were more liable to be degraded by endoenzymes of *Providencia rettgeri* L1. The degradation rates of single PAH ranged from 6.2% to 100%.

Enzymatic bioremediation is a valuable alternative especially in extreme environment. Researchers sought the degrading enzymes to generate less toxic products, identified their coding genes, developed preparation methods, optimized preparation conditions and obtained the target enzymes [26-28]. Previous studies have identified petroleum-degrading enzymes such as multiple alkane hydroxylases involved in alkane degradation and catechol dioxygenases responsible for aromatic ring cleavage in aromatic compounds degradation. These specific enzymes can degrade specific petroleum components. However, compare to crude enzymes directly obtained from the degrading microbes, they required longer production time and higher cost. The crude enzymes from *Providencia rettgeri* L1 in the study showed good degrading ability for both saturates and aromatics, indicating that hydroxylases for alkanes and dioxygenases for aromatics were both included. The crude enzymes can play different roles in the entire petroleum degradation process.

Source Tracing of *Providencia rettgeri* L1

Providencia rettgeri L1 belongs to *Providencia* (genus), Morganellaceae (family), Enterobacterales (order), γ-proteobacteria (class), Proteobacteria (phylum). The relative abundances of dominant species in bacterial community of original petroleum and enriched bacterial suspension were shown in Fig. 6. The relative abundance of *Providencia rettgeri* L1 and its corresponding taxonomic level to which it belongs were very different in the two samples.

In enriched bacterial suspension, Proteobacteria and γ-proteobacteria was the dominant phylum and class with relative abundances of 77.8%. *Providencia* was one of the dominant genera with the relative abundance of 25.9%, which was lower than *Virgibacillus*. In petroleum, Proteobacteria and Gammaproteobacteria were still the dominant phylum and class, but their relative abundance was lower than in enriched suspension by 36.4% and 51.7%. *Providencia* was not dominant genus and its relative abundance was only 0.02%. Bacterial community of petroleum were changed after enrichment in culture media. It is concluded that *Providencia rettgeri* L1 had advantage to compete with other genera in culture media and its rapid proliferation made it easy to be isolated.

Conclusions

A petroleum-degrading bacterium L1 was isolated from petroleum and identified as *Providencia rettgeri*. The strain was not dominant in bacterial composition in petroleum but dominant in enriched culture media. The intracellular crude enzymes of *Providencia rettgeri* L1 were supplemented with FDH to construct an enzyme cocktail. The cocktail can efficiently degraded the petroleum and its subfraction saturates and aromatics in a short time. This study can provide some insights into fast degradation of petroleum.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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