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Performance and community structure change of immobilized marine petroleum-degrading bacteria in the degradation process

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ABSTRACT

In order to further discuss the effect of diesel degradation and the biological diversity change in degradation process, the biodegradation effect and microbiological characteristics of marine immobilized petroleum-degrading bacteria were analyzed. In this paper, the biodegradations of diesel by petroleum-degrading bacteria immobilized on corn straw and free bacteria were compared. The diesel degradation rate of immobilized petroleum-degrading bacteria was up to 66% in artificial seawater on the 30th day, which was far higher than that of free petroleum-degrading bacteria (only 20%). The kinetics of diesel biodegradation of immobilized petroleum-degrading bacteria and free bacteria were also studied. The diesel degradation rate of diesel by immobilized petroleum-degrading bacteria is 3.5 times of that by free petroleum-degrading bacteria. Additionally, nutrients (e.g., nitrogen and phosphorus) released from corn straw were analyzed. The results indicated that a certain amount of nutrients released from corn straw plays a certain role in promoting the degradation of diesel by immobilized petroleum-degrading bacteria. The analysis of the microbial community structure showed that, although the population was changed in the degradation process, the dominant bacterial community was still composed of the *Pseudomonas* sp., *Stenotrophomonas* sp., *Achromobacter* sp., and *Alcanivorax* sp.

Keywords: Oil pollution; Immobilization; Nutrients; Marine environment; Microbial community

1. Introduction

More and more oil is released into the ocean because of the more frequent offshore oil transport and oil exploitation in recent years. Therefore, petroleum hydrocarbons are the most widespread contaminants in the marine environment [1,2]. Oil pollution has not only caused serious environmental and ecological problems, but also threatened human health and economic growth (e.g., marine fishery and tourism) [3,4]. Hence, it is necessary that a considerable remediation should be used in marine environment. Comparing with physical and chemical remediation methods, bioremediation is more cost-effective and environment friendly [5,6].

In marine environment, the biodegradation of hydrocarbon is mainly performed by microorganisms. However, factors, such as less petroleum-degrading bacteria and nutrients in marine environment, limit the biodegradation speed of diesel by indigenous bacteria. In order to readily improve the biodegradation of diesel, immobilization technology has been extensively studied [7,8]. Immobilized technology does not only increase the number of petroleum-degrading bacteria in per unit area, but also provide nutrients by carriers.

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Many studies have shown that the effect of degradation is different when immobilized carriers of different materials are used [9–11]. In the marine environment, especially open sea, the lack of nutrients leads to very few bacteria that can degrade petroleum hydrocarbons. Actually, some carriers, for example, agricultural wastes, can provide part of nutrients to bacteria. For most agricultural waste, they have relatively more porous structure, which does not only improve the density of immobilized bacteria floating in the seawater, but also absorbs more petroleum hydrocarbons. In addition, it is more important that some nutrients could be released, which is beneficial for the growth of petroleum-degrading bacteria [12].

Additionally, feasibility of petroleum-degrading bacteria immobilized on the carrier has been intensively studied. For example, community structure change of petroleum-degrading bacteria, which was considered an important evidence of the secondary pollution or not. Mac Naughton et al. [13] studied the changes in community structure of petroleum-degrading bacteria after adding oil and nutrients by a bioremediation experiment for oil spill. Nevertheless, there is a relative lack of research on the community structure change of immobilized petroleum-degrading bacteria in marine environment in the current report.

Therefore, it is important for discussing the effect of immobilized bioremediation to analyze both effect of diesel biodegradation and the biological community change in marine environment. In the paper, corn straw, with relatively more porous structure and some nutrients, is used as the immobilized carrier. The diesel degradation rate of immobilized petroleum-degrading bacteria was compared with free petroleum-degrading bacteria. The community structure of petroleum-degrading bacteria was analyzed in degradation process. This study could provide a basis for further optimization of immobilized bioremediation used in oil-contaminated seawater.

2. Experimental

2.1. Experimental materials and methods

2.1.1. Enrichment of petroleum-degrading bacteria

In this paper, the mixed petroleum-degrading bacteria were domesticated and enriched from fresh seawater, and the mixed petroleum-degrading bacteria were used in the experiment. The mixed petroleum-degrading bacteria were screened as follows: the seawater samples were collected from the Tang Island Bay, Qingdao, Shandong Province, China. First, 5 mL of fresh seawater sample and 1 mL of diesel filtered with a 0.22 μm pinhole membrane were synchronously added into 100 mL of autoclaved liquid mineral medium. Then, these samples were incubated at 30°C and under a rotary speed of 160 rpm for 7 d. Afterwards, 10 mL of incubated sample was taken from the medium and was then added into the fresh, autoclaved medium. The same steps were repeatedly operated three times and the gathered petroleum-degradation bacteria were used for the study.

2.1.2. Preparation of immobilized petroleum-degrading bacteria

The corn straw was selected as immobilized carrier in this study. First, these straws were broken into similar pieces, washed, and dried. Afterwards, these pieces were used as the carriers to adsorb bacteria. These experimental steps are described in detail in Ref. [14]. And the average concentration of immobilized bacteria was about 8.7×10^8 CFU (colony forming units) per gram carrier.

2.2. Experimental design

2.2.1. The release of nitrogen and phosphorus from carrier

The released amount of nutrients (nitrogen and phosphorus) into water was determined. The carriers were soaked in the artificial seawater without nitrogen and phosphorus, and the concentration of the total nitrogen (TN) and total phosphorus (TP) were measured in different time duration, subsequently.

2.2.2. Biodegradation of petroleum-degrading bacteria

A certain amount of immobilized petroleum-degrading bacteria and 1 mL of filtered diesel were added into the flasks containing 100 mL of autoclaved artificial seawater. Then, all flasks were subjected to incubation under a rotary speed of 130 rpm and at 32°C in a rotary shaker. Afterwards, the diesel degradation rate was measured in different degradation process. And the degradation kinetic of immobilized bacteria was analyzed. All groups were replicated in triplicate.

Meanwhile, the diesel degradation rate of the free petroleum-degrading bacteria was compared with that of the immobilized petroleum-degrading bacteria. In the experiment, the dosage of free bacteria was the same as that of immobilized petroleum-degrading bacteria. The same experimental procedure was used to determine the diesel removal rate of free petroleum-degrading bacteria. And the degradation kinetic of free petroleum-degrading bacteria was analyzed. All groups were replicated in triplicate.

2.2.3. Community analysis of immobilized petroleum-degrading bacteria

To further study the effect of diesel biodegradation for petroleum-degrading bacteria, samples were collected to analyze microbial community in the degradation process. The petroleum-degrading bacteria immobilized on carriers were collected at the 0, 15, and 30 d. Then, the Shannon index, the abundance of species, and the changes of community structure were analyzed.

2.3. Analysis method

2.3.1. Determination of the residual diesel

The *n*-hexane was used to extract diesel oil in sample, and the diesel degradation rate was measured by ultraviolet spectrophotometric method. Additionally, the diesel removal rates were calculated followed by Ref. [15], except diesel absorbed by carrier (about 3%).

2.3.2. Determination methods of nitrogen and phosphorus

The analysis of TN in water was conducted according to the UV spectrophotometric method of alkaline potassium persulfate digestion [16].

The analysis of TP in water was performed according to the ammonium molybdate spectrophotometric method [17].

2.3.3. Microbial community analysis

The petroleum-degrading bacteria were eluted from the immobilization samples, determined with sterile water, and then filtered through a 0.22 μm fiber filter membrane. These filter membranes were sent to Sangon Biotech (Shanghai) Co. DNA extraction, PCR amplification, and 16S rDNA sequencing were carried out by Sangon Biotech (Shanghai) Co., and the specific steps are as follows:

Pretreatment of the sample. The filter membranes were cut off which can make the bacteria better contacted with the lysate, this way can lead to more cell walls to rupture and release DNA. The scissors and tweezers used in the operation must be sterilized at high temperature in an alcohol lamp, suitably cooled and then used to operate quickly and reduce contamination of the bacteria in the environment.

Extraction of DNA. DNA was extracted using the OMEGA kit (E.Z.N.A™ Mag-Bind Soil DNA Kit).

PCR amplification. The first round of expansion: Quantitative quantification of the genomic DNA using the Qubit 2.0 DNA Assay Kit to determine the amount of DNA to be added to the PCR reaction. The PCR primers have been incorporated into the V3–V4 universal primers for the Miseq sequencing platform (341F Primer: CCCTACACGACGCT CTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG and 805R Primer: GACTGGAGTTCCTTGGCACCCGA GAATTCCAGACTACHVGGGTATCTAATCC). The second round of amplification, the introduction of Illumina bridge PCR-compatible primers.

DNA purification recovery. For the bacteria and archaea amplified PCR products and the normal amplification of more than 400 bp PCR products, the use of 0.6 times the magnetic beads (Agencourt AMPure XP) treatment. For fungal PCR products and other extended fragments of less than 400 bp of PCR products, the use of 0.8 times the magnetic beads treatment.

Quantitative mixing. The Qubit 2.0 DNA detection kit was used to quantify the recovered DNA so as to facilitate the mixing of the DNA by 10 ng each of the samples in order to facilitate the mixing of the same amount of 1:1 and the final on-machine sequencing concentration of 20 pmol.

Analysis. Rarefaction analysis of operational taxonomic unit using mothur software, and use the R Programming Language to make the rarefaction curve (Shannon index). The heatmap of genus was made by the R gplots package (abundance of species). And use R to classify species taxonomic statistics into a distribution barplot (changes of community structure).

3. Results

3.1. Biodegradation of diesel

First, in order to analyze the difference of diesel degradation rate between immobilized petroleum-degrading bacteria and free petroleum-degrading bacteria, diesel degradation rate are described in two systems from the 2nd to 30th day of the experiment, and the results are presented in Fig. 1. As seen from Fig. 1, the diesel degradation rate of immobilized petroleum-degrading bacteria was 66% on the 30th day, and

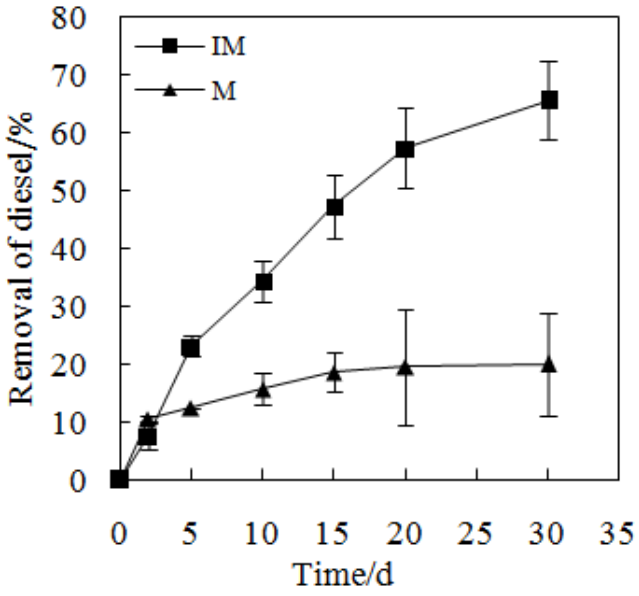

Fig. 1. Removal of diesel by immobilized bacteria and free bacteria. IM: immobilized petroleum-degrading bacteria; M: free petroleum-degrading bacteria.

that of free petroleum-degrading bacteria was 20% on the 30th day. And He et al. [18] studied the similar results: the different immobilized carriers, such as rice straw, corn straw, and montmorillonite, which showed the removal rate of straws were better than that of montmorillonite used as the carrier. Therefore, corn straw is suitable for use as the immobilized carrier.

To explore the relationship between immobilized petroleum-degrading bacteria and free degradable bacteria, the dynamic models were further studied as shown in Fig. 2. The kinetic equations for the degradation of immobilized petroleum-degrading bacteria and free petroleum-degrading bacteria are shown in Eqs. (1) and (2), respectively.

$$v = -0.028t + 0.961,\ R^2 = 0.976 \quad (1)$$

$$v = -0.008t + 0.941,\ R^2 = 0.775 \quad (2)$$

where v = Residual diesel (mL) and t = Diesel degradation time (d).

The results indicated the experimental data were fitted by a common first-order degradation kinetic model. Additionally, the diesel degradation rate of immobilized petroleum-degrading bacteria is 3.5 times of that of free petroleum-degrading bacteria, which showed that immobilized petroleum-degrading bacteria could more effectively degrade diesel than free petroleum-degrading bacteria. Simons et al. [9] used alginate and shell grit to immobilize bacteria and obtain better degradation rate of weathered oil, which is consistent with our obtained results.

3.2. Relationships between nutrients released from carrier and biodegradation

The amounts of nutrients (e.g., nitrogen and phosphorus) released from carrier are shown in Fig. 3. Taking 4 and 16 d

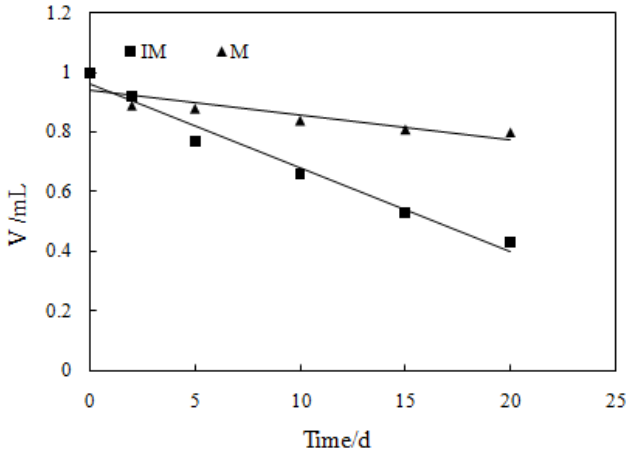

Fig. 2. First-order-kinetic equation curves of immobilized and free petroleum-degrading bacteria. IM: immobilized petroleum-degrading bacteria; M: free petroleum-degrading bacteria.

of nitrogen and phosphorus released amount as an example, the amount of nitrogen and phosphorus released from corn straw were 6.95 and 0.0708 mg/g, respectively, in 4 d; and those were 16.92 and 0.232 mg/g, respectively, in 16 d. It can be seen that a certain amount of nitrogen and phosphorus were released from the carrier, which can be used by petroleum-degrading bacteria. Additionally, the release rates of nitrogen and phosphorus were calculated in Tables 1 and 2. The released rate of nitrogen was the largest at 2–4 d, reaching a maximum of 1.91 mg/g d; the released rate of phosphorus was the highest at 4–7 d about 0.018 mg/g d. This is consistent with the change of diesel removal rate (the fastest diesel removal rate was in 0–5 d). Then, the removal rate decreased followed by the slow released speed of nitrogen and phosphorus. Many studies have shown that the addition of nitrogen and phosphorus can promote the diesel degradation, so corn straw as immobilized carrier has some advantages.

3.3. Analysis of microbial community

3.3.1. Diversity indices analysis

The diversity indices of immobilized petroleum-degrading bacteria in 0th, 15th, and 30th days were detected. Fig. 4 shows the Shannon index of immobilized petroleum-degrading bacteria in degradation process. The Shannon indexes of immobilized petroleum-degrading bacteria in 0th, 15th, and 30th days were 1.53, 2.19, and 2.14, respectively. It is indicated that the distribution of petroleum-degrading bacteria was influenced in the degradation process. Followed by the diesel degradation rate in different degradation times, the diversity index was improved. Additionally, compared with the diversity indices between 15th and 30th days, the results also indicate that, as the degradation time increases, the microbial diversity is limited.

3.3.2. Analysis of community structure at different degradation times

Fig. 5 shows the abundance of the immobilized petroleum-degrading bacteria in different degradation times at the genus level. The results only showed the top 50 species of the highest abundance classification information, the remaining species classification into other. The specie of the highest abundance was *Pseudomonas* sp. before degradation of diesel. After that, the abundance of *Pseudomonas* sp. was

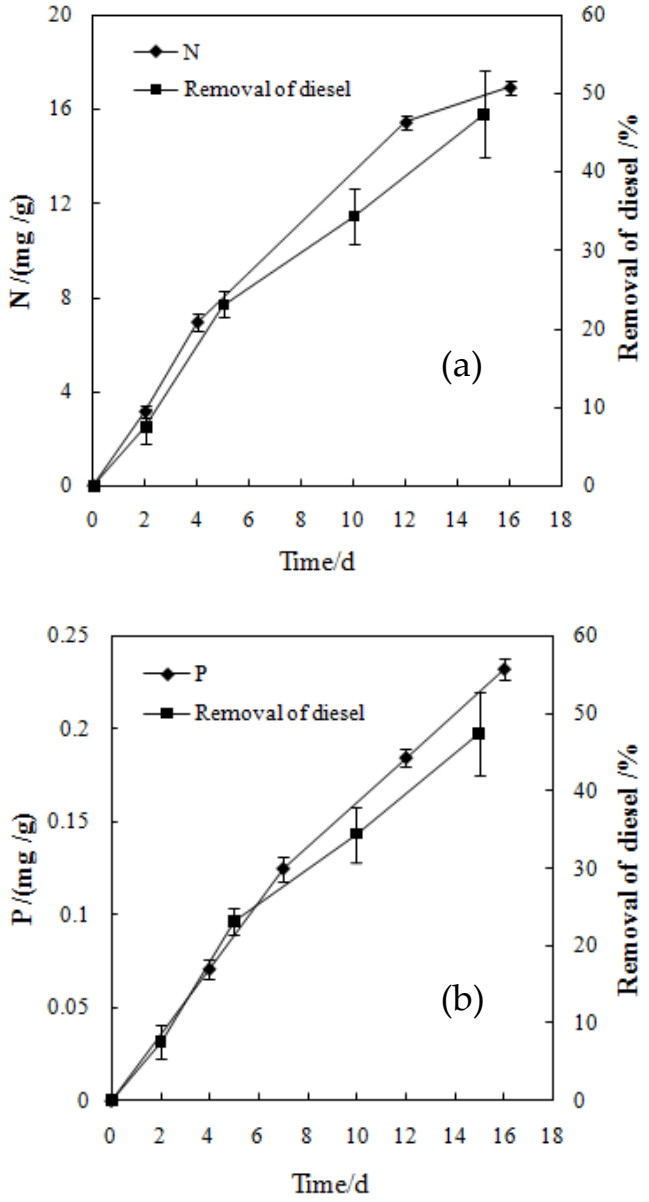

Fig. 3. (a) The amount of nitrogen released from corn straw, (b) The amount of phosphorus released from corn straw.

Table 1
The release rate of nitrogen

Time (d)	*K* (mg/g d)
0–2	1.57
2–4	1.91
4–12	1.06
12–16	0.37

Table 2
The release rate of phosphorus

Time (d)	*K* (mg/g d)
0–4	0.01770
4–7	0.01800
7–12	0.01192
12–16	0.01190

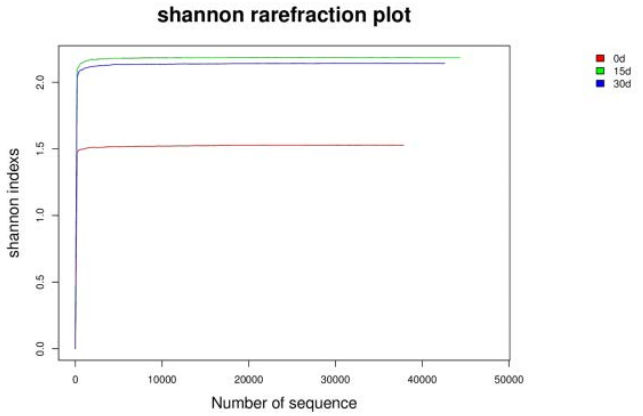

Fig. 4. Analysis of diversity indices of bacteria at different degradation times.

decreased, and the abundance of *Stenotrophomonas* sp. was increased.

Table 3 shows the main rank reads number of samples (immobilized petroleum-degrading bacteria) in different degradation times at genus levels. Taking the first four species of bacteria as an example, the most abundant content of species was *Pseudomonas* sp. in the mixed bacteria in initial process, the main rank reads were 21201. After 15 d of biodegradation, the amount of *Pseudomonas* sp. was decreased, the amount of *Stenotrophomonas* sp. was the highest (14,431). The most abundant strain was still *Stenotrophomonas* sp. (14,387) after 30 d.

Moreover, the community structure of petroleum-degrading bacteria was further analyzed. The community structure changes were analyzed according to Fig. 6. With the changes of degradation time, the microbial community structure has changed. In initial process, the most abundant strain was *Pseudomonas* sp. in the mixed bacteria. After 15 d of biodegradation, the amount of *Pseudomonas* sp. was decreased, and the amount of *Stenotrophomonas* sp. was the highest (32.55%). The most abundant strain was still *Stenotrophomonas* sp. (33.78%) after 30 d of biodegradation. The proportion of *Achromobacter* sp. was decreased with the increase of degradation time. Its proportion in the 0 d accounted for 18.44%, and the proportion after biodegradation for 15 and 30 d decreased to 14.48% and 15.01%, respectively. The proportion of *Alcanivorax* sp. was increased with the extension of the time. Its proportion in 0 d was only 0.17%, 15 and 30 d were increased to 14.38% and 15.13%, respectively. The populations of petroleum-degradation bacteria were changed in the degrading process, which indicated that diesel had a selective effect on dominant

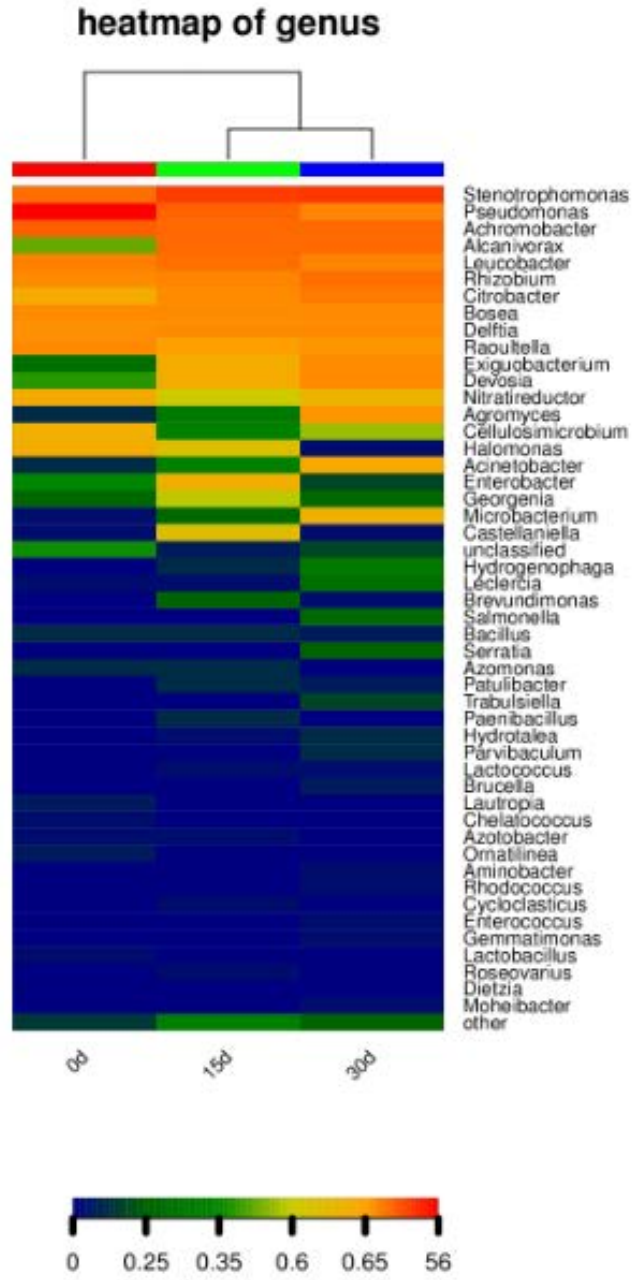

Fig. 5. Abundance of species in different degradation times at genus level.

Table 3
The main rank reads number of samples in different degradation times at genus level

Sample / Bacteria	0 d	15 d	30 d
Pseudomonas sp.	21,201	7,685	2,045
Stenotrophomonas sp.	4,348	14,431	14,387
Achromobacter sp.	6,981	6,420	6,393
Alcanivorax sp.	63	6,374	6,445

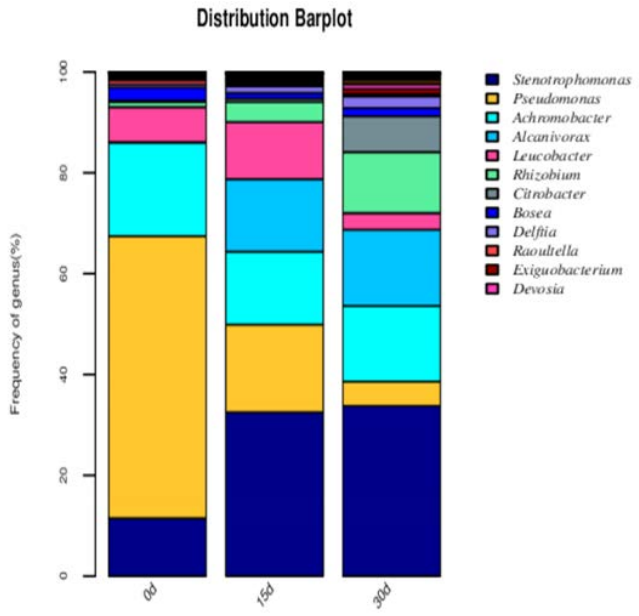

Fig. 6. Community structure changes at different degradation times.

bacteria. However, although the population was changed in the degrading process, the dominant bacterial community was still composed of the *Pseudomonas* sp., *Stenotrophomonas* sp., *Achromobacter* sp., and *Alcanivorax* sp.

4. Discussion

Nutrients play an important role in biodegradation. Nitrogen and phosphorus which are the essential nutrients for microbial growth and metabolism have a significant effect on bioremediation of petroleum pollution. Therefore, the process of oil degrading in undernourished water is very slow and hardly operable [19,20]. In this paper, nutrients released from the corn straw were utilized by immobilized petroleum-degrading bacteria. The results also showed that the degradation of diesel was promoted by immobilized petroleum-degrading bacteria.

Additionally, the main bacteria were composed of *Stenotrophomonas* sp., *Alcanivorax* sp., *Achromobacter* sp., and *Pseudomonas* sp. It is basis of studying second pollution or not that diversity indices analysis coupled with community structure analysis. The diversity of petroleum-degrading bacteria was first increased and then decreased. It is indicated that the distribution of petroleum-degrading bacteria was influenced in the degradation process. Followed by the degradation of diesel, the diversity index was improved. Diversity analysis illustrates the different characteristics of strains in degradation of hydrocarbons. The amount of some strains increased while the amount of some strains decreased in the degradation of diesel.

Many studies have analyzed the performance of individual petroleum-degrading bacteria. For example, *Stenotrophomonas* sp. a bacteria producing biosurfactant, can destroy the molecular structure of alkanes [21]. *Stenotrophomonas rhizophila* can degrade decane, hexadecane, engine oil, benzene, phenol, oleic acid, and naphthalene that represent various forms of hydrocarbons. The good performance of *Stenotrophomonas* sp. has been widely reported. In addition, *Alcanivorax* sp. and *Pseudomonas* sp. are marine-specific hydrocarbon-degrading bacterium that can utilize chain hydrocarbons and aromatic hydrocarbons [22]. However, there is little report about the degradation performance in the marine environment. In this study, results showed that *Stenotrophomonas* sp. was vigorous in marine environment polluted by diesel. Moreover, bacteria specie with the largest population after 15 and 30 d in the marine environment was *Stenotrophomonas sp*., which limit the higher degradation.

Many studies have indicated that *Pseudomonas* sp. can degrade alkanes, especially *n*-alkanes [23–26]. Furthermore, in some reports, the result also showed the growth of *Pseudomonas* sp. decreased at the end of the degradation process [27]. Results of this study shows the same changing tendency. The number of *Pseudomonas* sp. was more than that of *Achromobacter* sp. on the 15th day. However, the opposite result was observed on the 30th day.

In conclusion, the degradation of diesel and diversity of petroleum-degrading bacteria were improved due to the nutrient and characteristic of bacteria themselves. First, the nitrogen and phosphorus released from carriers could improve the growth of bacteria, which could improve the degradation of diesel. Second, the bacteria mainly consist of *Stenotrophomonas* sp., *Alcanivorax* sp., *Achromobacter* sp., and *Pseudomonas* sp. The distribution of petroleum-degrading bacteria was influenced in the degradation process. Followed by the degradation of diesel, the diversity index was improved. But, due to the characteristic of bacteria themselves, the amount of some strains increased or decreased in the degradation of diesel.

5. Conclusions

Biodegradation process and community structure of petroleum-degrading bacteria in degrading process were investigated in this study. The conclusions are as follows:

- The diesel degradation rate by immobilized petroleum-degrading bacteria (66%) was far higher than free petroleum-degrading bacteria (20%) on the 30th day. And, these were fitted by a common first-order degradation kinetic model in the degrading process.
- The amount of nitrogen released from carrier was 6.95–16.92 mg/g in 4–16 d, and the amount of phosphorus was 0.0708–0.232 mg/g in 4–16 d. Additionally, the released rates of nitrogen and phosphorus were the maximum in 0–4 and 0–7 d. This is consistent with the change of diesel degradation rate.
- By analyzing diversity indices of immobilized petroleum-degrading bacteria in different times, the Shannon index of immobilized petroleum-degrading bacteria in 0th, 15th, and 30th day is 1.53, 2.19, and 2.14, respectively. That means the diversity indices were improved, due to the degradation of diesel by immobilized petroleum-degrading bacteria.
- The populations of petroleum-degradation bacteria were changed in the degrading process, which indicated that diesel had a selective effect on dominant bacteria.

However, although the population was changed in the degrading process, the dominant bacterial community was still composed of the *Pseudomonas* sp., *Stenotrophomonas* sp., *Achromobacter* sp., and *Alcanivorax* sp.

Acknowledgment

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