Isolation, Identification and Characterization of Long-chain Alkane Degrading Bacteria

Yinsong Liu, Chi Dong, Jie Wang
School of Petroleum Engineering, Northeast Petroleum University, China

Abstract

With the development of industrial technology, oily waste water, coking wastewater, oil refining wastewater, papermaking wastewater which composition is complex, and there are many difficult biodegradable ingredients, especially the long chain alkane pollutants is poor biochemical and difficult to deal with. Biological reinforcement technique is to add the target pollution degradation bacteria to speed up the rate of degradation of organic pollutants. Water quality analysis showed that although the content of long chain alkane in coking wastewater was not high, it was the limiting factor for water quality to reach the standard. In this paper, the enrichment, isolation and purification of sludge samples were used to obtain three strains of long-chain alkane-degrading bacteria. All strains were identified as Gordonia sp.. The degradation rates of lys1-3, lys1-4 and lys2-1 on mixed long-chain alkanes were respectively 72.5%, 69.5% and 71.9%, respectively. And the strain characteristics and the environmental impact factors are studied, the optimum pH7.0, temperature 35 ℃, the shaking table speed of 180 r/min. It is hoped that the bioaugmentation of long-chain alkane-degrading bacteria accelerates the efficient biological treatment of refractory hydrocarbon contaminants in industrial wastewater

Keywords

Long-chain; alkane-degrading; treatment of refractory.

1. Introduction

Oil, as the main energy material, plays a vital role in the development of human society. However, it is hard to avoid leakage in the process of oil exploitation, transportation, loading and unloading, processing and use, resulting in increasingly serious environmental pollution [1]. Microbial technology is using microbial metabolic activity to the degradation of pollutants, reduce or eventually eliminate pollution, this method is one of the most promising method. Through the effective and environmentally friendly way to reduce pollution, it can not only reduce the petroleum hydrocarbon in the process of production, transportation, consumption particular harm to public health and natural resources, but also the cost is low and can reduce the secondary pollution. Microbial technology is economical, efficient and pollution-free in the treatment of petroleum pollution [2]. In this paper, long-chain alkanes are the main components of petroleum, so the study on microbial degradation of long-chain alkanes is of great importance.

Up to now, bacteria, actinomycetes, fungi, yeasts and algae have been found to degrade petroleum, totaling more than 70 genera and more than 200 species. Bacteria play a major role in hydrocarbon degradation, such as Pseudomonas [2-4], Acinetobacter, Flavobacterium, Corynebacterium, Achromobacter[5], Arthrobacter, Vibrio, Mycobacterium, Streptomyces[6], etc. Alcanivorax[7], Cycloclasticus, Oleiphilus, Oleispira, Thalassolituus, Planomicrobium, which were found in the oceans in the past decade, can use petroleum hydrocarbons as the sole carbon source and energy[8]. Alkanophagus, Oil-loving bacteria, Spirulina and Campylobacter can degrade. Straight-chain and
branched-chain alkanes, the genera Cyclolysis can utilize a large number of polycyclic aromatic hydrocarbons [9].

In the actual microbial remediation of petroleum hydrocarbon contaminated environment [10-12], different species of microorganisms have different ability to degrade petroleum hydrocarbons, and the degraded components of petroleum hydrocarbons are also different. Pseudomonas is the most widely studied gram-negative petroleum hydrocarbon-degrading bacteria, while Rhodococcus is the Gram-positive bacteria. Acinetobacter effectively degrades the most abundant alkanes in petroleum hydrocarbons, while Pseudomonas spp. has higher degrading efficiency for aromatic hydrocarbons. In recent years, although there have been some studies on the microbial degradation of long-chain alkanes, it is still not deep enough, especially on the degradation mechanism of long-chain alkanes. In this study, a highly efficient long-chain alkane degrading bacteria was screened and identified as Gordonia sp.. The degradation characteristics of long-chain alkanes were studied. In order to provide support for microbial remediation of petroleum hydrocarbon pollution [13-15].

2. State of the art

Alkanes are the main components of petroleum pollutants. Short-chain alkanes are volatile in natural environment. Medium-chain alkanes can be degraded by microorganisms. Long-chain alkanes have stable carbon-chain chemical bonds, strong hydrophobicity, solid state at room temperature and atmospheric pressure, and are not easy to be biologically utilized. Microbial remediation mainly relies on enzymes in microorganisms to decompose and metabolize petroleum pollutants. Compared with the traditional physical and chemical remediation methods, the microbial remediation method has the advantages of low cost, good purification effect, no secondary environmental pollution and strong environmental compatibility. It is the most potential remediation technology for removing petroleum hydrocarbons from soil [16].

Chen et al.[17] abstained Acinetobacter sp. XM-02 isolated from petroleum-contaminated soil samples. The strain could degrade petroleum effectively. After 10 days, 74.32% of petroleum was degraded, and the degradation rate could be increased to 87.29% by co-culture with Pseudomonas sp. XM-01. Bao et al.[18] had found that Acinetobacter sp. D3-2 separated from petroleum-contaminated soil can utilize various alkanes as the sole carbon source and energy source. The degradation rate of alkanes can reach 82% at 30 degree C and 3% NaCl concentration. Hassanshahian et al. [19] had found that Acinetobacter sp. BS and Acetobacter sp. PG3 separated from the Black Sea could degrade petroleum hydrocarbons separately to 82% and 65%. Further studies showed that they could degrade C9_C25 and other alkanes. Strain BS can degrade C9, C10, C21_C25 and other alkanes 100%, while strain PG3 can degrade C9_C16, C18 and C20_C25 100%.

Amund et al. [20] found that the strain of A. lwoffi could degrade not only linear alkanes(C12_C28), but also long-chain alkylbenzene (dodecylbenzene, tridecylbenzene and tetradecylbenzene). It was pointed out that the degradation mechanism of Dodecylbenzene was closely related to the pathway of decomposition and metabolism of aromatic amino acids. Yuan et al. isolated Acinetobacter strain USTB-X from contaminated soil of Beijing Coking Plant. Pyrene, naphthalene, fluorene, phenanthrene, benzene, toluene, ethyl benzene, ethanol, methanol and Tween-80 can be used as the only carbon and energy sources. The strain USTB-X can secrete surfactants and enhance the utilization of pyrene. Within 16 days, 100 mg/L pyrene can be removed by 63%[21].

The results show that there are obvious differences in the biodegradability of petroleum hydrocarbons between different species. Bacteria Acinetobacter calcoaceticus can only degrade C22-C30 petroleum hydrocarbons, while fungi Candida tropicalis can degrade C12-C32 petroleum substances. A strain of oil-degrading bacteria was screened out from oil-polluted seawater. It was identified as filamentous fungi, which can degrade many kinds of petroleum hydrocarbons quickly and is not restricted by phosphorus, nitrogen nutrients and dissolved oxygen. It has great development and application value. According to research, the conversion of filamentous fungi to petroleum hydrocarbons is a process of
combining biodegradation with biosorption, and its cell wall is directly related to deoiling [22]. Gas Chromatography was used to determine the degradation of petroleum hydrocarbons by bacteria. The results showed that the degradation rate of n-alkanes by bacteria was significantly higher than that of single bacteria, and the degradation rate of mixed bacteria was generally higher than that of single bacteria [23]. Five dominant strains were isolated from soil contaminated by Bombay High crude oil to form degradation bacteria. After 20 days treatment, the degradation rate reached 78%, and the highest single strain was 66%[24]. Four strains of oil-degrading bacteria were isolated and screened from the soil. These strains were mixed according to the principle of equal proportion. Among them, the oil removal rate of mixed strain G8 was nearly 30%[25] higher than that of single strain. However, there may be antagonistic or synergistic effects among different strains, and it is difficult to achieve the optimal effect by simple single bacterial. Therefore, how to obtain and utilize reasonable and efficient degrading bacteria remains to be further studied.

WENTZEL et al. [26] reported that Pseudomonas and Rhodococcus spp. could use n-C12-n-C3 alkanes as carbon and energy sources. In recent years, the microbial degradation of long-chain alkanes by Achromobacter xylosoxidans has also been studied, but it is not deep enough, especially the degradation mechanism of long-chain alkanes needs further discussion [27]. In this study, a highly efficient long-chain alkane degrading bacteria was screened and identified as Gordonia sp., and its degradation characteristics of long-chain alkanes were studied.

3. Methodology

3.1 Isolation of Long-chain Alkane Degrading Bacteria
(1) Enrichment of bacteria
10 ml activated sludge was added to 250 ml triangular flask containing 100 ml enrichment medium, and incubated for 7 days in a constant temperature shaking bed at 35°C and 180 rpm. The 5 ml culture medium was transferred to fresh enrichment medium, and then incubated for 7 days under the same conditions, so that the enrichment culture lasted for 28 days.
(2) Isolation of Long Chain Hydrocarbon Degrading Bacteria
The 200 ml enriched medium was gradiently diluted (10-1, 10-2, 10-3... 10-8, 10-9) and then coated on the separation medium. Each concentration was coated with three Petri dishes and cultured in an inverted incubator at 35°C. When the plate grew out of the plate, the single colonies were well distributed (usually 10-6 or 10-7 diluted concentration). Different single colonies were selected according to the shape, size and color of the colonies. Colony of bacteria.
(3) Purification of Long-chain Alkane Degrading Bacteria
Separated single colonies were cultured in 35°C incubator for 7 days by scribe method on the isolation medium plate. This was repeated three times until the purified single colony was obtained. The purified colonies were inoculated on the inclined preservation medium and stored in the refrigerator at 4°C for reserve.

3.2 Morphological characteristics of long-chain alkane-degrading bacteria
Three strains of bacteria were cultured on the isolation medium. After 5 days of inverted culture at 37°C, the morphology and characteristics of the colonies were observed, and each strain was stained with Gram's staining.

3.3 16SrDNA Identification and Phylogenetic Analysis of Long-chain Alkane Degrading Bacteria
The strain was identified by gram strain, morphology observation, 16SrRNA sequence analysis and other methods. The genome of hydrocarbon degrading bacteria was extracted and purification by bacterial genome extraction kit, which was regarded as a template to gain purpose gene fragment through 16SrRNA PCR amplification for conserved sequence. Then it was used to construct a
phylogenetic tree using the sequence alignment software ClustalX2.0 and system development analysis software MEGA5.1.

3.4 Characteristic Analysis of Long-chain Alkane Degrading Bacteria

Three strains of long-chain alkane-degrading bacteria were inoculated into the inorganic salt culture with 1000mg/L mixed long-chain alkane as the sole carbon source at a volume ratio of 2%. The degradation ability and growth of the bacteria were investigated under the conditions of pH 7.0, temperature 35°C and shaking speed 180 rpm for 12 days.

Seed liquid preparation: Microorganisms were cultured in LB solid medium at 30 for 3 days, then stored at 4°C for reserve. The OD600 value of single colony was about 0.8 when it was inoculated in LB liquid medium at 150 rpm and 30°C for 1 day. The culture medium was centrifuged for 5 minutes at 6000 rpm, then the bacteria were washed twice with sterilized inorganic salt medium, and the same volume inorganic salt medium was re-suspended as seed liquid.

3.5 Analysis of Degradation Characteristics of Long-chain Alkane Degrading Bacteria Affected by Environmental Factors

Long-chain alkanes are hydrophobic organic compounds with very low solubility in water, which reduces the growth rate of bacteria and the degradation rate of organic matter. The results show that the change of environmental factors can increase the content of hydrocarbons or the contact area between bacteria and hydrocarbons. For example, the increase of temperature and stirring speed can increase the solubility and contact area of long-chain alkanes, thus increasing their degradation rate. Therefore, optimizing environmental factors can improve the degradation efficiency of long-chain alkanes.

(1) Effect of pH on degradation characteristics of long-chain alkane-degrading bacteria

PH in the environment has a great influence on the growth of microorganisms. The main effect of pH is to cause the change of cell membrane charge (negative charge on the surface of bacteria) and the ionization degree of nutrients, thus affecting the availability of microorganisms to nutrients (such as ammonia, phosphate, etc. [1]). In this experiment, mixed long-chain alkanes (1000mg/L) were used as the sole carbon source and cultured for 12 days at 180 rpm and 35°C. The effects of different pH (2, 4, 6, 7, 8, 10, 12) on the degradation rate of long-chain alkanes-degrading bacteria were investigated.


(2) Effect of temperature on degradation characteristics of long-chain alkane-degrading bacteria

Because long-chain alkanes are mostly solid at room temperature and insoluble in water, they seriously affect the mass transfer efficiency of hydrocarbons and the degradation efficiency of hydrocarbon-degrading bacteria. Changing temperature has a great influence on the physical state of hydrocarbons, thus changing the solubility and degradation efficiency of hydrocarbons. In this experiment, mixed long-chain alkanes (1000mg/L) were used as the sole carbon source, and cultured at pH 7.0 and 180 rpm for 12 days. The effects of different temperatures (15, 25, 35, 45, 55°C) on the degradation rate of long-chain alkanes-degrading bacteria were investigated.

(3) The influence of shaker speed on the degradation characteristics of long-chain alkane-degrading bacteria

The contact area and dissolved oxygen content between hydrocarbon-degrading bacteria and long-chain alkanes can be changed by changing the rotational speed of shaker, thus affecting the degradation rate of hydrocarbon-degrading bacteria. In this experiment, mixed long-chain alkanes (1000mg/L) were used as the sole carbon source, and cultured at pH 7.0 and 35°C for 12 days. The effects of different rotational speeds (0, 90 rpm, 120 rpm, 150 rpm, 180 rpm, 210 rpm) on the degradation rate of long-chain alkanes-degrading bacteria were investigated.
4. Result Analysis and Discussion

4.1 Morphological characteristics of long-chain alkane-degrading bacteria

Activated sludge comes from the secondary sedimentation tank of Hayi coal gasification wastewater treatment process. After enrichment and separation, three strains of long-chain alkane degrading bacteria, named lys1-3, lys1-4 and lys2-1, were obtained. The results are shown in Table 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>morphology of Colony</th>
<th>Color of Colony</th>
<th>Shape of Bacteria</th>
<th>Gram staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>lys1-3</td>
<td>Round, regular edges, smooth texture, protruding surface</td>
<td>Turquoise, opaque</td>
<td>Short rod, flagellum-free</td>
<td>Positive</td>
</tr>
<tr>
<td>lys1-4</td>
<td>Round, irregular edges, smooth texture, protuberant surface</td>
<td>Turquoise, opaque</td>
<td>Short rod, flagellum-free</td>
<td>Positive</td>
</tr>
<tr>
<td>lys2-1</td>
<td>Small circle, neat edge, smooth texture</td>
<td>Turquoise, transparent</td>
<td>Short rod, flagellum-free</td>
<td>Positive</td>
</tr>
</tbody>
</table>

4.2 16SrDNA Identification and Phylogenetic Analysis of Long-chain Alkane Degrading Bacteria

According to the sequence homology of 16SrDNA proposed by Goodfellow and O'Donnell in 1993, all three strains belong to Gordonia sp. The results are shown in Table 2. The strain lys1-3 is named Gordonia sp. lys1-3 and its login number is KC211011.

<table>
<thead>
<tr>
<th>Strain</th>
<th>The most homologous reference bacteria (GenBank accession number)</th>
<th>Homology (%)</th>
<th>Genus</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>lys1-3, lys1-4, lys2-1</td>
<td><em>Gordonia sihwanensis</em>[^28] NBRC 108236 (BANU01000043)</td>
<td>100</td>
<td><em>Gordonia sp.</em></td>
<td>KC211011</td>
</tr>
</tbody>
</table>

Comparing lys1-3 sequence with 30 bacterial model strains with high homology in database, the 16S rDNA sequence of lys1-3 has high homology with 20 species of Gordonia genus, and the homology is over 97%. Among them, the homology with Gordonia sihwanensis NBRC 108236 (BANU01000043) is 100%. The results are shown in Figure 1. The phylogenetic tree showed that lys1-3 and Gordonia sihwanensis NBRC 108236 and Gordonia cholesterolivorans Chol-3 could be clustered together. Based on morphological characteristics, Gram staining and 16SrDNA sequence analysis, lys1-3 was classified as Gordonia and named Gordonia sp. lys1-3.

4.3 Degradation characteristics of long-chain alkane-degrading bacteria

According to Figure 2, the degradation rates of lys1-3, lys1-4 and lys2-1 were 72.5%, 69.5% and 71.9% respectively. Observing the growth curve of the bacteria, we can see that the growth delay of Gordonia sp. is longer, and it takes 5 days to reach the growth plateau. Most of the other genera can reach the maximum growth after 4 days and enter the growth platform stage. These differences may be due to different ways of uptake of long-chain alkanes. Because Gordonia sp. has three strains, in order to avoid overwork, in the follow-up study, the strain with the highest degradation rate was selected as the research object, namely lys1-3.
4.4 Effect of Environmental Factors on Degradation Characteristics of Long-chain Alkane Degrading Bacteria

(1) Effect of pH on degradation characteristics of long-chain alkane-degrading bacteria
As shown in Figure 3, lys1-3, a hydrocarbon-degrading bacterium, achieves the maximum degradation rate at pH 7.0. The bacteria could not grow under pH 2.0 or above, and the degradation rate was about 2% compared with the blank control. This indicated that too high or too low pH would cause damage to the bacteria, resulting in serious inhibition of the growth of the bacteria, thus reducing the degradation rate. The degradation rate of strains was higher in the range of pH 6.0-8.0. The lys1-3 still has a certain degradation rate when the pH is less than 2 or more than 12, which indicates that lys1-3 has a certain tolerance to adverse environment. During the experiment[29], it was also found that the pH of the solution after incubation decreased significantly (the data did not show), which may be due to the production of some long-chain fatty acids during the microbial degradation of long-chain alkanes, which reduced the pH of the solution. In addition, acidic or alkaline conditions have a certain resistance to degrading bacteria, but the strain can maintain certain activity in the range of pH 4.0-10.0, indicating that the strain can maintain the ability of degrading long-chain alkanes in a larger range of pH, has a strong ability to adapt to the environment, and can play a certain degradation role under adverse environmental conditions.

Fig. 3 Influence on the degradation rate of different pH to long chain alkane degrading bacteria

(2) Effect of temperature on degradation characteristics of long-chain alkane-degrading bacteria

Fig. 4 shows that lys1-3 can not grow at 15 to 55℃, and has almost no degradation activity. The reason may be that too low or too high temperature inhibits the activity of degrading enzymes, which leads to the inability of bacteria to grow and propagate using long-chain alkanes as carbon source. It was found that lys1-3 had degradation activity in the range of 25-45℃, but the degradation rate was the highest at 35℃. It indicated that the optimum temperature for degradation of hydrocarbons by lys1-3 was 35℃. At this temperature, the activity of degradation enzymes of long-chain alkanes was higher and the solubility of lys1-3 was certain, which provided favorable conditions for adsorption, uptake and degradation of long-chain alkanes by hydrocarbon-degrading bacteria.

Fig. 4 Influence on the degradation rate of different temperature to long chain alkane degrading bacteria

(3) The influence of shaker speed on the degradation characteristics of long-chain alkane-degrading bacteria

As shown in Fig. 5, the higher the rotational speed, the higher the degradation rate of lys1-3, but with the increase of rotational speed, the higher the hydrocarbon degradation rate, the lower the degradation rate.
rate. Especially when the rotational speed is 210 rpm, the degradation rate does not increase or decrease, which indicates that the higher the rotational speed, the better. The increase of rotational speed can effectively increase the interface area of long-chain alkanes, the contact area between long-chain alkanes and hydrocarbon-degrading bacteria, and the dissolved oxygen content in shaking flasks, so as to increase the degradation rate. However, when the rotational speed is too high, the mass transfer and stability of the system will be affected and the degradation rate will be reduced. Therefore, we set the rotational speed of the shaker to 180 rpm in the follow-up experiments.

Fig. 5 Influence on the degradation rate of different speed to long chain alkane degrading bacteria

5. Conclusion

(1) Three strains of lys1-3, lys1-4 and lys2-1 were obtained by enrichment, isolation and purification of sludge samples. Three strains were identified by morphological observation, Gram staining and 16SrDNA sequence analysis. The results showed that lys1-3, lys1-4 and lys2-1 belonged to Gordonia.

(2) The degradation characteristics of three strains of hydrocarbon-degrading bacteria were analyzed. The degradation rates of strains lys1-3, lys1-4 and lys2-1 were 72.5%, 69.5% and 71.9% respectively. The degradation rates of mixed long-chain alkanes by strains of Gordonia sp. were higher. But the growth delay of Gordonia sp. is longer, and it takes 5 days to reach the growth plateau.

(3) The environmental factors of long-chain alkane-degrading bacteria were optimized: the optimum pH was 7.0, the temperature was 35°C, and the shaking speed was 180 rpm.

Acknowledgements

Scientific Research Projects of Heilongjiang Education Department, 12541052.

References


[7] Seyyedeh Mandana SadrAzodi et al., Biodegradation of long chain alkanes in halophilic conditions by Alcanivorax sp. strain Est-02 isolated from saline soil, Biotech, 2019, 9:141


