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Identification of Petroleum Degrading Bacteria and Construction of Petroleum Degrading Agent

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Abstract

The aim of this study is to construct petroleum degrading agent (PDA) which can effectively degrade oil. By enrichment, domestication, and separation of culture from the soil sample of Qianjiang Guanghua Oilfield, the pure culture of three petroleum degrading bacteria G-40, G-53, and G-94 was identified from the medium supplemented with oil, which served as the sole source of carbon. The species of G-40 and G-53 were preliminarily identified and classified by morphological observation, physiological and biochemical determination, and sequence analyses of 16S rDNA. The species of G-94 was preliminarily identified and classified by morphological observation, physiological and biochemical determination, and sequence analyses of ITS rDNA. The optimal inoculation proportion of these three bacteria strains and bran proportion in composition of PDA were determined through orthogonal test. G-40, G-53, and G-94 were isolated and identified as *Brevibacillus laterosporus*, *Tsukamurella inchonensis*, and *Candida tropicalis*, respectively. To construct petroleum degrading agent, the optimum inoculation proportion of the three bacteria strains was A1B3C3 (G-40:G-53:G-94 = 1:4:4); and the optimum proportion of bran was D1E1F2 (soybean meal:corn flour:bran = 1:1:2). The oil removal rate of the constructed petroleum degrading agent reached to 42.32% on day 10 under the optimal proportion of bacteria inoculation and bran composition. Petroleum degrading bacteria can effectively degrade petroleum for its own growth. This study identified three petroleum degrading bacteria strains and proposed a petroleum degrading agent by studying the optimal inoculation proportion of the three bacterial strains and the accompanying bran. Our research could provide potential microbial resources for bioremediation of petroleum-contaminated soil.

Subject Areas

Environmental Sciences, Microbiology

Keywords

Petroleum Degrading Bacteria, Petroleum Degrading Agent, Degradation Characteristics

1. Introduction

Petroleum oil is a major energy source all over the world. It is a complex mixture of wide variety of different compounds including normal alkanes (n-alkanes), cyclic alkanes (c-alkanes), polyaromatic hydrocarbons (PAHs), and non-hydrocarbon compounds [1]. Petroleum oil contamination due to mismanagement of oil-production well poses serious environment problems which could also affect human health [2]. Petroleum hydrocarbons are considered very hazardous to living organism due to their toxicity, mutagenicity, and carcinogenicity [3].

Bioremediation technology including biodegradation is considered a noninvasive and relatively cost-effective approach to alleviate petroleum caused pollution of the environment [4]. Biodegradation by natural population of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment. In addition, it is cheaper than other remediation technologies, making it the most efficient and environment-friendly decontamination method [5] [6].

Bioremediation of petroleum-contaminated soils by natural population of microorganisms has been reported in many studies. There are about more than 30 genera and 200 species that can degrade oil in nature, including bacteria, fungi, and yeasts, among which bacteria are the main species identified. Oil degrading bacteria mainly include *Saccharomyces sp*, *Bacillus sp*, *Rhodococcus sp*, *Pseudomonas sp*, *Sphingomonas sp*, *Acinetobacter sp*, *Alcaligenes sp*, *Mirococcus sp*, and *Nocardia sp*, *et al.* [7] [8] [9] [10]. It is of great interest to utilize oil degrading bacteria to remove oil contamination in the environment. Although it is advantageous over bacterial fluid for its easier transportation and handling, bioremediation of petroleum-contaminated soils by solid degrading agent is rarely utilized in practice. In this study, we constructed petroleum degrading mixed bacteria agent, which showed effective oil-degrading capability.

2. Materials and Methods

2.1. Source of Bacteria Isolation

The G-40 (*Brevibacillus laterosporus*, accession number KY949476) and G-94 (*Candida tropicalis*, accession number KY949478) were isolated from Qianjiang Guanghua Oilfield in June 2015 and stored in the laboratory of College of Life Science, Yangtze University [11].

The G-53 was isolated from oil-contaminated soil sample. Soil samples were collected into 500 ml sterilized glass bottles from eight sites in Qianjiang Guanghua Oilfield in June 2015. After collection, they were immediately transported to the laboratory and stored in a refrigerator at 4 °C prior to use.

2.2. Sample

Oil-contaminated soil sample is from Qianjiang Guanghua Oilfield.

2.3. Bran Feed

Soybean meal, corn flour, and bran with no mildew, rot, or moth bite were used in this study.

2.4. Media

Oil medium (0.5% oil) was prepared according to [12].

Beef peptone liquid medium, beef peptone agar medium, and potato sucrose liquid medium were prepared according to [13].

2.5. Isolation of Bacteria

5 g oil-contaminated soil sample was inoculated in 100 ml oil medium in a 250 ml Erlenmeyer flask and cultured at 35˚C on a horizontal shaker (150 rpm). After 5-day incubation, 5 ml of sample from primary enrichment medium was subcultured to a fresh 100 ml oil medium and incubated at the same condition. After three rounds of subculturing in oil medium, 0.1 ml of enrichment culture was plated at appropriate dilution on beef peptone agar medium and incubated at 37˚C for 24 h. Single colony appeared on the medium plates were translated on a fresh beef peptone agar medium for incubation. Isolated single colony was stored at 4˚C on beef peptone agar medium and subcultured at 3-week intervals or mixed with 40% glycerol and stored at −80˚C for future use [12].

2.6. Strain Enrichment Culture

G-40 and G-53 were inoculated into beef peptone liquid medium and G-94 was inoculated into potato sucrose liquid medium and cultured on a horizontal shaker (150 rpm) at 35˚C for 48 h. The bacterial culture was stored at 4˚C for future experiments.

2.7. Determination of Biomass

Biomass was monitored by measuring the optical density at 420 nm with a TU-1900 spectrophotometer [14].

2.8. Determination of Petroleum Removal Rate

The oil removal rate was determined by gravimetric method [15].

2.9. Culture Characteristics and Cell Morphology

The colony characteristic of G-53 (3 d) was observed on the beef peptone agar

plate and the cell morphology was observed under microscope after gram staining.

2.10. Physiological and Biochemical Tests

The physiological and biochemical properties of the strain G-53 were determined according to the microbiological test method described by [13].

2.11. Molecular Biology Assay

The 16S rDNA gene of colony G-53 was amplified by PCR using the universal primers 27F and 1492R [16] and Thermo Scientific Phusion Flash High-Fidelity kit. The amplified 16S rDNA gene was sequenced by Sangon Biotech (Shanghai, China) Co. Ltd. The sequence similarity was analyzed by Blast on the National Centre for Biotechnology Information (NCBI). The phylogenetic tree was constructed with software MEGA5.0.

2.12. Determination of Bacteria Inoculation Proportion by Orthogonal Experiment

Three factors and three levels of orthogonal experiment (**Table 1**) of G-40, G-53, and G-94 were tested on a horizontal shaker (150 rpm) at 35˚C. After 5 days of incubation, biomass was determined by measuring the optical density at 420 nm with a TU-1900 spectrophotometer.

2.13. Determination of Bran Proportion by Orthogonal Experiment

Three factors and three levels of orthogonal experiment (**Table 2**) of soybean meal, corn flour, and bran were tested. 10% (v:v) bacterial fluid was added to the bran with 2% sucrose. After incubation for 10 days at 35˚C, the PDA was filtered through 0.45 mm sieve and stored in sterile bags. 1% (m:v) inoculum of PDA was added to oil medium for 5 day incubation on a horizontal shaker (150 rpm) at 30˚C. Biomass was monitored by measuring the optical density at 420 nm with a TU-1900 spectrophotometer.

Table 1. Factor levels table of bacteria inoculum proportion.

	A	B	C
1	0.5%	0.5%	0.5%
2	1.0%	1.0%	1.0%
3	2.0%	2.0%	2.0%

A: G-40; B: G-53; C: G-94..

Table 2. Factor levels table of bran proportion.

	D	E	F
1	10%	10%	10%
2	20%	20%	20%
3	30%	30%	30%

D: soybean meal; E: corn flour; F: bran.

2.14. Verification Test

The oil removal rate of PDA was tested after 10-day incubation under optimum inoculation proportion of bacteria and bran proportion.

3. Results and Analysis

3.1. Culture Characteristics

After 3 days of bacteria cultivation, colony morphology of G-53 is shown in (Figure 1).

3.2. Cell Morphology

The physiological and biochemical characteristic of G-53 is shown in (Table 3).

Figure 1. Morphology of G-53.

Table 3. Morphology and culture characteristics of G-53.

Number	G-53
Morphology	Rhizoidal
Colour	Orange
Texture	Rough
Protuberance	Flat
Edge	Crimp
Diameter(mm)	3.16
Area (mm^2)	6.71
Gelatin liquefaction	−
Indole	−
H_2O_2	+
Citrate	−
M.R.	−
V.P.	+
Starch hydrolysis	−
Lipid hydrolysis	−
Ammonia production	+
Nitrate reduction	−
Phenylalanine dehydrogenase	−
H_2S	+

+: positive; −: negative.

3.3. Molecular Identification

The 16S rDNA gene obtained from the isolates was amplified via PCR using a universal bacterial/fungal primer set. Based on the morphological observation, physiological and biochemical determination, and sequence analyses of 16S rDNA sequence, G-53 was identified as *Tsukamurella inchonensis* (**Figure 2**). Accession number is KY949477.

3.4. Determination of Bacteria Inoculation Proportion

The G-40 and G-53 were inoculated into beef peptone liquid medium and G-94 was inoculated into potato sucrose liquid medium. The bacteria culture was incubated on a horizontal shaker (150 rpm) at 35˚C for 48 h. The OD_{420} of G-40, G-53, and G-94 were 7.72, 2.00, and 9.87, respectively. Different inoculation proportion of the three bacteria strains showed different effect on the oil removal rate (**Table 4**). Maximum ratio of G-40 is the key factor affecting the oil removal rate, followed by G-94, while G-53 has the least effect. Therefore, the inoculation proportion of G-40, G-53, and G-94 in PDA for optimal oil removal was A1B3C3 (G-40:G-53:G-94 = 1:4:4).

3.5. Determination of Bran Proportion

Different bran proportion had obvious different effect on the oil removal rate (**Table 5**). Maximum range of corn flour is the key factor affecting the oil removal rate, followed by soybean meal, while bran has the least effect. Therefore, the bran proportion in PDA for optimal oil removal was D1E1F2 (soybean meal: corn flour:bran = 1:1:2).

3.6. Verification Test

The result of verification test of PDA was shown in (**Figure 3**). Under orthogonal

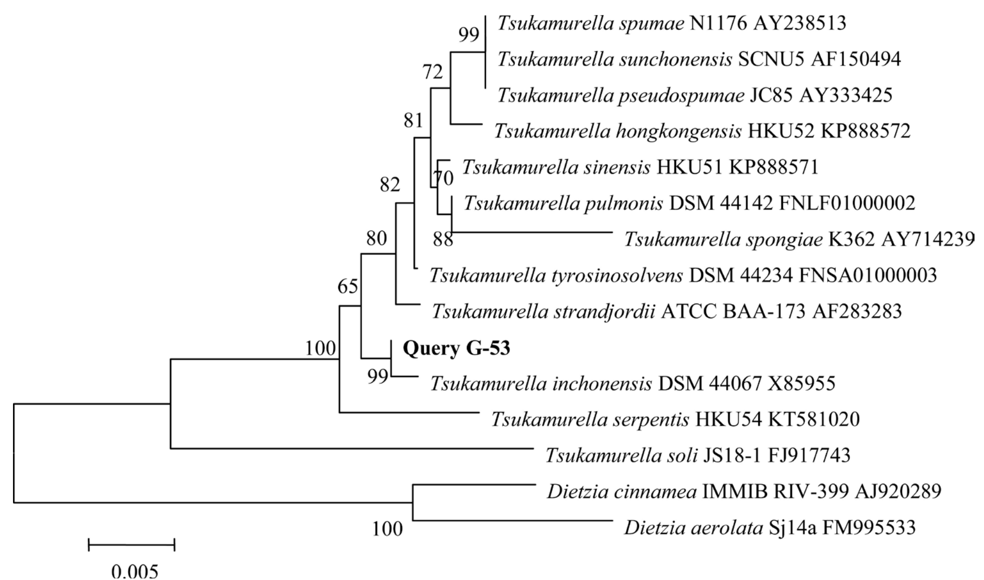

Figure 2. Neighbor-joining phylogenetic tree constructed from 16S rDNA gene sequence of G-53.

Table 4. The orthogonal design and analysis of bacteria inoculation proportion.

Number	A	B	C	Biomass
1	0.5%	0.5%	0.5%	0.161
2	0.5%	1.0%	1.0%	1.749
3	0.5%	2.0%	2.0%	2.046
4	1.0%	0.5%	1.0%	0.234
5	1.0%	1.0%	2.0%	0.263
6	1.0%	2.0%	0.5%	0.354
7	2.0%	0.5%	2.0%	0.469
8	2.0%	1.0%	0.5%	0.286
9	2.0%	2.0%	1.0%	0.380
K1	1.319	0.288	0.267	
K2	0.284	0.766	0.788	
K3	0.378	0.927	0.926	
R	1.035	0.639	0.659	

A: G-40; B: G-53; C: G-94.

Table 5. The orthogonal design and analysis of bran proportion.

Number	D	E	F	Biomass
1	10%	10%	10%	13.01
2	10%	20%	20%	9.04
3	10%	30%	30%	10.49
4	20%	10%	20%	10.30
5	20%	20%	30%	9.45
6	20%	30%	10%	12.10
7	30%	10%	30%	11.39
8	30%	20%	10%	6.25
9	30%	30%	20%	6.69
K1	9.413	10.133	9.020	
K2	9.117	6.747	9.343	
K3	6.610	8.260	7.513	
R	2.803	3.386	1.830	

D: soybean meal; E: corn flour; F: bran.

optimum conditions (the inoculation proportion of G-40:G-53:G-94 = 1:4:4, the bran proportion of soybean meal:corn flour:bran = 1:1:2), the oil removal rate of PDA on 10 d reached up to 42.32%.

4. Discussion

In this study, the optimal bacteria inoculation and bran proportion in petroleum

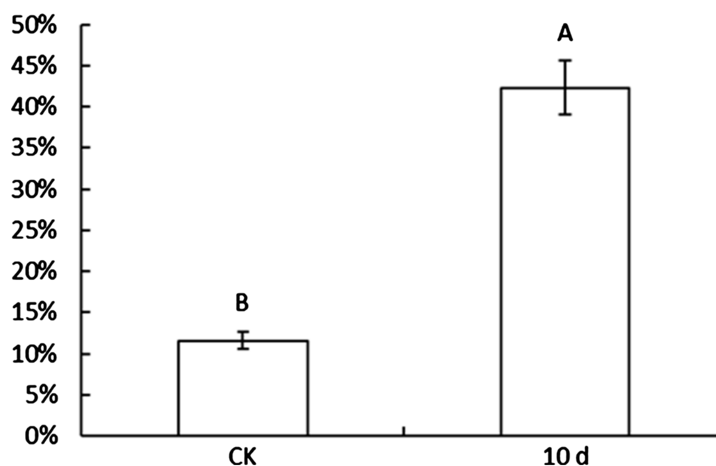

Figure 3. Oil degradation rate of mixed inoculum on 10 d.

degrading agent were determined according to the biomass of petroleum degradation microorganism instead of the oil removal rate. It is mainly because biomass is positively correlated with oil removal rate and more convenient to be measured and monitored than oil removal rate.

The degradation of oil in the environment is possible through several techniques including physical [17], chemical [18], or biological approaches [19]. The technology commonly used for soil remediation includes mechanical, burying, evaporation, dispersion, and washing. However, these technologies are expensive and can lead to incomplete decomposition of contaminants. The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities, is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry [20]. In addition, bioremediation technology is believed to be noninvasive and relatively cost-effective [4]. Biodegradation by natural population of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment and is cheaper than other remediation technologies, making it the most efficient approach for environment safe depollution [5] [6]. *Brevibacillus laterosporus*, *Tsukamurella inchonensis*, and *Candida tropicalis* were used for environmental management, biological medicine, and food fermentation [21] [22] [23] [24] [25]. However, the bacterial fluid is inconvenient for transportation. Therefore, we developed microbial agents with G-40, G-53, and G-94. The optimal proportion of the three bacteria strains as well as the bran was determined with orthogonal designed experiments to effectively degrade petroleum. When the inoculation proportion of G-40:G-53:G-94 is 1:4:4 and the bran proportion of soybean meal:corn flour:bran is 1:1:2, the oil removal rate of the constructed PDA reached up to 42.32% on day 10 after bacteria cultivation.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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