Chinese Journal of Chemical Engineering 24 (2016) 353–359

Contents lists available at ScienceDirect

Chinese Journal of Chemical Engineering

journal homepage: www.elsevier.com/locate/CJChE

Separation Science and Engineering

Novel method for separation and screening of lubricant-degrading microorganisms and bacterial biodegradation☆

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ARTICLE INFO

Article history:
Received 3 May 2015
Received in revised form 3 August 2015
Accepted 31 August 2015
Available online 11 November 2015

Keywords:
Lubricant
Degradation
Separation
24-well plate
Enzyme

ABSTRACT

With the rapid increase of lubricant consumption, oil contamination becomes more serious. Biotreatment is an important method to remove oil contamination with some advantages. In this study, acclimatized oil-contaminated soil and used lubricating oil were sampled to isolate lubricant-degrading strains by several methods. 51 isolates were obtained and 24-well plates were employed to assess bacterial potential in high-throughput screening. The method was noted for the prominence of oil–water two-phase system with saving chemicals, shortening cycles and lessening workloads. In order to decrease inaccuracy, subculture and resting cells were inoculated into mineral salt medium with 200 μl oil in well plates for the cultivation at 37 °C for 5 and 7 days, and the biodegradation potential was characterized by the changes of oil film and cell density. With appropriate evaluation by shaking flask tests, 5 isolates were retained for their potentials with the maximum biodegradation from 1500 to 2200 mg · L^{-1} and identified as *Acidovorax citrulli*, *Pseudomonas balearica*, *Acinetobacter johnsonii* (two isolates with different biodegradation potentials) and *Acidovorax avenae* using 16S rRNA sequencing analysis. Also, lipase activity was determined using indicator titration and *p*-nitrophenyl palmitate (*p*-NPP) methods. The results indicated that only *p*-NPP was successful to test lipase activity with the range of 1.93–6.29 U · ml^{-1}. Although these five strains could degrade 1000 mg · L^{-1} lubricating oil in 158–168 h, there existed distinct difference in enzyme activity, which demonstrates that lipase activity could not be used as the criterion to evaluate microbial biodegradation potential for petroleum hydrocarbons.

1. Introduction

The lubricant consumption in China is the 2nd in the world. Lubricant hastens the rapid development of modern industries at the cost of prevalent environmental oil pollutions due to accidental leaking and improper disposal [1,2]. It is reported that discharges of used lubricating oil in land and waterway account for about 40% of total oil inputs, and considering the restrictions of recycling channels and regeneration techniques, the discharge is far more than that. The remediation of oil-contaminated sites raises a growing concern in many countries [3,4].

Physical and chemical methods are widely employed for lubricant treatment, but these methods are often grossly inadequate and ineffective, easily causing secondary pollution [5]. Biological techniques have been developed as an efficient, economic and eco-friendly treatment [3,6]. Lee *et al.* [7,8] focused on biodegradation of specific substrates of two recalcitrant lubricant ingredients, cyclohexane and hexane, with the maximum specific degradation rate of 246 and 361 μmol · (g DCW · h)$^{-1}$, respectively, by *Rhodococcus* sp. isolated from oil-contaminated soil. The strain could utilize many other hydrocarbons under optimal conditions, such as PAHs and BTEX. Khondee *et al.* [2] made use of a bioreactor containing chitosan-immobilized *Sphingobium* sp. P2 to remove automotive lubricants from emulsified wastewater. It is worth noting that an internal loop airlift bioreactor and immobilized cells were utilized in the tests, which are regarded as the advanced technologies in the field. However, the removal rate of 200 mg · L^{-1} total petroleum hydrocarbons only reaches 80%–90%. These studies mainly concentrate upon two aspects, specific substrate biodegradation and bioremediation of contaminated sites and wastes by petroleum hydroxycarbons, with the microbial strains. Currently, many microorganisms with the ability to degrade crude oil, diesel oil and heavy oil, as well as their compositions have been isolated and identified from pollution sites and oily sludge. They are usually screened from a large quantity of isolates by traditional shaking flask tests with such disadvantages as chemical consumption, heavy load and long cycle. Since biodegradation occurs in oil–water phase, well plate method would be preferred for high-throughput isolate screening. Lubricating oils float on water, so it is easy to evaluate

☆ Supported by the National Natural Science Foundation of China (21376285), Chongqing Natural Science Foundation (CSTC2013jcyjA20014), Open Funding Project of the Key Laboratory of Systems Bioengineering, Ministry of Education, and Scientific Platform Project, Ministry of Education (FYKF201506).
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http://dx.doi.org/10.1016/j.cjche.2015.11.001
1004-9541/

the biodegradation through oil film change. Presently, well plate tests have been applied in fermentation experiments for different purposes, but seldom employed in oil–water systems [9].

It is a growing trend to add exogenous microorganisms in bioremediation, which hinges on bacterial activity for lubricating oil degradation. As the ingredients in lubricating oil are a complex mixture of hydrocarbons including linear and branched paraffins, cyclic alkanes and aromatic hydrocarbons, activity of effective lubricant-degrading microorganisms reflects their biodegradation abilities for multisubstrate in the aspect of lubricant biodegradation [10]. Study on lubricating oil as substrate is of practical significance for the treatment of lubricating oil contamination.

The objectives of this study are to isolate and identify lubricant-degrading bacteria from used lubricating oil and acclimated oil-contaminated soil separately, to investigate different methods on bacterial isolation and explore the possibility of well plate method in high-throughput screening, to describe bacterial biodegradation characterization by shaking flask experiments, and to evaluate lipase activity by comparison of two different methods.

2. Materials and Method

2.1. Acclimation of soil sample

Soils perennially contaminated by lubricating oil were collected from a used lubricant recycling enterprise in Chongqing, China. Soils were sampled 0–20 cm underneath the regolith, dried at 37 °C in a constant temperature incubator to be easily comminuted, and sieved to 2 mm. Then it was enriched acclimation at 37 °C using lubricating oil as sole carbon source for a period of 37 days. In order to avoid the death of indigenous microorganisms owing to the side effect of high-concentration petroleum hydrocarbons, 30 g soil was reserved as isolation sample according to five-point sampling method before each gradient acclimation. Table 1 summarizes the acclimation method. 2.5% and 5.0% urea and 0.2 mol · L^{-1} dipotassium phosphate were served as fertilizer to provide nutrients N and P in order to stimulate indigenous microorganisms [11]. Their addition depended on lubricant concentration in soil and acclimation stage [12]. Besides, moisturizing and turning over the soil were conducted twice every day. In samples 3 and 4 with high-concentration lubricating oil, 0.5% (v/v) Tween 80 was introduced as surfactant in order to enhance indigenous microorganism biodegradation.

2.2. Bacterial isolation, identification and cultivation

Bacterial strains were isolated from acclimated soil and used lubricating oil. 5 g soil and lubricating oil were inoculated into shaking flasks with LB medium (peptone 10 g · L^{-1}, yeast extract 5 g · L^{-1}, sodium chloride 10 g · L^{-1}) separately. After three enrichment cultures, the cultures were inoculated into mineral salt medium (MSM), composed of (g · L^{-1}): 1 NH_4NO_3, 0.8 K_2H0PO_4, 0.4 KH_2PO_4, 0.8 NaCl, 0.05 $CaCl_2$, 0.05 $MgSO_4$ and 0.05 $FeSO_4$, and 150 mg · L^{-1} lubricating oil was used in this test. The initial pH was adjusted to 7.2 before sterilization at 121 °C for 20 min. Incubation was done in a rotary shaker with 200 r·min^{-1} at 37 °C. After two selective cultures, 100 μl cultures were plated onto agar plates. In order to improve the likelihood to obtain highly active strains and decrease the disturbance from untargeted strains, such methods were designed aiming at the plates as LB, LB and MSM containing lubricant, and LB and MSM with oil film on the surface for the isolation from soil sample, and LB, LB and MSM with oil film on the surface for the isolation from lubricant sample. In each of the eight methods, 5 plates were continually coated. The individual colonies were inoculated on nutrient agar slants and evaluated for the potential to degrade lubricating oil through 24-well plates and shaking flask tests.

The isolates screened were identified based on 16S rRNA sequencing (Shanghai Sunny Biotechnology Co., Ltd, China) followed by BLAST analysis (NCBI) with closed match with available sequences.

2.3. 24-Well plate tests

The isolates from slants were cultivated overnight in 10 ml LB medium. 0.4 ml of this culture was inoculated to 20 ml fresh medium. Subculture with $OD_{600} = 1.2 \pm 0.02$ was harvested to inoculate into oil–water system. Immediately after measurements of optical density, this subculture was injected into 1.5 ml sterilized centrifugal tubes to centrifuge for 10 min at 6000 r·min^{-1} and the supernate was discarded. Cells were re-suspended using 1.5 ml phosphate buffer (pH = 7.2). After cells were washed, cell suspension was centrifuged. The operation above was repeated twice. Resting cells were prepared by cell resuspension with 0.2 ml phosphate buffer, which also served as inoculum to conduct well plate tests.

2.5 ml MSM was added into sterile 24-well plates, 0.2 ml different inoculum was inoculated in each hole, and immediately 0.3 ml used lubricating oil was combined. 24-well plates were cultivated for 5 days for three subcultures as inoculum and 7 days for three resting cells at 37 °C. Samples without inoculation and without oil were used as two different controls. All tests including controls were repeated three times. Biodegradation of isolates was evaluated mainly based on the change of oil film supplemented with cell density determination.

2.4. Shaking flask tests

A total of 15 isolates were obtained after preliminary screening and further tested by shaking flasks. 2% (v/v) subculture ($OD_{600} = 1.2 \pm 0.02$) as inoculum was inoculated into shaking flasks supplemented with 25 ml MSM and lubricant concentration of 100 mg · L^{-1} for 30 h cultivation at 37 °C and 200 r·min^{-1} in a rotary shaker. In order to compare and choose the more effective *Acinetobacter johnsonii*, a series of shaking flasks were applied to evaluate their biodegradation for 400 and 800 mg · L^{-1} lubricating oil at 5% inoculum volume. Moreover, 5 screened isolates were tested for the biodegradation potential at

Table 1
Acclimation of soil sample

Time/day	Soil sample	Additives	Residual oil/%
Initial	300 g	2.5% urea + 0.2 mol · L^{-1} dipotassium phosphate	6.92
5th	Isolate 30 g as sample 1 without oil		Not measured.
37th			1.25
5th	The rest 270 g + oil	20 g lubricant + 2.5% urea + 0.2 mol · L^{-1} dipotassium phosphate	13.52
12th	Isolate 30 g as sample 2 without oil		12.97
37th			10.09
12th	The rest 240 g + oil	20 g lubricant + 5% urea + 0.2 mol · L^{-1} dipotassium phosphate + Tween 80	20.51
22nd	Isolate 30 g as sample 3 without oil		20.06
37th			18.85
22nd	The rest 210 g + oil	20 g lubricant + 5% urea + 0.2 mol · L^{-1} dipotassium phosphate + Tween 80	29.96
37th	Isolate 30 g as sample 4		29.22