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Optimization of nutrient component for diesel oil degradation by *Acinetobacter beijerinckii* ZRS

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ABSTRACT

A new bacterial strain that is capable of using diesel as the sole carbon source was isolated from the petroleum-contaminated soil of Xinjiang oil field, Northwest China. This bacterium was identified on the basis of its morphological and physiological characteristics and 16S rRNA gene sequence analysis, and it showed the greatest similarity with *Acinetobacter beijerinckii* 302-PWB-OH1 (99.86%). In order to enhance degradation efficiency, single-factor experiments and response surface methodology (RSM) were employed to optimize the nutrients used in artificial seawater. The results of this study revealed that 2.05 g L^{-1} $(NH_4)_2SO_4$, 1.46 g L^{-1} Na_2HPO_4, and 14 mg L^{-1} yeast extract in artificial seawater resulted in increasing the diesel degradation rate of the bacterial strain from 20.87% to 80.40% within 7 days. The actual experimental results were in agreement with the prediction.

1. Introduction

Due to the global production, transportation, use, and disposal of petroleum, petroleum pollution has become a widespread and serious problem in many environments, particularly the oceanic environment. Crude oil poses many risks when released into the environment. The toxic compounds present in crude oil, such as polycyclic aromatic hydrocarbons, benzene, and its substituted and cycloalkane rings, which are present in relatively high concentrations, cause physical, chemical, and biological harm to the marine environment. Statistical analyses have revealed that oil pollution accounts for approximately 80% of all marine pollution. With the growing concern for the marine environment, marine oil pollution and its prevention have become an urgent problem.

Microbial biodegradation has become the main mechanism to eliminate petroleum from the environment. Bioremediation presents countless advantages over other processes employed to remove pollutants (such as solvent extraction and chemical oxidization), and it has been considered to be one of the most efficient methods to treat polluted environments (Van Gestel et al., 2003; Gogoi et al., 2003; Nano et al., 2003; Morelli et al., 2005; Demnerova et al., 2005). Many dominant petroleum-degrading bacteria have been isolated for use in the biological treatment of oil-contaminated areas, and the factors influencing petroleum biodegradation have been studied (Liu et al., 2012; Wang, 2012; Guang et al., 2011; Lee et al., 2012).

Bioremediation is a multi-variable process, and optimization of this process through classical methods is inflexible, unreliable, and time-consuming. Response surface methodology (RSM) was used to overcome these disadvantages. RSM is typically used to explore the relationships between several explanatory variables and one or more response variables. The main idea of the RSM is to use a set of experiments designed to obtain an optimal response (Adinarayana et al., 2003; Elibol, 2004). This statistical technique has been successfully applied in many fields, including media optimization (Bustos et al., 2004; Wang and Lu, 2004; Liong and Shah, 2005), fermentation conditions (Ratnam et al., 2003; Rigas et al., 2005), and enzyme-catalyzed reactions in lab experiments (Murthy et al., 2000).

In this research, a diesel-degrading bacterium was isolated from oil-contaminated soil samples obtained from Xinjiang oil field, China. On the basis of phenotypic and phylogenic analyses, this strain was identified as *Acinetobacter beijerinckii*. The nutrition requirements of this strain were optimized using RSM. Currently, there are only few reports about the use of this species in marine hydrocarbon degradation. Therefore, this strain has potential development possibilities and is of practical significance in the bioremediation of marine oil pollution.

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2. Material and methods

2.1. Microorganisms and culture conditions

A diesel-degrading strain (designated strain ZRS) was isolated from oil-contaminated soil sampled from Xinjiang oil field, China, and the culture conditions were based on our previous work (Huang et al., 2008). Number 0 diesel oil was purchased from Tianjin Science and Technology Co., Ltd. (China). *n*-Hexane and squalane were purchased from Sigma (USA) or Fluka (Switzerland). All the other reagents were of analytical grade and were obtained from various commercial sources.

2.2. Species identification

Species identification was carried out using the Biolog identification system, the DNA sequence of a 1500-bp fragment of the 16S rRNA gene of strain ZRS, and by physiological and biochemical characterization. For sequencing, the 16S rRNA gene was amplified from genomic DNA of strain ZRS using the following primer pair: 27f, 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1541r, 5′-AAG GAG GTG ATC CAG CC-3′. The sequences were subjected to a similarity search on the BLAST database (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/), and deposited into the DDBJ/EMBL/GenBank database under the accession no. JQ839143.

2.3. Diesel degradation test

ZRS was cultured in Luria-Bertani (LB) medium overnight at 25 °C, and the cells were collected by centrifugation of the overnight culture at 12,000 g for 5 min, and washed twice with minimal M9 salt medium or modified artificial seawater. Culture suspension was adjusted at an optical density of 0.05 at 600 nm in 50 mL minimal M9 salt medium or in modified artificial seawater (Austin, 1993) supplemented with $(NH_4)_2SO_4$, Na_2HPO_4, and yeast extract. The cells were incubated at 25 °C with shaking at 120 rpm in a medium containing 0.5% (*w*/*v*) diesel oil as the sole carbon source. The remaining hydrocarbons were extracted with an equal volume of hexane at 12-h intervals. Each sample contained 20 ppm (*w*/*v*) squalane as an internal standard. Each data point was the average of triplicate diesel degradation experiments.

Gas chromatography (GC) was performed using an Agilent Technologies 6820 N gas chromatograph equipped with an on-column injector, flame ionization detector (FID), and SPBTM-5 capillary column (internal diameter, 30 m × 0.53 mm; thickness, 1.5 μm). Nitrogen gas was used as a carrier and set at a constant flow rate of 0.6 mL min^{-1}. Oven temperature was set at 150 °C for 5 min and then programmed to increase from 150–280 °C at 15 °C min^{-1}. The injector and detector temperatures were 280 and 350 °C, respectively (Huang et al., 2008).

2.4. Screening of significant medium components using single-factor design

Here, the single-factor design was used to evaluate the relative importance of the following nutrients that influenced the responses: $(NH_4)_2SO_4$, Na_2HPO_4, and yeast extract. This design does not consider the effects of interaction among the variables. The strain was incubated for 7 days in modified artificial seawater medium containing $(NH_4)_2SO_4$, Na_2HPO_4, and yeast extract, and the diesel degradation was measured using GC.

2.5. Central composite designs and media optimization using response surface method

Adoption of a central composite design (CCD) implied prior knowledge of the upper and lower parameter limits and awareness of the nutrient amendment process and its factors (Poorna and Kulkarni, 1995). Preliminary trials indicated that $(NH_4)_2SO_4$, Na_2HPO_4, and yeast extract in the medium significantly affected the degradation rate. Therefore, these three variables were chosen to obtain the optimum levels. The lowest and highest concentrations of each supplement added during the study are listed in Table 1.

A 2^3 factorial CCD was designed with six star points and six replicates at the center points leading to 20 runs. The variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X} \quad i = 1, 2, \ldots, k$$

where x_i is the dimensionless value of an independent variable, X_i the real value of an independent variable, the value of X_i at the center point, and ΔX is the step change.

The second-order polynomial model was fitted to response, giving the equation:

$$\widehat{Y}_i = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii}x_i^2 + \sum_{i,j=1}^{3}\beta_{ij}x_i x_j$$

where $\widehat{Y}_i$ is the predicted response, x_i and x_j are the input variables, β_0 is the intercept term, β_i is the linear effect, β_{ii} is the squared effect, and β_{ii} is the interaction term.

Statistical software was used for regression and graphical analysis of the data. The optimum concentrations of $(NH_4)_2SO_4$, Na_2HPO_4, and yeast extract in the nutrient medium were obtained by solving the regression equation and analyzing the response surface contour plots.

3. Results

3.1. Isolation and identification of hydrocarbon-degrading bacteria

Hydrocarbon-degrading strains were enriched and separated by culturing oil-contaminated soils sampled from Xinjiang oil field. The obtained isolate was found to be aerobic, gram-negative, and nonmotile. Colonies on LB agar were approximately 1.0–2.0 mm in diameter, circular, convex, smooth, and slightly opaque with entire margins. Sequence analysis of the 16S rDNA gene revealed the least evolving distance and the maximum similarity value between strain ZRS and *Ac. beijerinckii* 302-PWB-OH1 (GenBank accession no. HQ425646) to be 0.000357% and 99.86%, respectively (Fig. 1). Physiological and biochemical tests revealed 90% similarity between the isolated strain and the standard strain *Ac. beijerinckii* CCUG 51249^T (Nemec et al., 2009) (Table 2). The strain isolated in this study was thus identified as an *A. beijerinckii* strain and was termed *A. beijerinckii* ZRS.

Table 1
Variables and their levels used in the experiment design.

Variables	Range and levels				
	−1.68	−1.00	0.00	+1.00	+1.68
X_1: $(NH_4)_2SO_4$ (g L^{-1})	0.42	1.32	2.64	3.96	4.86
X_2: Na_2HPO_4(g L^{-1})	0.24	0.75	1.5	2.25	2.76
X_3: Yeast extract (mg L^{-1})	6.28	9	13	17	19.72