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Biodegradation of Crude Oil by an Arctic Psychrotrophic Bacterium *Pseudoalteromomas* sp. P29

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Abstract A psychrotrophic petroleum-degrading bacterium *Pseudoalteromonas* sp. P29 was isolated from marine sediment, which was collected during 2nd Chinese Arctic Scientific Expedition. The phenotypic character and biodegradation efficiency on mixed oil or vacuum oil were tested at low temperature. The strain *Pseudoalteromonas* sp. P29 grew in a range of temperature from 5 to 35°C and the optimum temperature was 25°C. Gas chromatography analysis indicated that the strain might preferentially metabolize shorter-chain alkanes. The biodegradation efficiency were nearly 90 and 80%, respectively, after incubation at 5°C for 28 days in the mineral medium supplement with mixed oil or vacuum oil as the sole carbon and energy source. The results showed a possible exploitation of the strain in future biotechnological processes especially in cold contaminated environments.

Introduction

More and more pollutions and hereby toxicity to biological system are increasing with the rapid development of exploitation, production, and transport of crude oil on the earth. Biodegradation by naturally occurring populations of microorganisms is a major mechanism of oil removal from the environment [8, 13]. In seawater, weathering processes contribute to the natural attenuation of discharged oil, including surface evaporation, hydrocarbon dissolution from oil film and droplets, photo-oxidation, emulsion, dispersion, and biodegradation. Several processes among them are affected by seawater temperature. For example, the increase in the viscosity of hydrocarbon mixture reduces the degree of its spreading and degrading in water system [4].

Recently, the increasing human activities in Antarctic, Arctic and other cold regions have led to the demand of the research into hydrocarbon degradation processes in cold environments. Interests in microorganisms with high petroleum-degrading efficiency at low temperature have been increased in the field of marine biotechnology. Bacteria, yeast, and filamentous fungi have been reported as transforming agents for their ability to degrade a wide range of pollutants. Investigations of hydrocarbon spills in Polar Regions indicate that hydrocarbon degraders, typically bacteria, are widely distributed and their numbers are usually enhanced following hydrocarbon spillage [3]. For example, in "pristine" ecosystems, hydrocarbon utilizers may make up less than 0.1% of the microbial community; and in oil-polluted environments, they can constitute up to 100% of the viable microorganisms [18]. Furthermore, biodegradation of petroleum by indigenous cold-adapted microbial populations has been observed at low temperature in hydrocarbon-contaminated soils [2, 15, 20].

Although the majority of hydrocarbon-derived contaminations occurs in cold marine environments, most investigations have been performed at higher temperatures, namely between 20 and 35°C [5]. In cold climates, psychrophilic and psychrotolerant microorganisms play an important role in biodegradation of the organic matter. Former studies on degradation of hydrocarbons by psychrotolerant bacteria were conducted with single bacterial strain and in the presence of complex compounds such as yeast extract [6, 9, 11, 17, 19].

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Based on the fact that hydrocarbon-derived pollutions often occur in cold area, attempts were made to enrich a microbial consortium effective in crude oil degradation in cold habitats. In this study, the biodegradation efficiency of a psychrotrophic petroleum-degrading bacterium *Pseudoalteromonas* sp. P29, which was isolated from Arctic marine sediment, was investigated at low temperature (5°C).

Materials and Methods

Strain and Medium

The petroleum-degrading microorganism *Pseudoalteromonas* sp. P29, deposited in China General Microbiological Culture Collection Center (CGMCC No 2913), was isolated from marine sediments collected during 2nd Chinese Arctic Scientific Expedition.

The medium was mineral medium (MM) containing (per liter of filtered seawater): NH_4SO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.025 g, NH_4NO_3 0.2 g, NaH_2PO_4, 0.5 g, $K_2HPO_4 \cdot 3H_2O$ 1.0 g, and mixed oil 2 g (the ratio of crude oil and diesel was 1:4) supplied as single carbon and energy source.

Growth Requirement on Temperature

The strain *Pseudoalteromonas* sp. P29 was cultivated at a range of temperature from 5 to 35°C in ZoBell 2216 E medium, and their growth rate was calculated by optical density at 550 nm using colorimeter.

Phenotypic Properties Analysis

Phenotypic properties of strain *Pseudoalteromonas* sp. P29 was analyzed with the API 20 NE system of bioMérieux SA which was a standardized system for non-fastidious, non-enteric Gram-negative rods. The performance followed the instruction strictly.

Growth Requirement on Nitrogen Source

The growth and biodegradation efficiency (BE) of strain *Pseudoalteromomas* sp. P29 were tested in the mineral medium with different nitrogen sources (10 mmol N l^{-1}) containing 2 g l^{-1} mixed oil as sole carbon and energy source.

Biodegradation Activity Assay

Biodegradation of crude oil was monitored by measuring the loss of oil weight in culture medium: Following the different incubation periods, the culture were extracted with 10-ml volume of *n*-hexane as a solvent by using separatory funnel to remove the cellular material for two times. Uninoculated control flasks were incubated and cultured in parallel to monitor abiotic loss of crude oil. The biodegradation efficiency (BE), based on the decrease in the total weight of crude oil, was evaluated by using following expression: $BE = ((M_0 - M) - (M_{C0} - M_C))/M_0 \times 100\%$, where M_0 = the weight of crude oil of sample before inoculation, M = the weight of crude oil of sample after inoculation, M_{C0} = the weight of crude oil of control, M_C = the weight of crude oil of control after the same treatment as that of the sample without inoculation.

Gas Chromatography

An Agilent 6890N gas chromatography instrument coupled with an Agilent 5973N mass spectrometer and an Agilent ChemStation software (Agilent Technologies, Palo Alto, CA). Compounds were separated on a 30 m × 0.25 mm i.d. capillary column coated with 0.25 μm film 5% phenyl methyl siloxane. The column temperature was at 50°C for injection, then programmed at 10°C min^{-1} to 280°C. Helium was used as carrier gas of 1.0 ml min^{-1} flow-rate. The spectrometers were operated in electron-impact (EI) mode, the scan range was 50–500 amu, the ionization energy was 70 eV. The inlet, ionization source temperatures were 280 and 300°C, respectively.

Results

Phenotypic Properties of *Pseudoalteromonas* sp. P29

Molecular identification and phylogenetic analysis of the strain, based on 16S rDNA (GenBank accession number EF628009), showed that it was a member of genus *Pseudoalteromonas* [7]. Phenotypic properties of the strain were summarized in Table 1 and Fig. 1.

Strain *Pseudoalteromonas* sp. 29 grew in a temperature from 5 to 35°C, with an optimum temperature of 25°C. The growth of P29 was inhibited markedly when cultured at 35°C. The strain P29 was found to be psychrotrophic rather than psychrophilic as it was capable of growing at 35°C. According to the most widely accepted definition of cold-adapted microorganisms of Morita [12], *Pseudoalteromonas* sp. was a psychrotrophic species.

Growth Requirements on Nitrogen Sources

The growth and BE of strain *Pseudoalteromonas* sp. P29 in the mineral medium containing 2 g l^{-1} mixed oil as sole carbon and energy source with different nitrogen sources after incubation for 7 days at 5°C were shown in Table 2.

Table 1 Morphological, biochemical, and physiological characters of *Pseudoalteromonas* sp. P29

Characteristics	Results
Station	169°59′37″W, 75°00′24″N
Depth	263 m
Gram staining	−
Cell morphology	Rod
Growth temperature	5–35°C
Assimilation of	
D-glucose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, malic acid, trisodium citrate	+
L-arabinose, adipic acid, phenylacetic acid	−
Enzymes	
Urease	+
β-Galactosidase, protease, β-glucosidase, arginine dihydrolase, glucose, indole production	−

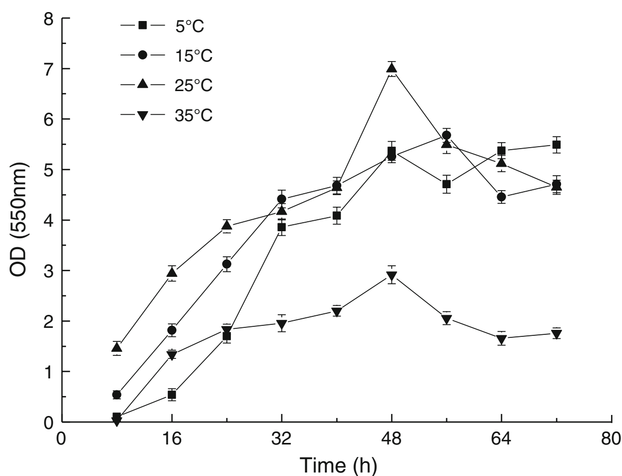

Fig. 1 Effects of temperature on the growth of *Pseudoalteromonas* sp. P29. (the data has been analyzed by average of three times)

Table 2 Effects of different nitrogen source on the growth and biodegradation of *Pseudoalteromonas* sp. P29 (the data has been analyzed by average of three times)

Nitrogen source	OD value of culture (550 nm)	BE (%)
$(NH_4)_2SO_4$	1.412 ± 0.092	31.3 ± 3.57
NH_4NO_3	1.027 ± 0.081	30.8 ± 4.01
Urea	2.642 ± 0.098	41.2 ± 4.23
$NaNO_3$	0.876 ± 0.067	21.6 ± 3.29
Yeast extract	2.364 ± 0.088	38.6 ± 3.96

Strain *Pseudoalteromonas* sp. P29 showed the highest growth and biodegradation efficiency by using urea as nitrogen source, while it showed the lowest growth and BE when using $NaNO_3$ as nitrogen source.

Growth and Biodegradation Efficiency on Mixed Oil and Vacuum Oil

Growth and biodegradation efficiency of strain *Pseudoalteromonas* sp. P29, respectively, using mixed oil or vacuum oil as sole carbon and energy source in the mineral medium were tested at 5°C with continuous shaking (150 r min^{-1}). The results were shown in Figs. 2 and 3. Although strain *Pseudoalteromonas* sp. P29 showed an ability to utilize both mixed oil and vacuum oil as sole carbon and energy source, it showed a higher utilization ability on mixed oil. The BE was up to 60% when strain *Pseudoalteromonas* sp. P29 was cultured with mixed oil after 14 days while it was at the stationary growth phase with a OD value of 1.280. The BE was close to 90% after 28 days. When using vacuum oil as sole carbon and energy source, the BE was up to 76% after 21 days while it was at the stationary growth phase with a OD value of 1.479. The BE was close to 80% after incubation for 28 days.

Gas Chromatography of Mixed Oil as Substrate

The gas chromatography (GC) of strain *Pseudoalteromonas* sp. P29 during the degradation process using mixed oil as sole carbon and energy source was shown in Fig. 4. GC analysis provided a clear indication of the degradation of diverse components in mixed oil at 5°C. The composition change of *n*-alkanes during the time experiment was shown in Table 3. With the prolongation of culture time, the proportion of longer-chain alkanes (C_{21}–C_{28}) increased from 24.63 to 33.56%, which might suggest that shorter-chain

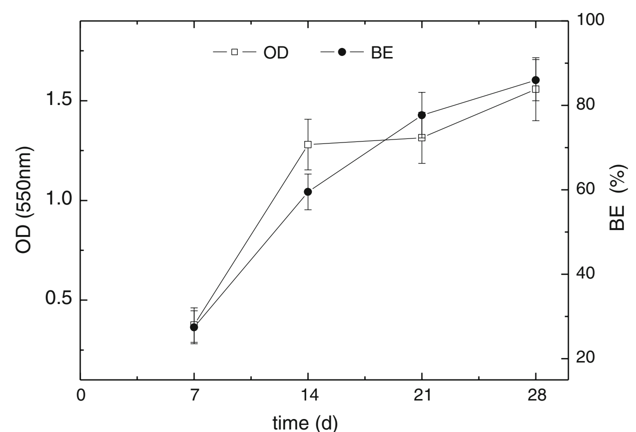

Fig. 2 Utilization of mixed oil by strain *Pseudoalteromonas* sp. P29. (the data has been analyzed by average of three times)

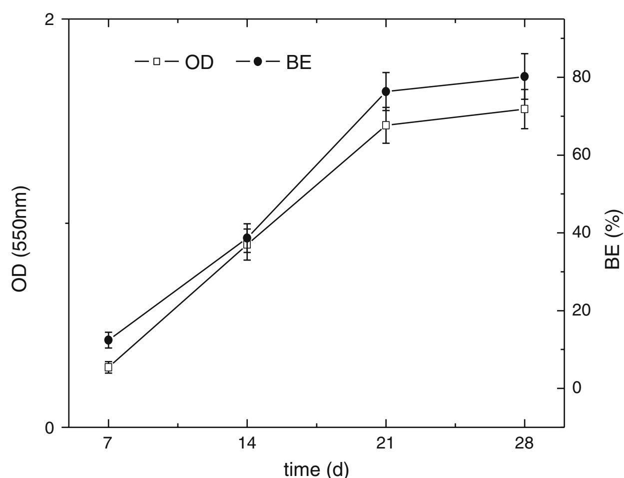

Fig. 3 Utilization of vacuum oil by strain *Pseudoalteromonas* sp. P29. (the data has been analyzed by average of three times)

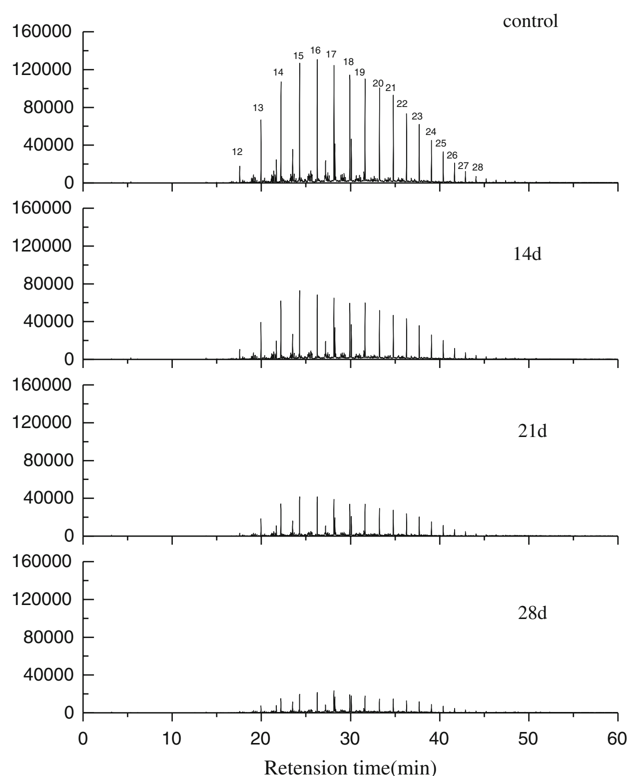

Fig. 4 Gas chromatography (GC) profiles of crude oil extracted from aqueous phase after incubation in different time at 5°C

Table 3 Composition change of *n*-alkanes distribution of mixed oil during the time experiment[a]

Alkane	Culture time			
	Control	14 days	21 days	28 days
C_{12}	1.44	1.34	0.75	0.73
C_{13}	5.69	5.49	4.75	3.92
C_{14}	9.15	8.29	7.83	6.00
C_{15}	10.84	11.67	11.63	10.56
C_{16}	11.15	9.61	8.38	7.50
C_{17}	10.61	10.37	9.24	10.07
C_{18}	9.76	9.53	9.49	10.31
C_{19}	8.15	8.59	9.46	9.64
C_{20}	8.58	8.30	8.18	7.71
Shorter-chain alkanes (C_{12}–C_{20})	75.37	73.19	69.71	66.44
C_{21}	6.05	6.79	7.69	7.93
C_{22}	5.49	6.00	6.62	6.82
C_{23}	4.04	4.24	5.14	5.69
C_{24}	3.37	3.75	4.25	4.75
C_{25}	2.82	2.84	3.23	3.81
C_{26}	1.69	1.88	1.97	2.58
C_{27}	1.04	1.16	1.22	1.62
C_{28}	0.13	0.15	0.17	0.36
Longer-chain alkanes (C_{21}–C_{28})	24.63	26.81	30.29	33.56

[a] Data shown in table were the proportion of total *n*-alkanes

alkanes (C_{12}–C_{20}) were preferentially metabolized by strain *Pseudoalteromonas* sp. P29 compared to longer-chain alkanes.

Discussion

Psychrotrophic/psychrophilic bacteria from polar seas were reported to have substrate limitations at low temperature, showing lower affinities for substrate uptake at 2°C than at 16°C [4]. However, several microbes from cold environments have significant potentials for hydrocarbon biodegradation at seawater temperature close to 0°C, including bacterial and fungal species [10].

In cold climates, psychrophilic and psychrotolerant microorganisms play an important role in the biodegradation of organic matter. The ability to degrade petroleum hydrocarbons is ubiquitous among marine bacteria. Biodegradation is considered as a major mechanism for removal of spilled petroleum hydrocarbons from aquatic environments [11]. The prevalence for hydrocarbon biodegradation exists in pristine cold environments, and alkane monooxygenases and hydrocarbonoclastic bacteria have been detected or characterized in both Arctic and Antarctic environments [4]. Studies on oil-polluted polar environments have demonstrated abundance of α-Proteobacteria, γ-Proteobacteria and Acitinobacteria [1, 4, 16]. The bacteria from polar surface soils so far are usually psychrotrophic, probably reflecting summer in situ temperatures that may range from below 0 to about 20°C [3]. Our results demonstrated this further. Twenty-six species of petroleum-degrading bacteria with distinct morphological character were isolated from marine sediments, which

were collected during 2nd Chinese Arctic Scientific Expedition by procedure of enrichment and screening [7].

Generally speaking, rates of degradation decrease with the decreasing of temperature which seems to be primarily related to the decrease in the enzyme activity [11]. According to Whyte et al. [19], psychrotrophic microorganisms seem to possess an intrinsic advantage in the degradation of contaminants at low temperature. The strain *Pseudoalteromonas* sp. P29 was observed to grow in a range of temperature from 5 to 35°C, which indicated the strain had wider temperature range for potential biodegradation. In fact, all the physical, chemical, and biological factors which characterize any natural ecosystem are basically impossible to replicate in laboratory. However, based on the high biodegradation efficiency, *Pseudoalteromonas* sp. P29 could be utilized in the bioremediation of hydrocarbon contaminated environments in cold area.

Former studies concerning degradation of hydrocarbons by psychrotrophic bacteria were performed with single bacterial strain and in the presence of complex compounds such as yeast extract, the obtained cell yields under these conditions in most cases do not correspond to the degradation of hydrocarbons [5]. In our study, the biodegradation efficiency of hydrocarbon was generally in line with the growth, probably due to the reason that hydrocarbon was sole carbon and energy source in the mineral medium used, and there was no complex compounds such as yeast extract which might be used as both carbon and nitrogen source.

As in agreement with former studies [14, 19], strain *Pseudoalteromonas* sp. P29, as a pure culture, seemed to metabolize a limited range of hydrocarbons for its preferentially biodegradation for shorter-chain hydrocarbons. However, mixed populations are necessary for effective biodegradation for complex mixtures of hydrocarbons such as crude oil. In a consortium, single strain can complement each other, e.g., by co-metabolic turnover reactions or by interactions with substrates and finally carry out a more effective degradation process.

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