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Research Article

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Identification and biodegradation potential of two newly isolated hydrocarbon-degradating microorganisms

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ABSTRACT

Two hydrocarbon-degradating bacteria were isolated from petroleum contaminated soils and identified. These two strains are all Pseudomonas sp. and designated as ptr13 and ptr20. Their aromatic hydrocarbon degradation ratios were 80% and 69%, respectively. GC-MS assays showed that the light components of the crude oil were disappeared after degradation, and at the same time, the "unresolved complex material (UCM)" emerged. Enzyme assays indicated that ptr13 and ptr20 present significant catechol 2, 3-dioxygenase activity.

Key words: crude oil, hydrocarbon, biodegradation, *pseudomonas*, catechol 2, 3-dioxygenase

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INTRODUCTION

Petroleum is a complex mixture of hydrocarbons composed by carbon-hydrogen, variable quantities of oxygen and sulfur, trace amount of nitrogen, metals and other elements. It is estimated that there is about 1×10^9 t petroleum and oil products entered into underground water, aboveground water and soil through storage tanks, improper disposal of petroleum wastes, accidental spills routes and so on[1],[2], [3]. Degradation of petroleum is a microbial process whereby the petroleum is used as an organic carbon source and many microorganisms can excrete extracellular enzymes to degrade petroleum hydrocarbons, resulting in the breakdown of petroleum components to compounds of lower molecular weight [4] , [5] , [6] . Microbial utilization of these compounds as sole carbon sources is highly dependent on the chemical nature of the compounds[7]. Different components of crude oil are degraded at different rates: n-paraffins are oxidized more rapidly than either aromatics or naphthenes [8] . Aromatic hydrocarbon is the component of petroleum and exists widely, only fewer than glucose group in nature. It is toxic, carcinogenic and inducing material. Now more than 400 aromatic hydrocarbons and their ramifications which could induce cancer had been found. In this paper, we report the isolation and characterization the strains which could degrade the petroleum, and the degradation ability of the different components of petroleum, as well as the changes of aromatic hydrocarbon before and after degradation were also assayed.

EXPERIMENTAL SECTION

Isolation and culture conditions

Mineral salts medium(M1) containing 1% crude oil(w/v) was used for culture enrichment, the medium consisted of 1g K_2HPO_4•$3H_2O$, 1g KH_2PO_4, 0.5g $MgSO_4$•$7H_2O$, 1g NH_4NO_3, 0.02g $CaCl_2$ per liter, at pH7.2-7.4. Mineral salts medium (M2) containing 0.1% Crude oil(w/v) was used for microbial growth and degradation studies at 37℃. The M2 medium consisted of 0.5g $(NH_4)SO_4$, 0.5g $NaNO_3$, 0.02g $CaCl_2$, 0.2g $MgSO_4$, 1.0g KH_2PO_4, 1.0g NaH_2PO_4•H_2O per liter, at pH7.2.

To screen petroleum-degradating bacteria, soil samples mixed with water and some sand/mud from Dagang oil field (China) were incubated in the Mineral salts medium(M1) for culture enrichment. Cultures were incubated with

shaking at 37℃ for 7 days at 180 rpm. 2 mL cultured medium was taken out and transferred to another fresh medium. This process was performed continuously for two months. Finally, 0.2 mL cultured medium was taken out and spread onto beef-peptone plate and thus the pure culture of microorganisms were obtained which were capable of utilizing the petroleum as source of carbon and energy.

Crude oil and its components aliphatic hydrocarbons, aromatic hydrocarbons, resin and asphaltene extracted from it using routine methods were tested for microbial degradation ability.

Strains identification

The strains were identified by their morphological, physiological and biochemical features using the Bergey's Manual of Systematic Bacteriology and the 16S rRNA gene sequence from each strain. The 27f and the 1492r universal primers (Lane 1991) were used to amplify the 16S rRNA gene from strain ptr13 and ptr20 by PCR. High fidelity *Pyrobest*™ DNA Polymerase (Takara, Dalian) was used for PCR amplifications. The 16S rRNA gene sequences of the strains were compared to all sequences available from the GenBank database by using the BLAST program. Phylogenetic tree was constructed by DNAMAN software (Lynnon Corporation).

Hydrocarbon degradation assays

Strains from the slants were inoculated into 100-ml flasks containing a 50 ml M2 medium. Cultures were incubated with shaking at 37℃ for 7 days at 180 rpm. Then the cultures were filtrated through absorbent cotton pretreated by $CHCl_3$, after washed cotton several times by water, drying the absorbent cotton at 60℃ until constant. Recollected oil from cotton using $CHCl_3$, drying the oil at natural temperature and weighed the residue weight of oil. The changes of oil components were assayed using GC-MS.

The catechol 2,3-dioxygenase activity assay

The extracellular catechol 2,3-dioxygenase activity was spectrophotometrically measured in 100 mM phosphate buffer (pH7.5) containing 0.3 mM catechol at 30℃. The reaction time was 3 min and measured the absorbance changes at 375 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of meta cleavage compound from substrate per min.

RESULTS AND DISCUSSION

The isolation and characterization of the strains

A total of 24 bacterial isolates were showed to degrade petroleum. Two of them with the better degradation ability were further identified and designated strain ptr13, ptr20, respectively. These two strains were Gram-negative rod with a long and fine polar flagellum. The morphology characteristic and physiological and biochemical tests showed that ptr13 and ptr20 belonged to *Pseudomonas* sp.(Table 1).

Table 1 The identification of the strains

characters	ptr13	ptr20
Morphology	Short rod	Short rod
Gram stain	-	-
spore	-	-
Nonaqueous-solubility pigment	-	-
Motility	+	+
Flagellum	One flagellum in pole	One flagellum in pole
Oxidase	+	little
Catalase	+	+
Glucose oxidation	-	-
Ethanol oxidation	+	+
acetic acid oxidation	+	+
Nitrate reduction	+	-
pH4.5 growth	-	-

Sequences analysis of the 16S rRNA gene from Strain ptr13 and ptr20 indicated that Strain ptr13 has 97% sequence identity to *Pseudomonas resinovorans*. It was considered that it may be belonged to a same genus and different species with 16S rRNA gene sequence identity lower than 98%, and it may be belonged to different genus with 16S rRNA gene sequence identity lower than 93-95% [9], [10]. So it could be concluded that strain ptr13 may be belonged to a new species of *Pseudomonas*, which could be conformed from the distance of phylogenetic tree, but more works need to carry out to define which species it belong to. Strain ptr20 had 100% sequence identity to *Pseudomonas thermaerum*. So, it was concluded that strain ptr20 may be a new strain of *Pseudomonas thermaerum* (Fig. 1).

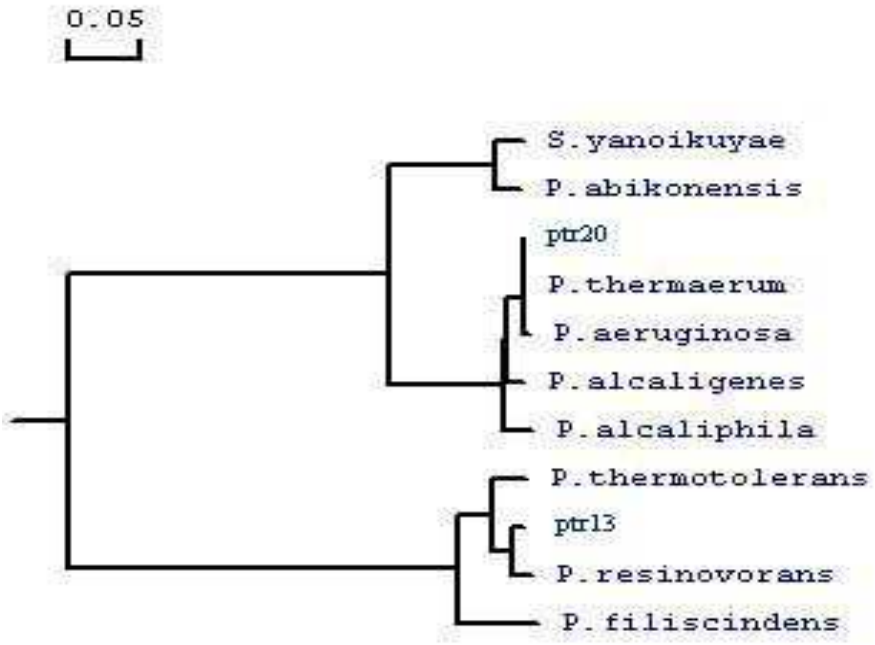


Fig. 1 Phylogenetic tree of strains ptr13 and ptr20 based on 16S rRNA gene sequences

Hydrocarbon degradation assays

It is well known that petroleum contains numerous compounds of varying structural complexities. It is composed mainly of 95-99.5% hydrocarbon and some bitumen and other materials. Hydrocarbons involved with alkanes, aromatic hydrocarbon, cyclic alkanes, fused ring compounds and heterocyclics [7], [11], [12]. Many microorganisms possess the capability to degrade petroleum. Microorganisms have different degradation ability for different components of petroleum[13], [14]. Often the normal alkanes in the range C10 to C26 are viewed as the most readily degraded, and low-molecular-weight aromatics, such as benzene, toluene and xylene, which are among the toxic compounds found in the petroleum, are also readily biodegraded by many microorganisms. More complex structures are more resistant to biodegradation. The greater the complexity of the hydrocarbon structure, i.e. the higher the number of methyl branched substituents or considered aromatic rings, the slower the rates of degradation [15]. Our research also supported this conclusion. It could be seen that different petroleum components had different degradation degree (Table 2). Aromatic hydrocarbon degradation rate was highest, reaching 80%, asphaltene's maximal degradation rate was 53%, the nonhydrocarbon's degradation rate was lowest, only 30%. The same strain also had different degradation ability for different components. Strains ptr13 and ptr20 had the maximal degradation ability for aromatic hydrocarbon, which were 80% and 69% (as to the petroleum, were 41% and 32%, respectively), respectively. Among all the isolates, ptr20 had the best degradation ability for nonhydrocarbon, which was 53%，13% more than the formerly reported [16], and the time was also shortened to a half. Furthermore, all the strains had high degradation ability to aromatic hydrocarbon and nonhydrocarbon, according with their structure analogue.

Table 1 The degradation ratio of fractions of petroleum after 30 days incubation

Strains	Degradation rate (%)		
	Aromatic hydrocarbon	Non-hydrocarbon	asphaltene
ptr13	80	20	44
ptr20	69	18	53

The GC-MS of aromatic hydrocarbon

Aromatic hydrocarbon was the main pollutant and difficult for biodegradation. To define the changes of the crude oil before and after degradation by strains ptr13 and ptr20, GC-MS analysis was performed. Total ion chromatogram (TIC) showed (Fig. 2) that the main components of aromatic hydrocarbon were light components before degradation.

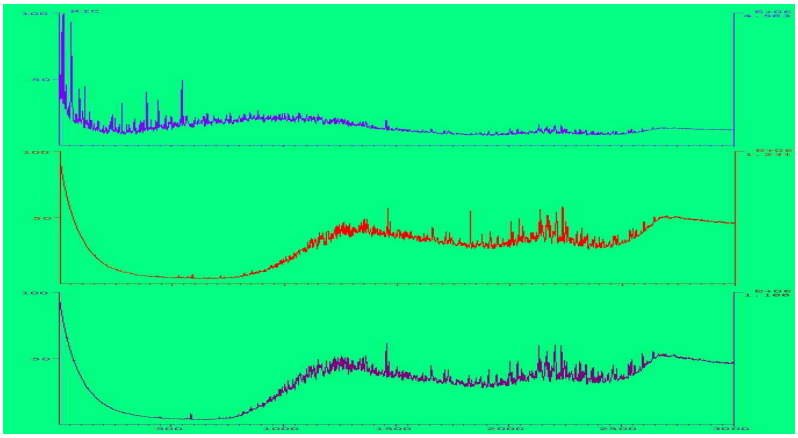
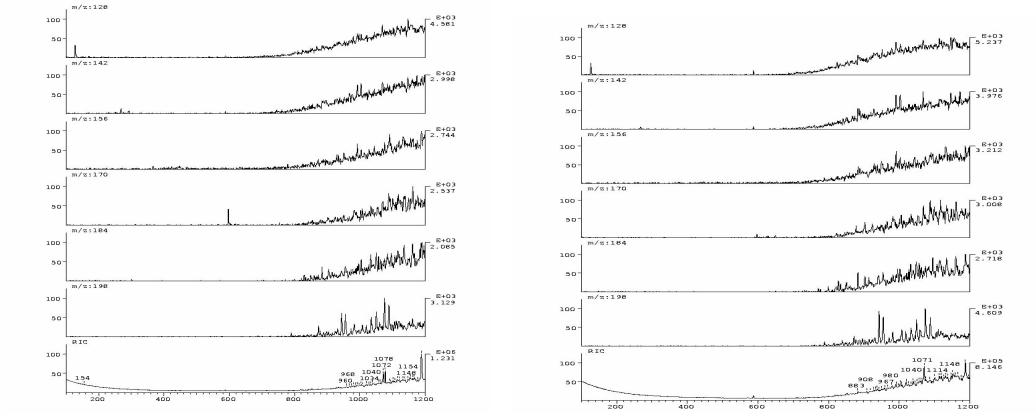


Fig. 2 Total ion current of petroleum by GC-MS

After biodegradation, light components disappeared, and emerged clear "unresolved complex material (UCM)" both for strain ptr13 and ptr20. m/z128, m/z142, m/z156 and m/z 170 distribution character of mass chromatogram before and after degradation showed that there had intact naphthalene, methylnaphthalene and C2, C3 position substitutional naphthalenes before degradation. After degradation, there was only minimal naphthalene, alkylnaphthlanene had almost disappeared (Fig. 3).



(a) strain ptr13 **(b) strain ptr20**

Fig. 3 m/z128, m/z142, m/z156 and m/z170 analysis of aromatic hydrocarbon fractions of strain ptr13 and ptr20 by GC-MS

m/z178, m/z192, m/z252 mass chromatogram showed that there were not distinct degradation effect for resin, methylphenanthrene and benzopyrene for strains under this condition. Furthermore, their degradation ability had no significant difference (Fig. 4, Fig. 5)

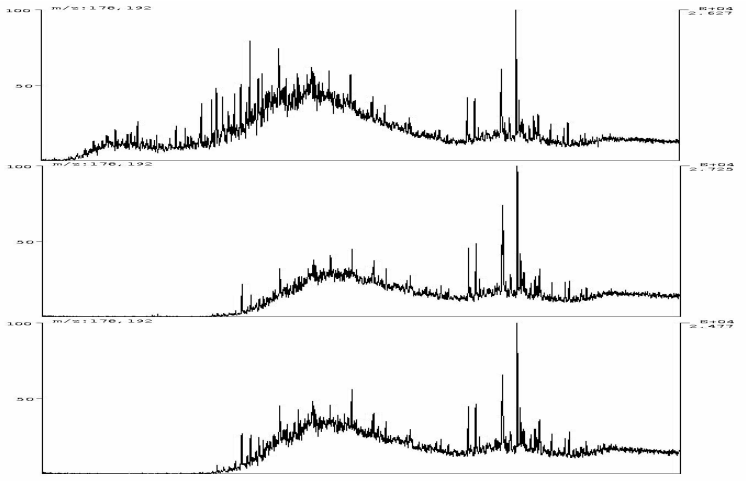


Fig. 4 m/z178 and m/z192 analysis of aromatic hydrocarbon fractions of strain ptr13 and ptr20 by GC-MS

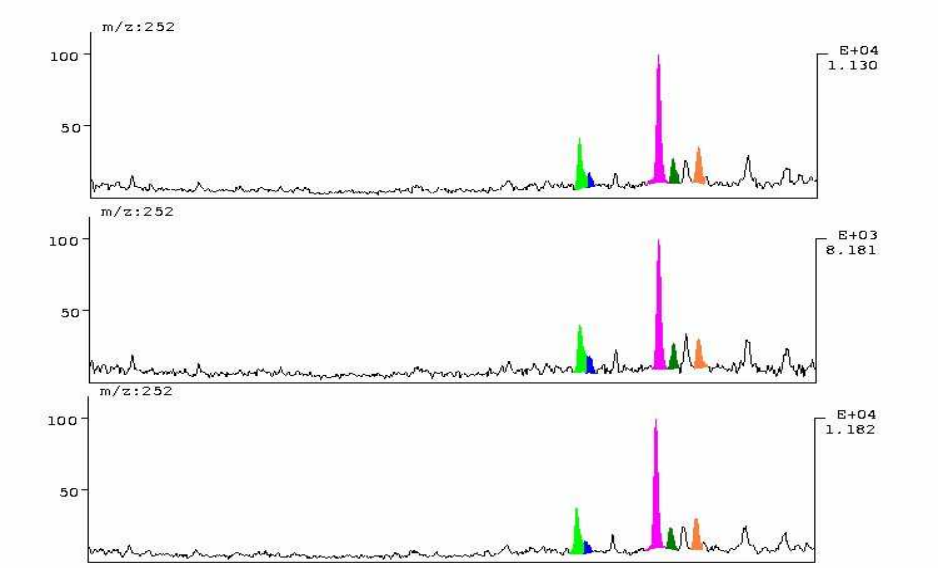


Fig. 5 m/z252 analysis of aromatic hydrocarbon fractions of strain ptr13 and ptr20 by GC-MS

The production of enzyme

The enzyme assay showed that ptr13 and ptr20 could excreted catechol 2,3-dioxygenase. For strain ptr13, its extrocellular catechol dioxygenase activity reached highest when cultured 24h (2.1U/mL), lagged than the maximum microorganism concentration and decreased slower in whole microorganism stability phase. For strain ptr20, the maximum enzyme activity and microorganism concentration reached highest at 18h. The time of enzyme production was some quick than strain ptr13 (Fig. 6).

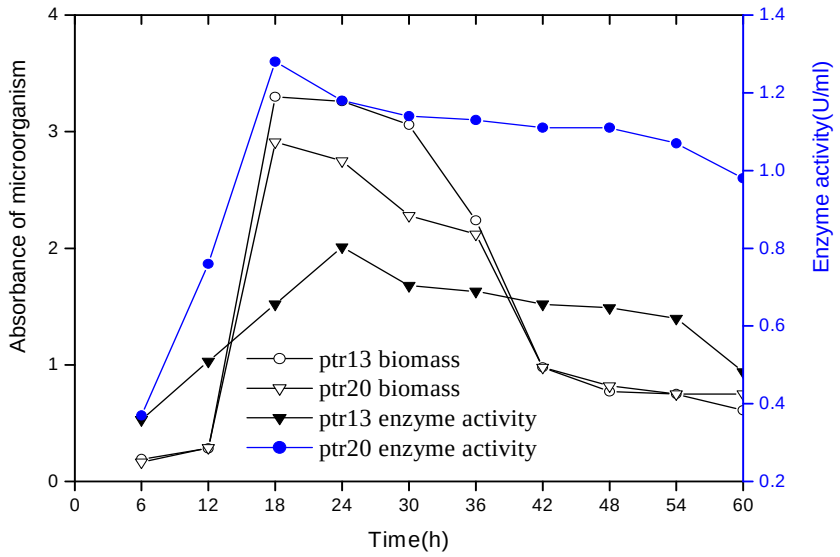


Fig.6 The time course of enzyme production

CONCLUSION

This study, we have showed that the characters and the degradation ability of the two hydrocarbon-degradating *pseudomonas* sp. which were isolated from China. This not only offers their potential in bioremediation of contaminated sites, but also the progressing work to investigate the genetic basis or degradation pathway of some special crude oil components (phenanthrene et al) degradation about these two strains, which will give us better understand the mechanism about the petroleum biodegradation.

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