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Screening And Identification Of Petroleum Hydrocarbon Degrading Bacteria

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Abstract:
34 strains were obtained by enrichment culture, isolation and purification of petroleum hydrocarbon contaminated soils around Baoding, among which 13 strains could grow with diesel oil, gasoline or kerosene as the only carbon source. Six strains named AY12, BY4, BY6, CY8, CY9 and CY10 with high degradation performance of diesel, gasoline or kerosene were screened by degradation rate measurement. Physiological, biochemical and 16SrDNA molecular identification, Strains AY12 were identified as Klebsiella pneumoniae, BY4 as Enterobacter coli and BY6 as Klebsiella oxytoca and CY8 were identified as Enterobacter cloacae, CY9 as Bacillus welchii and CY10 as Bacillus cereus.

Key words: *Petroleum hydrocarbon pollution；Degradation bacteria；Separation and identification*

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I.Introduction

Petroleum hydrocarbons are widespread pollutants in the environment, including diesel, gasoline, paraffin, lubricating oil, paraffin and bitumen, and are mixtures of a variety of hydrocarbons (n-alkanes, branched alkanes, cycloalkanes, aromatics) and small amounts of other organic substances such as sulphides, nitrides and naphthenic acids(Yuriy et al., 2016; Abdullah et al., 2020; Lan et al., 2023). With the rapid economic development, human demand for fuel oil such as diesel, gasoline and paraffin has been expanding, making it one of the main sources of energy for human beings. The approximately 100,000 existing petrol stations in China may all be sources of soil groundwater pollution. Wholesale storage enterprises of refined oil products, petrol stations and refuelling points, and the soil around these sites inevitably leads to the entry of petroleum and its products into soil and water bodies, which is difficult to eliminate, causing serious hazards to society, the economy and human health(Wang et al., 2016; Wang et al., 2018; Chen et al., 2019). According to statistics, millions of tonnes of petroleum hydrocarbon pollutants enter the soil, surface water and groundwater every year, causing serious damage to the natural environment, ecosystems and agricultural farming. Without human intervention, natural remediation is extremely slow, takes a long time and is not effective(Wojtowicz et al., 2022; Mendoza-Burguete et al., 2023). At present, there are four remediation methods to manage petroleum hydrocarbon pollution: chemical remediation, physical remediation, microbial remediation and combined remediation. Physical and chemical remediation methods were used more often in the early days and have the

characteristics of fast results and short remediation cycle, but they have fatal defects such as causing secondary pollution of the polluted land and high remediation costs(Ebadi et al., 2018; Siti et al., 2020; Sattar et al., 2022). Microbial remediation, on the other hand, does not cause secondary contamination and is cost effective and environmentally friendly. It is the mainstream direction of research at present. Microbial remediation is the use of microorganisms, which can use petroleum hydrocarbon pollutants as a carbon source to grow and reproduce, through a series of biochemical reactions to metabolize and degrade petroleum hydrocarbon material components, and then produce carbon dioxide, water and other harmless inorganic substances(Mazarji et al., 2021; Li et al., 2023). Microbial remediation technology is a green and healthy, ideal remediation method in line with the development of society, and has a very broad development prospect(Wang et al., 2022; Lan et al., 2023).

In this experiment, we collected soil polluted by petroleum hydrocarbons around Baoding and Baiyangdian watershed, enriched the degrading bacteria, isolated the petroleum hydrocarbon degrading bacteria in the polluted soil, and conducted molecular biology identification, aiming to obtain the dominant petroleum hydrocarbon degrading strains.

II. Materials and methods

Culture medium

Inorganic salt media: NaCI 5g/L、$Na_2HPO_4 \cdot 12H_2O$ 4.0 g/L、KH_2PO_4 2 g/L、、$MgSO_4$ 0.5 g/L、NH_4CI 0.5 g/L.

Carbon Free Media: NH_4Cl (0.2g/L), $MgCl_2 \cdot 6H_2O$ (0.2 g/L), K_2HPO_4(0.5g/L)and $CaCl_2 \cdot 2H_2O$ (0.2g/L), pH 7.0.

Oil plate: The liquid carbon source medium is added to agar to make a solid medium, and 0.5mL of filtered and de-bacterised oil is added to each plate to disperse it evenly over the surface.

Enrichment cultures of strains

10 g of petroleum hydrocarbon-contaminated soil samples were weighed into triangular flasks pre-filled with 100 mL of inorganic salt liquid medium containing diesel, gasoline and paraffin respectively, and incubated in a constant temperature shaking shaker at 30°C and 180 r/min for 6 d. Every 6 d, the culture solution was taken at 10% and added to fresh inorganic salt liquid medium containing oil for a second enrichment culture, and transferred twice in succession. Table 1 shows details of the main substances added to the enrichment medium.

Tab.1 The parameter setting of enrichment

Media number	Soil sample addition/g	Oil addition/mL
1	10	3
2	10	3
3	10	3
4	0	3
5	0	3
6	0	3

Note: No. 1,4 add diesel; No. 2,5 add petrol; No. 3,6 add paraffin.

Isolation and purification of petroleum hydrocarbon degrading bacteria and screening

Primary screening. Using the gradient dilution method. Three enrichment cultures were diluted to appropriate dilutions of 10^{-1}, 10^{-2}, 10^{-3}, $10^{-4,}$ 10^{-5}, 10^{-6} and 10^{-7}, and then 0.1mL of each of the three dilutions of 10^{-5}, 10^{-6} and 10^{-7} were applied to LB solid plates, ten plates for each dilution. The plates were incubated at 37°C for 2 d. Single colonies were repeatedly picked and purified until the colonies in the same plate were of uniform size and shape. The isolated strains were stored in 30% glycerol in an ultra-low temperature refrigerator at -80°C.

Rescreening. The diesel-degrading bacteria, gasoline-degrading bacteria and kerosene-degrading bacteria obtained from the primary screening were scribed on oil plates with diesel, gasoline and paraffin as the only carbon source and incubated in a constant temperature incubator at 30°C for 2 d. The strains that could grow on the oil plates and had good growth were selected for subsequent experiments.

Determination of degradation rates of complex sieved petroleum hydrocarbon degrading bacteria. The petroleum hydrocarbon degradation rate was measured by weighing. The culture tubes were centrifuged and frozen at -20°C for a few hours and then the top layer of liquid oil was weighed on an electronic scale using a pipette, and the data was recorded. Liquid culture tubes without inoculated bacteria were used as controls.

Calculation formula：Degradation rate=(m_1-m_2) / $m_1\times100\%$

m_1 is the amount of oil remaining in the blank culture tube（g）， m_2 is the amount of oil remaining in each culture tube (g).

Strain identification

16SrDNA molecular identification

The strains were individually inoculated onto LB solid medium and incubated in a constant temperature incubator at 37°C for 14h, then single colonies were picked and placed in sterilised centrifuge tubes of size 1.5mL and 10uL of lysate was added for DNA extraction.

PCR reaction procedures: Pre-denaturing at 94°C for 5min，Denaturing at 94°C for 30s，Annealed at 55°C for 30s，Extension at 72°C for 1min，30 cycles；Extension at72℃ for 5min. The PCR products were detected by 1% agarose gel electrophoresis, and the products were sent to Shanghai Biotechnology for sequencing, and the sequencing results were matched for BLAST homology in the NCBI database.

Tab 2 PCR reaction system

Reagent	Volume
Template DNA	1
Primer ITS1	0.5
Primer ITS4	0.5
Mix	10
ddH_2O	8
Total volume	20

III. Results and analysis

Results of primary screening for petroleum hydrocarbon degrading bacteria

A total of 34 strains of petroleum hydrocarbon degrading bacteria and 14 strains of diesel bacteria were screened in three enrichment cultures，numbered as AY1、AY2、AY3、AY4、AY5、AY6、AY7、AY8、AY9、AY10、AY11、AY12、AY13、AY14；9 strains of petrol bacteria，numbered as BY1、BY2、BY3、BY4、BY5、BY6、BY7、BY8、BY9；11 strains of paraffin bacteria，numbered as CY1、CY2、CY3、CY4、

CY5、CY6、CY7、CY8、CY9、CY10、CY11。Photographs of single colonies of some of the petroleum hydrocarbon degrading bacteria from the initial screening are shown in Figures 1, 2 and 3。

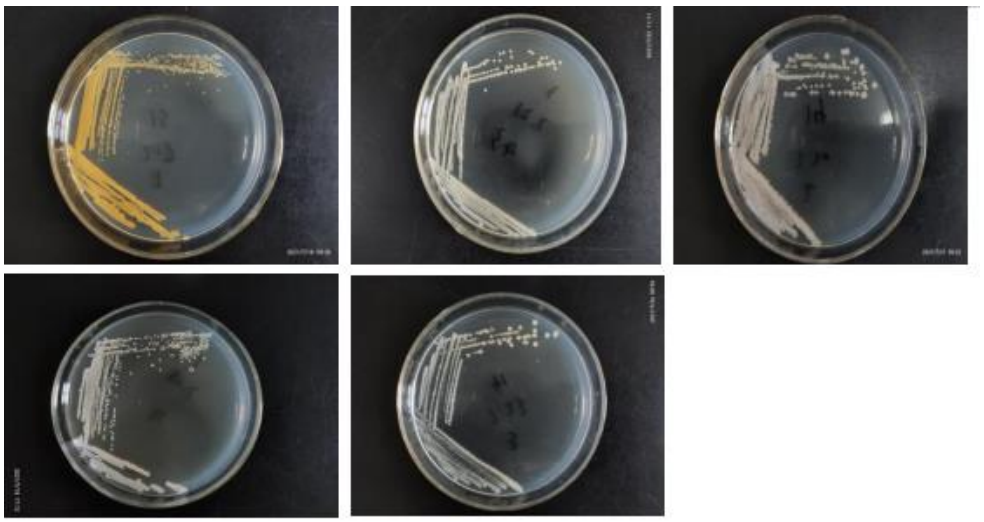

Fig 1 Photo of single colony of some diesel degrading bacteria

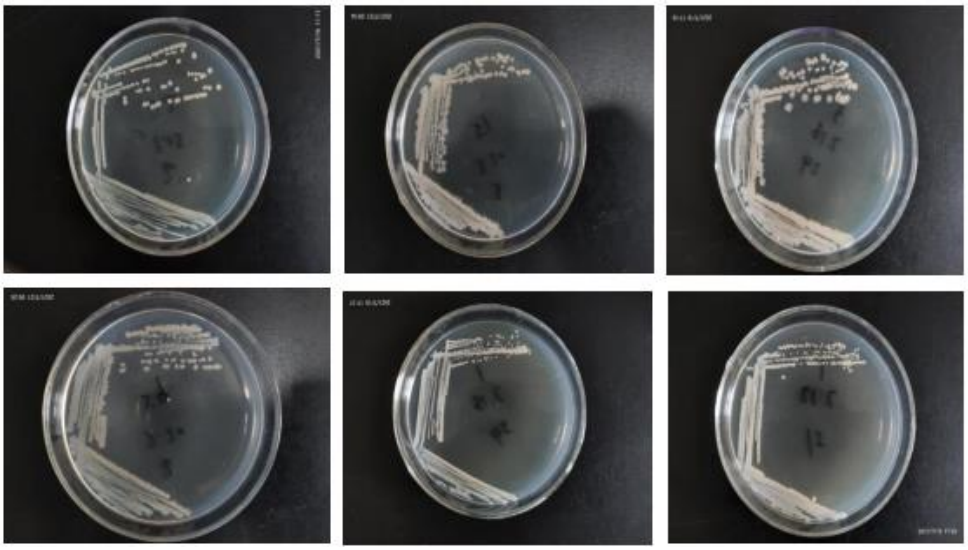

Fig 2 Photo of single colony of some gasoline degrading bacteria

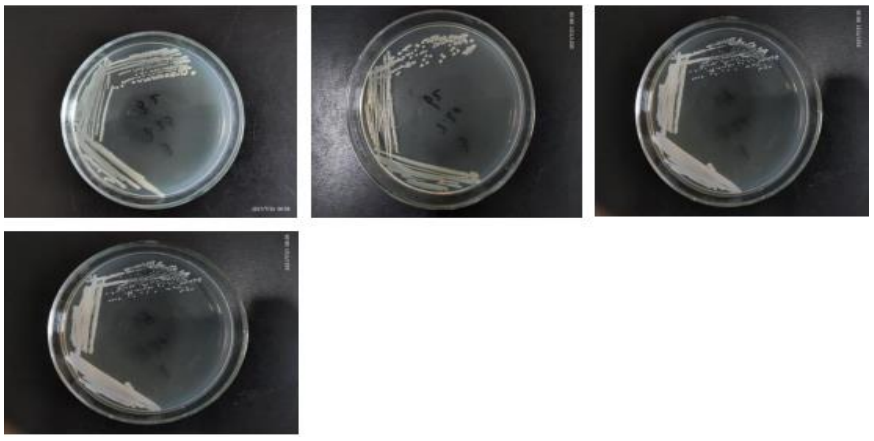

Fig 3 Photo of single colony of some kerosene degrading bacteria

Results of re-screening of petroleum hydrocarbon-degrading bacteria

The 13 strains obtained by oil plate screening are shown in Figures 4, 5 and 6，Three strains of diesel degrading bacteria AY9, AY12 and AY13, four strains of gasoline degrading bacteria BY4, BY5, BY6 and BY9 and six strains of paraffin degrading bacteria CY4, CY6, CY7, CY8, CY9 and CY10 grew vigorously on the oil plates and the colonies were clearly visible.

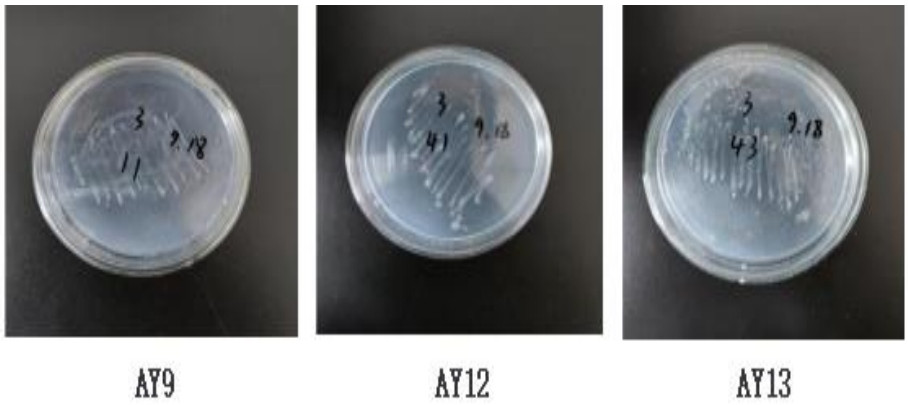

Fig 4 Diesel degrading bacteria can grow well on the oil plate

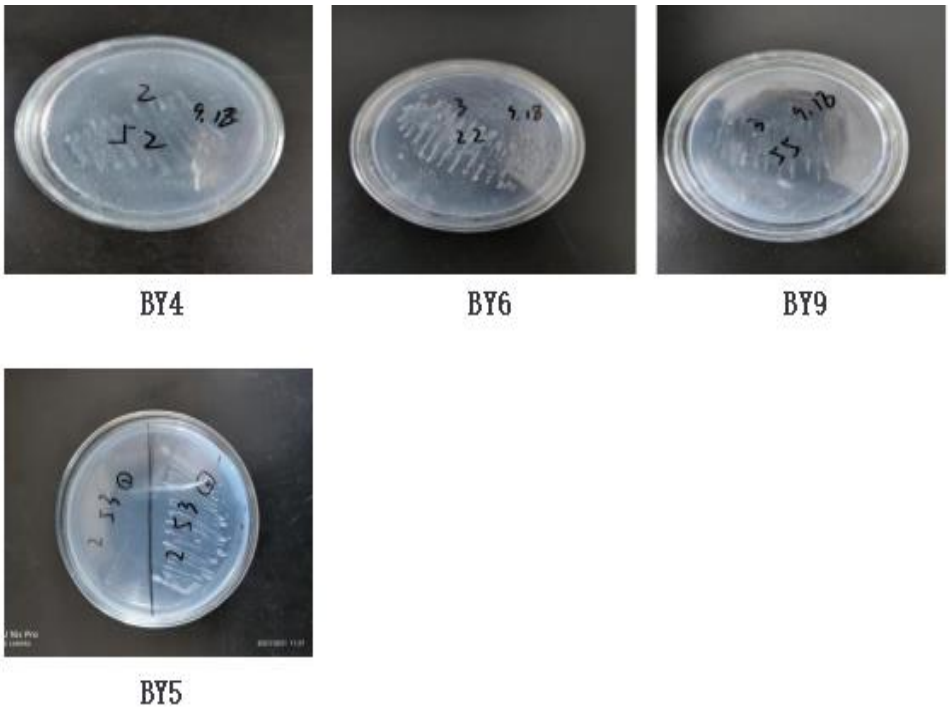

Fig 5 Gasoline degrading bacteria can grow well on the oil plate

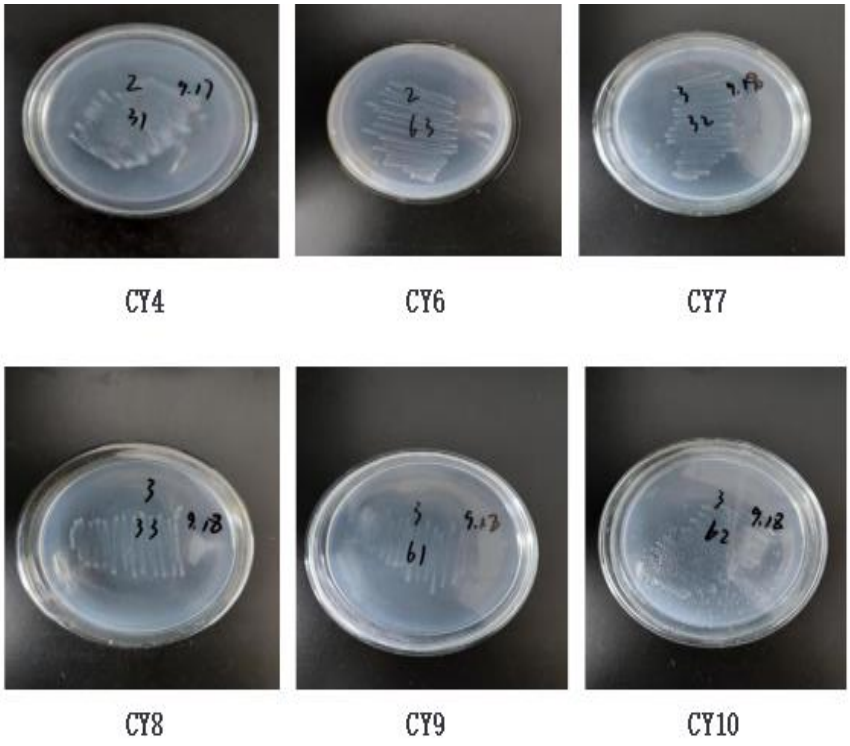

Fig 6 Kerosene-degrading bacteria can grow well on the oil plate

Determination of petroleum hydrocarbon degradation rates by petroleum hydrocarbon degrading bacteria

The degradation of the corresponding oils by the 13 strains obtained from the re-screening is shown in the figure below. three strains of diesel degrading bacteria, AY9, AY12 and AY13, degraded 4.88%, 31.71% and 4.90% of diesel oil respectively. Four strains of gasoline degrading bacteria, BY4, BY5, BY6 and BY9, degraded 20.59%, 11.76%, 17.65% and 8.82% of gasoline respectively, The degradation rates of paraffin by six strains of paraffin degrading bacteria CY4, CY6, CY7, CY8, CY9 and CY10 were 15.38%, 17.95%, 15.38%, 20.51%, 23.08% and 21.74%, respectively. The six bacteria, strains AY12, BY4, BY6, CY8, CY9 and CY10, were the dominant strains for the degradation of petroleum.

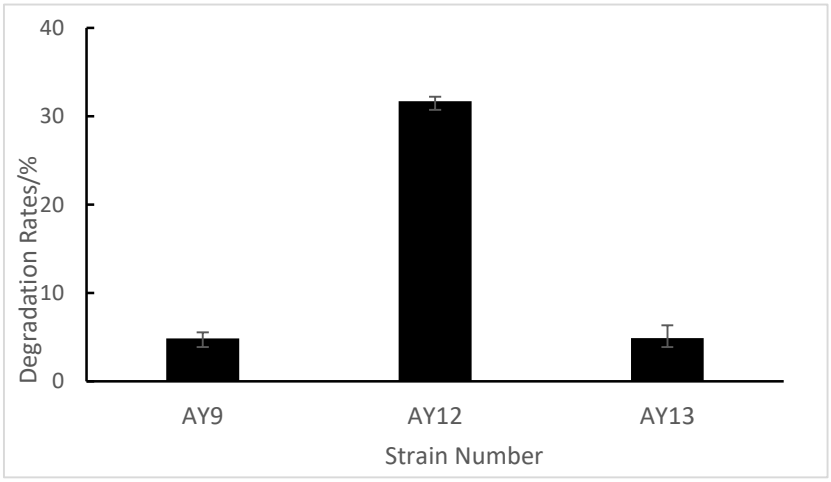

Fig 7 Degradation of diesel oil by three strains of diesel oil degrading bacteria

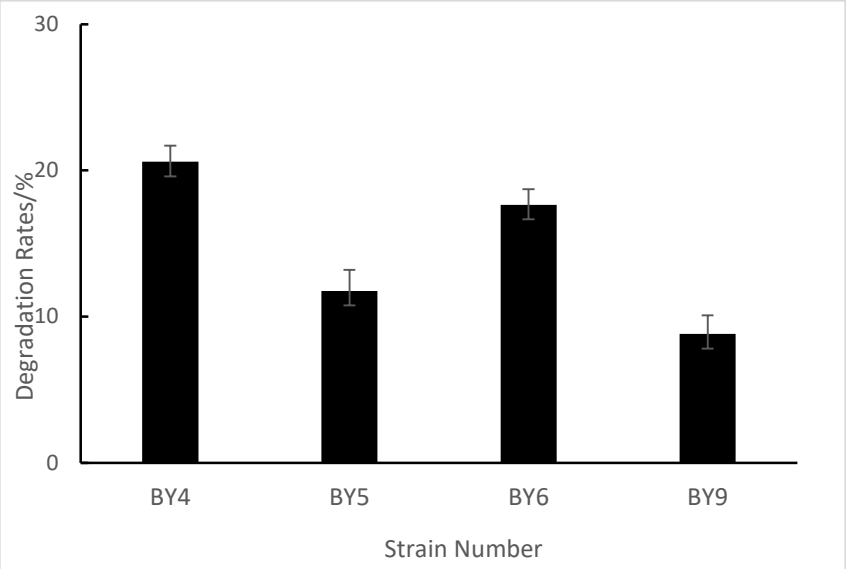

Fig 8 Four strains of gasoline degrading bacteria degrade gasoline

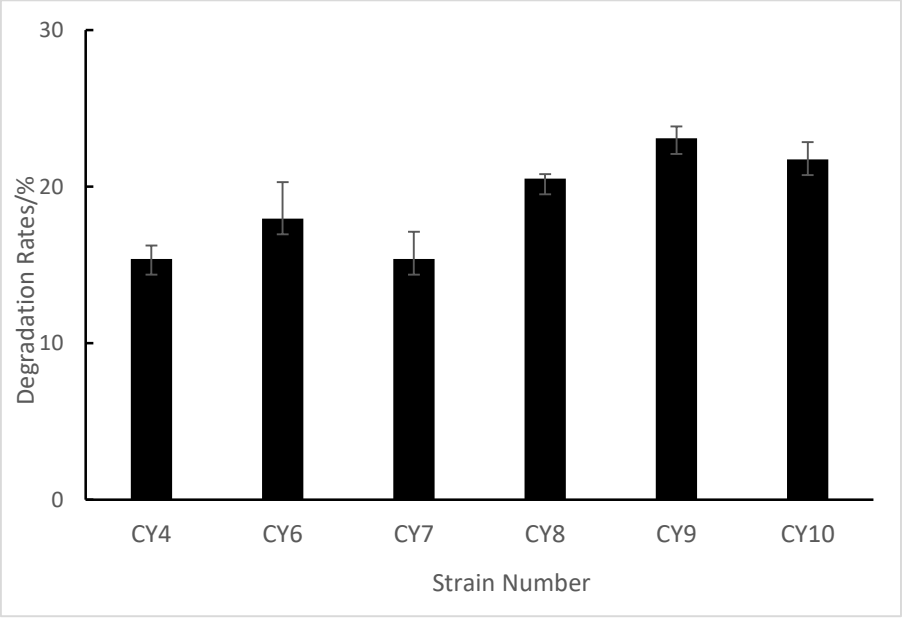

Fig 9 Six strains of kerosene degrading bacteria degrade kerosene

Strain identification results

PCR amplification results

The extracted strain DNA was amplified and detected by 1% agarose gel electrophoresis and the electrophoretic bands are shown in Figure 10.

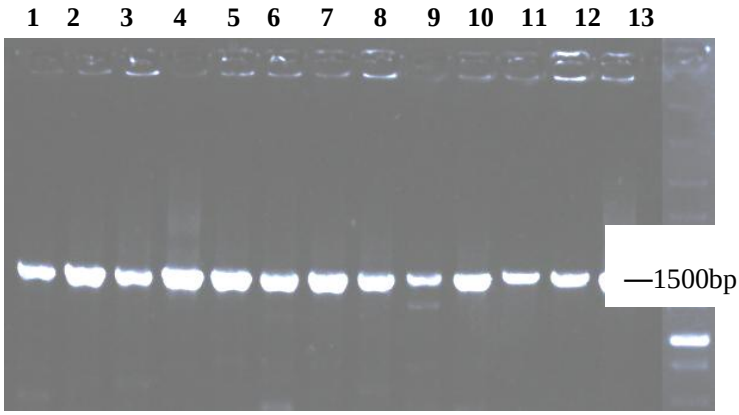

Fig 10 Bacterial 16S rDNA amplification electrophoresis numbers 1 to 13 represent CY4、BY4、BY5、CY6、AY9、BY6、CY7、CY8、AY12、AY13、BY9、CY9、CY10

Sequence Comparison Results

The sequence information was submitted to the NCBI network GenBanK database for BLAST homology comparison and combined with the results of physiological and biochemical experiments as shown in Table 7. From the comparison results, it can be seen that:BY4, CY7 and CY8 all belong to the genus Enterobacter, BY6, CY4 and AY12 belong to the genus Klebsiella, BY5, AY9, BY9, CY9 and CY10 Bacillus and AY13 belong to the genus Pseudomonas. The genera Klebsiella, Pseudomonas and Enterobacter are often mentioned in the literature and have been found to be highly efficient in degrading petroleum hydrocarbons, with good degradation properties for the petroleum hydrocarbon representatives cetane and phenanthrene, and promising applications. CY6 (Bordetella petrii) may be a newly discovered species that can degrade petroleum pollutants.

Tab 3 Results of strain identification

BacteriaNumber Strain No	Scientific Name	Similarity in16s
CY4	*Klebsiella quasipneumoniae*	98.14%
BY4	*Enterobacter coli*	100%
BY5	*Bacillus altitudinis*	99.38%
CY6	*Bordetella petrii*	98.63%
AY9	*Bacillus thuringiensis*	99.86%
BY6	*Klebsiella oxytoca*	98.02%
CY7	*Enterobacter ENB-bsy7*	98.53%
CY8	*Enterobacter cloacae*	98.52%
AY12	*Klebsiella pneumoniae*	98.10%
AY13	*pseudomonas aeruginosa*	98.51%
BY9	*Bacillus subtilis*	99.80%
CY9	*Bacillus welchii*	100%
CY10	*Bacillus cereus*	99.60%

IV Summary

From the petroleum hydrocarbon-contaminated soil samples, 34 strains of bacteria were isolated and purified through enrichment culture, including 14 strains of diesel degrading bacteria, 9 strains of gasoline degrading bacteria and 11 strains of paraffin degrading bacteria, and these strains were re-screened to obtain a total of 13 strains that could grow with petroleum hydrocarbons as the only carbon source. Among these，there are three strains of diesel degrading bacteria, numbered AY9, AY12 and AY13, four strains of gasoline degrading bacteria, numbered BY4, BY5, BY6 and BY9, and six strains of paraffin degrading bacteria, numbered CY4, CY6, CY7, CY8, CY9 and CY10.The petroleum hydrocarbon degradation rates of these 13 strains were also determined.The results showed that strains AY12, BY4, BY6, CY8, CY9 and CY10 had a good effect on petroleum hydrocarbon degradation. Identified by physiological, biochemical and 16SrDNA molecules, strain AY12 was Klebsiella pneumoniae, strain BY4 was Enterobacter coli, strain BY6 was Klebsiella oxytoca, strain CY8 was Enterobacter cloacae, strain CY9 is Bacillus welchii and strain CY10 is Bacillus cereus.

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