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Abstract. Enrichment culture method, A highly efficient functional bacterium B4 was isolated from polycyclic aromatic hydrocarbon contaminated soil by gradient domestication with pyrene as a single carbon source. The bacterium has strong tolerance to high concentration of polycyclic aromatic hydrocarbons and good degradation ability in a certain concentration range. The degradation rate of pyrene by the strain was 85% under the condition of pyrene concentration of 100 mg/L and inoculation amount of 5%. The degradation effects of the strain on polycyclic aromatic hydrocarbons (PAHs) such as fluorene, naphthalene, fluoranthene and phenanthrene were also investigated. The results showed that the strain B4 had good broad-spectrum degradation performance for PAHs.

Key words: Polycyclic aromatic hydrocarbons; pyrene; degradation Functional.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hydrocarbons consisting of two or more benzene rings arranged in a certain order. They have strong teratogenic, carcinogenic and mutagenic properties and are by-products of pyrolysis or incomplete combustion of organic compounds [1]. PAHs, as a global organic pollutant, is one of the 129 priority pollutants formulated by the U.S. Environmental Protection Agency (EPA). It has significant "three-way" toxicity. PAHs are widely distributed in the environment and generally exist in the natural environment, but most of them accumulate in the soil [3]. Under suitable environmental conditions, PAHs will migrate to the surrounding soil environment and expand the scope of soil pollution.

2. Materials and methods

2.1. Site conditions of tested strains

This research strain originated from the agricultural pollution site of an agricultural demonstration area test base in Shenbei New Area, Shenyang. The area of agricultural farms in this region is about 700,000 mu. Mainly grow a variety of flowers and crops.

2.2. Microbial culture methods

LB medium: 5g yeast extract, 10g peptone, 10g sodium chloride, 1L deionized water at constant volume, pH value 7.0, sterilization under high pressure and humid heat at 121 C for 20 minutes.

Inorganic Salt Base Medium (MSM) [15]: 0.8gNa_2HPO_4, 1.0g$(NH_4)_2$ SO_4, 0.2gKH_2PO_4, 0.1g$CaCl_2 \cdot 2H_2O$, 0.2g$MgSO_4 \cdot 7H_2O$, 1.0mg$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 5.0mg$FeCl_3 \cdot 6H_2O$, pH =7 .2, sterilization at 121 C for 20 min. Different concentrations of pyrene were added to the conical bottle after cooling (the concentration was gradually increased by gradient domestication).

2.3. Major PAHs reagents

Fluorene, naphthalene, phenanthrene, pyrene, and fluoranthene, products of German Fluka Company, purity > 97%; n-hexane, dichloromethane, products of Tianjin Kangke Technological Co., Ltd; M-610, 16 kinds of polycyclic aromatic hydrocarbons mixed standard samples (MeOH: CH_2Cl_2=1: 1 medium), products of American Supelco Company.

2.4. Instruments and chromatographic conditions

Agilent GC-6890 (n) gas chromatography. Chromatographic conditions: non-shunt mode temperature 250 C at the inlet; HP-5 elastic quartz capillary column (30 m×0.25 mm×0.25 μm); column flow rate 1.0 ml min-1; furnace temperature 80 ℃ for 1 min, Keep 1 minute at 255 with 15 min-1 ,Then raise it to 265 at 1 min-1 and keep it for 1 minute, Finally, the temperature rises from 2.5 min-1 to 295 and remains for 2 minutes; Hydrogen Flame Ionization Detector (FID) 300 ℃.

2.5. Isolation and screening of dominant strains and spectroscopic test of PAHs degradation

2.5.1. Strain culture. (1) Enrichment culture: 1 g of soil samples 5 cm deep beneath the surface of the test site were collected and put into a conical flask with 300 mL enrichment medium after sterilization. The samples were placed in a shaking table at 30 ℃ and cultured at 120 r/min for 72 hours.

(2) Directional selective culture: 50 mL mixed bacterial solution after enrichment culture was inoculated into 200 mL inorganic salt medium. Pyrene was used as the sole carbon source. The bacterial turbidity (A600 nm) was determined at a certain interval by oscillating culture at 30 C for 120 r/min. Gradient domestication was used to control the pyrene concentration in the range of 30-300 mg/L. Each time the medium was obviously turbid, 50 mL mixed bacterial solution was transferred to fresh medium, and pyrene concentration was increased at the same time. For each gradient concentration, the mixed bacterial solution in the stable period of culture was stored at 4 ℃ for reserve.

2.5.2. Isolation of strain. In order to isolate the functional bacteria which can degrade PAHs efficiently, the highest concentration of mixed bacterial culture medium which can degrade PAHs pyrene was used to isolate and screen the functional bacteria on the solid culture plate containing 300 mg/L PAHs. The single bacterial colonies with different morphological characteristics were separated and purified through repeated line separation on the plate until pure colonies were obtained.

2.5.3. Strain screening. The different isolated pure bacteria were expanded and cultured, then the centrifuges were washed out with sterile water to prepare the pure bacterial solution. The content of bacteria is about 10^8~10^9CFU mL, the bacteria are inoculated into the culture medium containing 100 mg/L pyrene and 200 mL pyrene in a conical flask at 30 ~120r/min. The residual pyrene content is determined by regular sampling. The efficient functional bacteria are selected according to the degradation effect of pyrene.

2.5.4. Preliminary identification of selected strains. The single strain characteristics of the selected strains were studied. The species were identified primarily by physiological and biochemical experiments and morphological observation.

2.5.5. Broad-spectrum degradation of polycyclic aromatic hydrocarbons by strains. The bacterial suspensions were inoculated into 200 mL inorganic salt medium containing only 100 mg/L fluorene, naphthalene, fluoranthene and phenanthrene, respectively, and cultured in shaking bed at 120 r/min 3 and 0 ℃. Quantitative timing sampling was conducted to determine the degradation capacity of the selected strains to four PAHs.

3. Results and Discussions

3.1. Gradient selective culture

In the range of pyrene concentration from 30 to 60 mg/L, the bacterial strains grew well, and the obvious turbidity could be observed within 12 hours; in the range of 90 to 120 mg/L, the adaptability period of bacterial strains was slightly longer than that of low concentration, and no turbidity could be observed at 12 hours. After 18 hours, the bacterial turbidity began to increase significantly, then showed logarithmic growth, and the bacterial strains grew well in the range of 150 to 240 mg/L. During the process, bacterial turbidity decreased in varying degrees, and then increased gradually. At the same time, the initial time of medium turbidity became longer and longer, and the highest bacterial turbidity gradually decreased during the stable period. This indicated that with the increase of pyrene concentration, a small number of microorganisms died in the adaptive period, and the growth of strains was inhibited to varying degrees. Moreover, pyrene concentration gradually reached the tolerance limit of mixed bacteria. The species and quantity of pyrene-degrading functional bacteria decreased, but it also showed that the surviving and growing microorganisms had stronger tolerance and better degradation ability to pyrene. When pyrene concentration increased to 270 mg/L for 48 hours, the bacterial turbidity increased slightly, and the phenomenon of visual culture medium turbidity was not obvious. When pyrene concentration reached 300 mg/L, the bacterial turbidity did not increase and the bacterial strains died. The results showed that under this condition, the bacterial strains were intolerant and the pollutants could not be degraded.

3.2. Isolation and Screening of Functional Bacteria

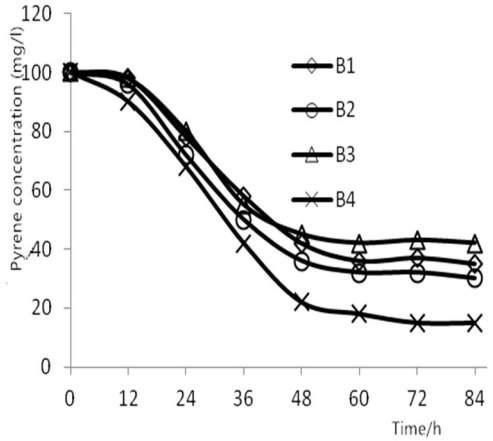


Fig.1 Degradation of Pyrene by Different Functional Backeria

Four strains of pure bacteria named B1, B2, B3 and B4 were obtained from the inorganic solid medium plate containing 300 mg/L pyrene. Four kinds of pure bacteria were inoculated into four groups of culture medium with pyrene concentration of 100 mg/L after expanded culture. The degradation ability of pyrene by four kinds of pure bacteria was compared. The results are shown in Fig1

Fig. 1 shows that the adaptation period of B4 is the shortest in batch culture. Substrates have been removed at 12h. Strains B1, B2 and B3 have a certain adaptation period compared with B4. With the passage of time, pyrene has been removed well by four strains. After 48h, pyrene degradation basically

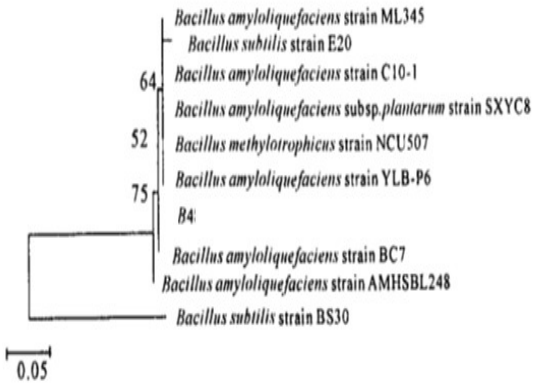
reaches its limit, and pyrene concentration tends to balance in subsequent determination. Ultimately, the degradation rates of 100 mg/L pyrene by B1, B2, B3 and B4 were 65%, 70%, 58% and 85%, respectively. In the process of pyrene degradation, strain B4 could degrade substrate faster and had the highest degradation efficiency, so it was preferred to carry out follow-up experiments on behalf of the strain.

3.3. *Morphological Observation and Physiological and Biochemical Test of Strain B4*
The microbial morphology and physiological and biochemical tests of the selected strain B4 were carried out. The results are shown in Table 1.

Table 1. Microbial Morphological Characteristics and Physiological and Biochemical Test Results of Strain B4

Study on Colony-related Morphology		Study on Physiological and Biochemical Characteristics	
Mycelium morphology	Short rod shape	Gram staining	+
Colony shape	rule	Spore dyeing	+
Uplift shape	Low convex	Capsular staining	-
Edge shape	rule	Flagellum dyeing	-
colour	Canary yellow	Nutrition Requirement Test	Facultative anaerobic
Transparency	translucent	Glucose Fermentation	
Viscosity	More viscous	V.P test	+

According to the colony characteristics and Gram staining experiments, combined with physiological and biochemical experiments, strain B4 was identified as spore stem, and the specific species still need further molecular biology diagnosis.



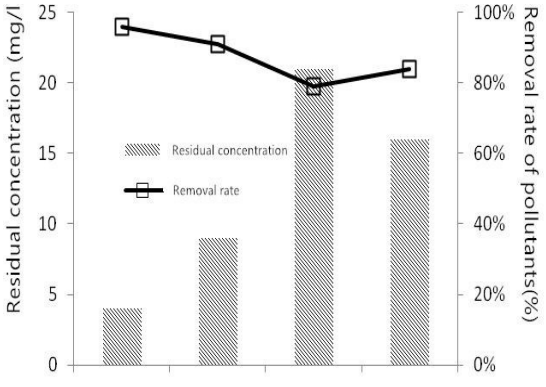


Fig. 2 Phylogenetic location of B4 in 16S rDNA analysis. **Fig.3** Removal of PAHs by strain B4

The 16S rDNA sequences were Blast-aligned in GenBank, and the homologous sequences were all Bacillus genus. The homology with Bacillus amyloliquefaciens strain YLB-P6 was as high as 96.8%. The phylogenetic tree was constructed to determine the phylogenetic relationship of the strain in its species and genera, as shown in Figure 2. From the comparison of the figures, we can see that B4 belongs to Bacillus.

3.4. Broad-spectrum degradation of polycyclic aromatic hydrocarbons by selected strains
Bacterial strain B4 was cultured for 48 hours under the same conditions as pyrene degradation.

Theoretically, polycyclic aromatic hydrocarbons (PAHs) are more difficult to degrade as the number of benzene rings increases. Fig. 3 shows that under the condition that the initial concentration of several

substrates are 100 mg/L, the optimal strain B4 has the best degradation effect on naphthalene, up to 96%, phenanthrene, 91%, fluorene, 84% and fluoranthene, 79%. Although strain B4 is a functional strain selected from pyrene as a single carbon source. But the other four representative polycyclic aromatic hydrocarbons have good degradation effect. The experimental study shows that the strain has broad-spectrum characteristics for the degradation of polycyclic aromatic hydrocarbons, and the degradation efficiency is very high. The experimental results are in good agreement with the theory.

4. Conclusion

(1) A pyrene-degrading strain B4 was isolated from agricultural contaminated soil samples. The optimum strain had a high pyrene-degrading efficiency of 85% under the conditions of inoculation amount of 5%, initial pyrene substrate concentration of 100 mg/L, 120 r/min and shaking-bed culture at 30 ℃. The strain had a short adaptation period to the culture medium. The strain B4 was identified as Bacillus by microbial morphology observation and physiological and biochemical tests.

(2) The degradation effects of strain B4 on fluorene, naphthalene, fluoranthene, phenanthrene and other typical polyaromatic hydrocarbons were investigated under the same conditions. The experimental results showed that the removal efficiencies of fluorene, naphthalene, fluoranthene and phenanthrene by strain B4 were 84%, 96%, 79% and 91%, respectively. The results showed that the strain B4 had good broad-spectrum degradability to polycyclic aromatic hydrocarbons and could remediate the polycyclic aromatic hydrocarbons contaminated sites. Providing bacterial sources has good prospects for engineering remediation of contaminated sites.

Acknowledgments

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