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Study on the Breeding and Characterization of High-Efficiency Oil-Degrading Bacteria by Mutagenesis

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Abstract: In the present study, a high-efficiency petroleum hydrocarbon-degrading bacterium MX1 was screened from petrochemical wastewater sludge, and MX1 was identified using morphological, physiological, and biochemical experiments and combined with 16S rDNA. Results showed that the the MX1 strain belongs to *Enterobacter* sp. The degradation conditions were an incubation time of 18 days, temperature of 30 °C, pH of 7, and salinity of 2% (*w*/*v*), and the degradation proportion was 37.41% for 7 days. The combination of microwave and ultraviolet mutagenesis yielded the strain MXM3U2. The mutant strain had a petroleum hydrocarbon breakdown efficiency of 56.74% after 7 days of culture, and this value was 51.66% higher than the original strain. The number of strains and the rate of degradation of n-alkanes (C16, C24, C32, and C40) decreased steadily with the increase in carbon chains in the degradation test. GC/MS (Gas chromatography mass spectrometry) results showed that in the process of degrading crude oil, the hydrocarbons with carbon number C < 24 were degraded first, followed by hydrocarbons with carbon number C > 24. The strains had a good degradation effect on pristane, naphthalene, and phenanthrene. In this study, a high-efficiency petroleum hydrocarbon-degrading bacterium was screened via microwave-ultraviolet composite mutagenesis technology.

Keywords: crude oil; bioremediation; oil-degrading bacteria; mutation

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1. Introduction

Water is the source of life [1] and the most important component of living organisms and an important resource for human survival. Oil is an organic substance that is insoluble in water and is also important to life and production [2]. With the continuous development of industrialization, especially in heavy industry, large-scale industry, new industry, and a series of indispensable links such as post-processing assistance, organic solvents and surfactants are discharged together with oily wastewater [3], thus increasing the oily wastewater emissions. The total amount of oily wastewater in the world has reached 1.0–1.5 billion cubic meters, which will continue to increase [4]. Oily wastewater is mainly composed of water, hydrocarbons [5], oils [6], and soaps, and all kinds of oil cause serious damage to the natural environment and ecological environment [7,8], pollute water bodies, and affect the safety of drinking water for residents. Some aromatic hydrocarbon oils are biologically teratogenic and carcinogenic [9].

Conventional methods for the treatment of oily wastewater include physical, physical-chemical, chemical, and biochemical methods [10]. Physical methods mainly include oil traps and coarse-grained, air flotation, and hydro-cyclone methods [11], etc. It uses the incompatibility and specific gravity of oil and water, which are different, suitable for removing large particle size oil slicks and dispersed oil [12,13], as well as most of the solid

particles in wastewater. The oil separator aims to separate oil and water by gravity. The coarse-grained method uses porous media to absorb oil droplets in the oily wastewater . The air flotation method injects air into the water to form small bubbles, which are affected by interfacial tension, buoyancy, and hydrostatic pressure. Under the combined action of various forces, the air bubbles adhere to the oil particles suspended in the water [14], making the oil droplets and air bubbles float to the surface together to form a scum layer, and finally separate the oil from water. The physical and chemical methods commonly used for the treatment of oily wastewater are flocculation and adsorption [15]. A certain proportion of flocculant is added to the sewage to form flocs to absorb tiny oil droplets, causing the oil droplets to become unstable. Then, the oil droplets are separated and removed by sedimentation or air flotation, which is called flocculation. Adsorption uses adsorbents such as activated carbon to remove pollutants in water [16,17]. Considering the high cost of use and the difficulty in regeneration, these materials are often used for the advanced treatment of wastewater [18].

Physical and chemical methods are commonly used to remove petroleum pollutants in the environment [19]. However, these treatment technologies encounter problems, such as high operating and maintenance costs, sludge that needs to be further treated after sewage treatment [20], high energy demand, limited pH controllable range, long operation and assembly time, and the need for pre-processing. Considering the treatment process and the formation of fouling, scaling, and foaming, bioremediation technology has received increasing attention because of its great potential for the remediation of oil-contaminated environments [21].

Microbial degradation can effectively remove various types of organic pollutants [22]. Under suitable conditions, microorganisms such as bacteria, protozoa, algae, and fungi can biologically oxidize soluble organic substances in wastewater into carbon dioxide and water through their metabolic activities [23]. Considering that many macromolecular refractory substances are present in oily wastewater [24], ordinary microorganisms hardly exert their functions, thus greatly reducing the efficiency of biological remediation [25]. Therefore, microbial degradation aims toward the screening and domestication of microorganisms, which are among the more economical and effective processes for removing pollutants [26], although these processes take a long time. Microorganisms that can survive in oily wastewater use pollutants in the water as a nutrient source for growth [27], decompose macromolecular organic compounds such as petroleum hydrocarbons into small molecules, and release chemical energy for their metabolism. With the continuous development of the economy, the demand for oil in the process of industrialization is also increasing, and oily wastewater is produced when oil is exploited and used [28,29]. The biological treatment method is less likely to cause secondary pollution [30], incurs a low cost, and has high treatment efficiency, making it the ideal sewage treatment technology [31]. However, the oily wastewater contains various complex organic substances that inhibit the metabolic function of bacteria, thus increasing the difficulty of biological treatment [32]. Therefore, the key to biological treatment of oily wastewater is the screening and domestication of degrading bacteria that can efficiently degrade organic pollutants [33].

In the present study, ultraviolet and microwave composite mutagenesis technologies were first investigated and compared, the optimal mutagenesis conditions of the strain were finally determined, and a high-efficiency and salt-tolerant petroleum hydrocarbon-degrading bacterium was screened. This method can be used as a breeding advantage. This method can be used as a reference for petroleum hydrocarbon-degrading strains. This study also investigated the characteristics of the strain to degrade petroleum and speculated that it degraded some common petroleum hydrocarbon components, thus providing some experimental and theoretical basis for the further research and application of the strain.

2. Materials and Methods

2.1. Materials

The excess sludge of the Yangzi Petrochemical Wastewater Treatment Plant (Nanjing, China) was used as a bacterial source for screening petroleum hydrocarbon-degrading bacteria. The crude oil used in the experiment was derived from laboratory-simulated crude oil ($0^{\#}$ diesel oil: heavy oil = 1:1 mixture).

The reagents and dye solutions used for bacterial identification are as follows. The gram stain includes Lugol's iodine solution, oxalic acid amine crystal violet mixture, 0.5% safranine counterstain solution, and 95% ethanol. The methyl red reagent includes methyl red, distilled water, and 95% ethanol. Oxidase reagents include 1% α-naophenol-ethanol solution and 1% xylene hydrochloride p-phenylenediamine solution. The catalase reagent contains 4% H_2O_2. The contact enzyme activity reagent contains 3% hydrogen peroxide reagent. The sugar fermentation reagent contains 1% acidic fuchsin aqueous solution. The abovementioned reagents and dye solutions were of analytical grade.

The medium used in the experiment is as follows. LB (Luria–Bertani) medium contains the following (g/L): tryptone, 10; NaCl, 10; yeast extract, 5; sterile water, 1 L. The pH was adjusted to 7.4–7.6, and the reagent was used after autoclaving (1×10^5 Pa, 20 min). Solid medium requires the addition of 1.5%–2.0% agar. Inorganic salt medium contains the following (g/L): $CuCl_2$, 0.0005; $FeSO_4$, 0.005; $ZnCl_2$, 0.005; $MnCl_2 \cdot 4H_2O$, 0.05; $CaCl_2$, 0.02; K_2HPO_4, 4.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 1.0; NaCl, 1.0; sterile water, NaOH to adjust pH to 7.5. The primary screening medium for the petroleum hydrocarbon-degrading bacteria was prepared by adding sterilized crude oil to the inorganic salt medium to a final concentration of 500 mg/L. Agar (1.5%–2%) was added to the solid medium. The rescreening medium for petroleum hydrocarbon-degrading bacteria was prepared by adding sterilized crude oil to the inorganic salt medium to a final concentration of 2000 mg/L. Agar (1.5%–2%) was added to the solid medium. The degraded crude oil test medium was prepared by adding 1% (*w*/*v*) sterilized crude oil to the inorganic salt medium. Agar (1.5%–2%) was added to the solid medium.

2.2. Isolation and Culture of Petroleum Hydrocarbon Degrading Bacteria

Approximately 1 mL of each oily sludge sample was diluted, applied to the screening medium for primary screening, and incubated at 35 °C for 48 h. A single colony that grows vigorously was selected and transferred to 100 mL of re-screening medium for re-screening. Three shake flasks were incubated at 35 °C and 180 rpm for 72 h. The content of petroleum hydrocarbons in the fermentation broth was detected by UV (Ultraviolet) spectrophotometry, and the degradation proportion was calculated. The strains with higher degradation ability were selected and cultured on LB slant medium at 4 °C. The sample was saved for backup. The screened strains were inoculated on the plate medium and incubated at 30 °C for 72 h, and the bacterial morphology of the strains was observed. The strains were identified by physiological and biochemical tests. The identification methods included Gram staining, glucose oxidative fermentation test, VP, methyl red test, oxidase test, and indole test, etc. Bacteria were identified in the Manual of Systematic Identification.

Generally, the growth of microorganisms is very sensitive to changes in the environment, and changing environmental factors affect the growth of microorganisms. Therefore, this study focused on the following factors that may affect the degradation ability of the strain: (1) Culture time (3, 6, 9, 12, 15, 18, and 21 days); (2) Temperature (20, 30, 40, and 50 °C); (3) pH value (5, 6, 7, 8, and 9); (4) NaCl concentration (1%, 2%, degradation proportion 3%, 4%, and 5%). The control group consisted of the culture medium without bacterial liquid under the same conditions. The OD_{600} (absorbance value of bacterial culture solution at 600 nm wavelength) value of the fermentation broth and the of petroleum hydrocarbons was determined [34].

2.3. Mutagenesis Test of Petroleum Hydrocarbon Degrading Bacteria

The MX1 strain was inoculated into LB liquid medium and cultured in a constant-temperature shaking box at 30 °C and 150 rpm for 48 h. After centrifugation for 15 min, the cells were washed twice with sterile saline to prepare a bacterial suspension. A hemocytometer was used to adjust the concentration of bacterial suspension to approximately 108 CFU/mL for later use. For ultraviolet mutagenesis, 5 mL of bacterial suspension was obtained during the logarithmic growth phase, and then transferred into a sterilized Petri dish with a diameter of 9 cm. The ultraviolet lamp was turned on and preheated for 20 min, and the Petri dish was placed directly under the ultraviolet lamp for 35 cm, and irradiated for 1, 2, 3, 4, 5, and 6 min. For microwave mutagenesis, 5 mL of bacterial suspension was obtained during the logarithmic growth phase, and then transferred to a 9 cm-diameter sterilized Petri dish. Every 5 s, the Petri dish was placed on an ice pack for cooling to room temperature, and the process was carried out alternately for 10, 30, 50, 70, 90, and 110 s.

For compound mutagenesis, microwave mutagenesis was performed on the bacterial suspension in the Petri dish, which was placed on an ice pack for cooling to room temperature every 5 s for a total of 110 s. Approximately 5 mL of the bacterial suspension was obtained during the logarithmic growth phase, and then transferred to a sterilized Petri dish with a diameter of 9 cm. The UV lamp was turned on and preheated for 20 min, and the Petri dish 35 cm was placed directly below the UV lamp for irradiation for 3 min. For post-mutation treatment, 0.1 mL of the mutagenized bacterial suspension was obtained and diluted with sterile water to 10^6, 10^7, and 10^8. Approximately 0.1 mL of sample was obtained for plating, and three parallel samples were prepared for each gradient. The unmutated bacterial suspension was used as the control and placed in a constant-temperature incubator at 30 °C for 72 h in the dark. The plates were then counted, and the average of the three groups of results was used to calculate the lethality of the strains.

2.4. Degradation Test of Pollutants by Mutant Strain MXM3U2

The utilization of crude oil by mutant strain MXM3U2 was tested using n-hexadecane, n-tetracosane, n-tridodecane, and n-tetradecane as the only carbon sources of the strain. MXM3U2 was inoculated into 100 mL of liquid LB medium and cultured at 30 °C with shaking at 180 rpm for 3 days. The LB broth was removed after centrifugation at 8000 rpm for 5 min. After washing twice with sterile saline, 5 mL of bacterial suspension (10^9 CFU/mL) was transferred using a pipette into 100 mL of liquid inorganic salt medium. The concentration of the carbon source added was 0.3% (v/v) for liquid hydrocarbons and 0.05% (w/v) for solid hydrocarbons. The abovementioned experiments were performed in parallel in six groups, and two blank experiments were added, where bacteria were added without a carbon source and a carbon source was added without bacteria to calculate the growth of strains and the degradation of carbon sources. After the 7th, 14th, and 21st days of the experiment, a gas chromatograph was used to detect the residual amount of alkanes in the fermentation broth of four different carbon sources. In addition, the number of bacteria in the fermentation broth was determined by colony counting every 2 days, in which an aliquot of the fermentation broth was diluted, spread on solid LB medium plates, and cultured at 30 °C for 2 days.

2.5. Analysis of Petroleum Hydrocarbons

2.5.1. Determination of the Degradation Proportion of Petroleum Hydrocarbons by UV Spectrophotometry

Hydrocarbon compounds in crude oil have characteristic absorption in the ultraviolet region [35]. The concentration of hydrocarbons can be measured by UV spectroscopy at (λ = 256 nm) and rate of degradation can be calculated.

Petroleum hydrocarbon degradation proportion = (measured value Q_0 of control group—measurement value Q_1 of experimental group)/measured value of control group Q_0.

2.5.2. Determination of the Growth of Petroleum Hydrocarbon-Degrading Bacteria by UV Spectrophotometry

Bacterial suspensions with different growth amounts have different OD values (optical density values) [36]. Therefore, the absorbance of the bacterial suspension can be used to measure the concentration of bacteria to evaluate its growth [37]. The absorbance value (OD_{600}) was measured at the wavelength of 600 nm.

Petroleum ether was used as the extractant and solvent, and the absorption value of petroleum ether was used as a reference for measurement. The standard curve was made as shown in Figure S1. The fermentation broth to be tested should be centrifuged before extraction. We added 0, 2.00, 4.00, 8.00, 12.00, 20.00, and 25.00 mL of standard oil working solution to seven 50 mL volumetric flasks and diluted them with petroleum ether (30–60 °C boiling range) to the mark. At a wavelength of 256 nm, the absorbance was measured with petroleum ether as a reference, and a calibration curve was generated after blank correction (Figure S1).

For sample pre-treatment, the fermentation broth containing alkanes was extracted with 100 mL of n-hexane, and the volume was adjusted to 100 mL for injection. The crude oil degradation fermentation broth was extracted twice with 100 mL of n-hexane, and then passed through a 0.22 μm organic filter for injection. The pre-treatment of cyclic aromatic hydrocarbon fermentation broth was carried out as described by Gutman et al. [38].

An Agilent GC7890A 5975C MSD gas chromatography mass spectrometer with HP-5MS quartz capillary column (30 m × 0.32 mm × 0.25 μm) was used for GC/MS analysis. The carrier gas was high-purity helium, the injection volume was 1 μL, and the injection was splitless. The temperature program of the n-hexadecane sample is as follows: initial temperature of 140 °C was maintained for 1 min, and then increased to 280 °C at 20 °C/min; the injection temperature was 260 °C; the detector temperature was 280 °C. The temperature program of n-tetracosane and n-tetradecane samples was as follows: the initial temperature was 155 °C for 2 min, and then increased to 280 °C at 25 °C/min, the injection temperature was 300 °C, and the detection temperature of the sample was 350 °C. The initial temperature of the n-docosane sample was 155 °C for 2 min, which was then increased to 280 °C at 25 °C/min; the injection temperature was 300 °C, and the detector temperature was 350 °C. For the crude oil sample, the initial temperature was 50 °C, which was maintained for 1 min; the temperature was increased to 310 °C at 6 °C/min, the injection temperature was 310 °C, and the detector temperature was 320 °C. For the naphthalene and phenanthrene samples, the initial temperature was 80 °C, which was held for 2 min; the sample was heated at 3 °C/min to 120 °C, 5 °C/min to 200 °C, and 7 °C/min to 290 °C, and this temperature was held for 15 min; the inlet temperature was 280 °C, and the detector temperature was 300 °C.

3. Results and Discussion

3.1. Screening and Characteristics of Petroleum Hydrocarbon Degrading Bacteria MX1 Strain

The single colonies MX1, MX2, and MX3 of the primary screening petroleum hydrocarbon-degrading bacteria were selected and placed in a liquid medium containing 100 mL of petroleum hydrocarbon-degrading bacteria for re-screening. Each strain was connected to three shake flasks and cultured at 35 °C and 180 rpm for 21 days. After 21 days of continuous culture, as shown in Figure 1, the average degradation proportion of petroleum hydrocarbons by the MX1 strain reached 61.08%. The degradation proportions of petroleum hydrocarbons by strains MX2 and MX3 were relatively low. The highest degradation proportion of MX2 was 47.35 % within 21 days, and the highest degradation proportion of strain MX3 was 45.72%.

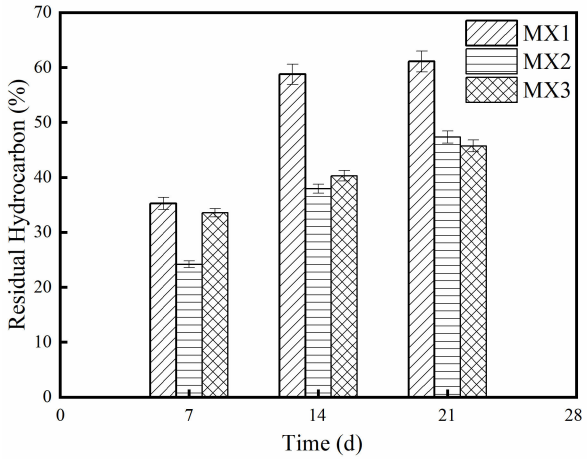


Figure 1. The screening of the oil-degrading bacteria.

The colony morphology and physiological and biochemical tests of the MX1 strain (Table 1) showed that the MX1 strain was a facultative anaerobic Gram-negative Enterobacter aerogenes, with a straight rod-shaped cell, size of 0.6–1.0 μm × 1.2–3.0 μm, and motility of pericyte flagella. Fermented glucose produces acid and gas, has catalase, can use citrate and malonate as the only carbon and energy source respectively, does not produce H_2S from thiosulfate, can slowly liquefy gelatin, yields a positive VP test and negative MR test, and can grow in 4% NaCl. Therefore, the MX1 strain should be classified as Enterobacter. The morphological characteristics of MX1 in LB plate medium and under microscope are shown in Figure S2.

Table 1. Morphological, physiological, and biochemical characteristics of MX1 (Note: "+" means positive, "−" means negative).

Bacteria	**Straight Rod-Shaped, 0.6~1.0 μm × 1.2~3.0 μm in Size, Gram-Negative, with Pericytic Flagella.**		
colony	Round or nearly round, 1–4 mm in diameter, milky white. The surface is slightly moist and shiny, with a slightly raised center and a nearly crenellated edge.		
exercise observation	+	Gelatin Liquefaction Test	+
Anaerobic growth	+	Citrate Utilization Test	+
aerobic growth	+	Fluorochrome	−
VP test	+	KCN	+
indole test	−	contact enzyme	−
oxidase	+	H_2S production test	−
MR test	−	Sugar fermentation test	+
methyl red test	+	Urease	−

3.2. 16S rDNA Sequence Homology and Phylogenetic Analysis

Primer synthesis, PCR (Polymerase Chain Reaction) product purification, and sequencing were completed by Sangon Bioengineering (Shanghai) Co., Ltd., Shanghai, China. The 16S rDNA gene sequence of MX1 was compared with the 16S rDNA sequence (the NCBI (National Center for Biotechnology Information) sequence number in brackets) of the known type strains belonging to the genus Enterobacter in GenBank by BLAST (Basic Local Alignment Search Tool), and MEGA 5.1 software (an open source software, version No.5121019) was used. A phylogenetic tree was constructed, and it was inferred that the MX1 strain belonged to the genus Enterobacter (Figure 2).

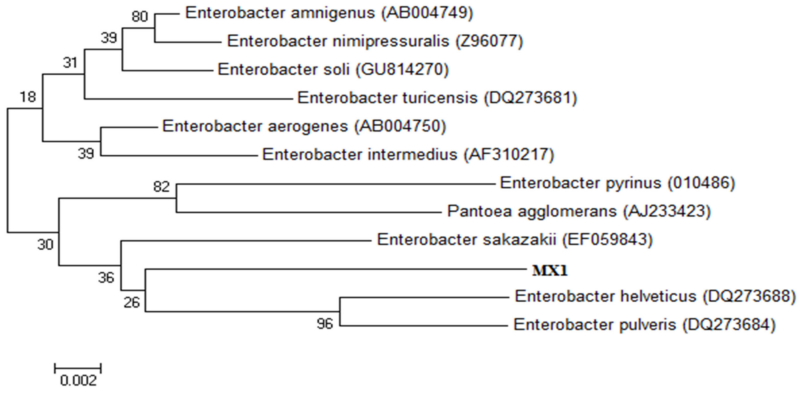


Figure 2. Rootless phylogenetic tree of 16S rDNA sequences between MX1 and related species of model strains in Enterobacter.

3.3. Determination of Optimal Growth and Degradation Conditions of the MX1 Strain

As shown in Figure 3a, with the increase of culture time, the degradation proportion of petroleum hydrocarbons also increased rapidly, and the OD_{600} in the fermentation broth increased gradually. During the growth period, a high degradation activity was observed. When the culture time was 18 days, the degradation proportion of petroleum hydrocarbons reached 58.8%, and the OD_{600} value was 1.15. Afterward, the increase in the degradation proportion of petroleum hydrocarbons slowed down and became stable, possibly because the strain had reached the stable growth phase and gradually began to transition to the decay phase, resulting in a decrease in its overall degradation activity. When the fermentation time reached 21 days, the degradation proportion of petroleum hydrocarbons did not increase significantly. Therefore, the optimal culture time of the MX1 strain is 18 days.

Culture temperature affected the degradation capacity of MX1. The 2% bacterial suspension was inoculated into the medium of the experimental group, and the pH value was controlled at 7.0, cultured at 20, 30, 40, and 50 °C, and shaken at 180 rpm for 21 days. Temperature affects the chemical properties of petroleum hydrocarbons and the physiological properties of microorganisms, thus indirectly affecting the ability of microorganisms to degrade petroleum hydrocarbons. As shown in Figure 3b, the degradation proportion of petroleum hydrocarbons first increased and then decreased gradually with the increase of temperature, and the maximum degradation proportion was 65.19% when the culture temperature was 30 °C. Therefore, the optimum growth temperature of the MX1 strain was 30 °C. This finding was obtained possibly because a very high or very low temperature will reduce the enzyme activity, and a very high temperature will enhance the volatility of light hydrocarbons, resulting in bias in the experimental results.

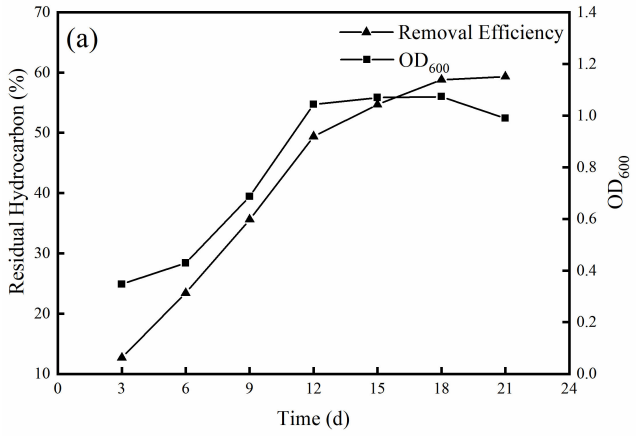


Figure 3. *Cont.*

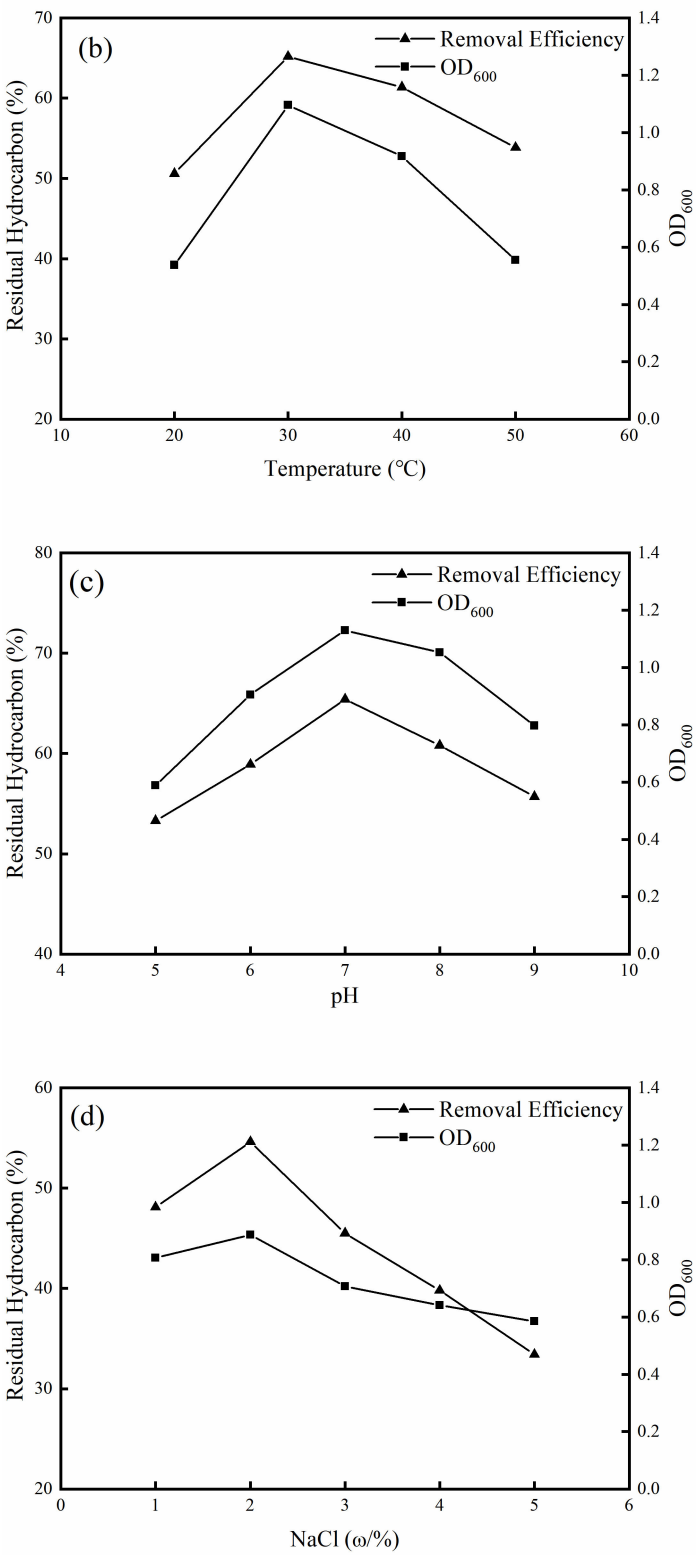


Figure 3. TPHs removal efficiency of the MX1 strain under different culture conditions. (**a**) Culture time; (**b**) temperature; (**c**) pH; (**d**) the concentration of NaCl.

The 2% bacterial suspension was inoculated into the medium of the experimental group, the pH values were controlled to 5.0, 6.0, 7.0, 8.0, and 9.0, and the bacteria were cultured at 30 °C and 180 rpm in a constant-temperature shaker for 21 days, as shown in Figure 3c. The pH value affects the absorption of nutrients by microorganisms, the adsorption of microorganisms, and the activity of metabolic enzymes, which are important

to the biodegradation of petroleum hydrocarbons. With the increase in pH, the degradation of petroleum hydrocarbon first increased and then decreased. The degradation proportion and OD_{600} of the strain reached the maximum at the same time when the environmental pH was 7.0, and the degradation proportion of petroleum hydrocarbon was 65.4%. Therefore, the optimum pH value of the MX1 strain for degrading petroleum hydrocarbons was 7.0.

The 2% bacterial suspension was inoculated into the medium of the test group, 1%, 2%, 3%, 4%, and 5% NaCl were added to the medium, the pH value was controlled to 7.0, and the temperature was kept at 30 °C and 180 rpm. Shakers were cultured for 21 days, as shown in Figure 3d. With the continuous increase in NaCl concentration, the degradation of petroleum hydrocarbons first increased slightly, and then gradually decreased. Experiments showed that the optimum NaCl mass concentration for the degradation of MX1 was 2%. At this time, the degradation proportion of petroleum hydrocarbons was 54.6%, which may be related to the salinity in the environment of the domesticated strain. In addition, when the mass concentration of NaCl was 5%, the degradation proportion of petroleum hydrocarbons decreased to 33.4%, which indicated that a higher NaCl concentration had an inhibitory effect on the degradation of petroleum hydrocarbons, possibly because higher ion concentration would hinder the degradation of petroleum hydrocarbons. Mass transfer of petroleum hydrocarbons occurred between solution and cells.

3.4. Microwave-UV Mutagenesis of Petroleum Hydrocarbon Degrading Bacteria

Considering the strong bactericidal ability of ultraviolet light, long mutagenesis time may lead to the complete inactivation of the strain, but short mutagenesis time will decrease the lethality, thus affecting the positive mutation rate of the strain. Therefore, ultraviolet mutagenesis strains must be controlled within a certain time frame. To ensure that the bacteria can mutate to the greatest extent, the UV lethality is usually controlled between 70% and 90% in the process of mutagenesis breeding.

Figure 4a shows the lethality of the bacterial suspension of the MX1 strain irradiated by ultraviolet rays at different times. The lethality of the strains increased continuously with the duration of UV irradiation. When the irradiation time was 3 min, the lethality rate was 84.09%. Therefore, 3 min was selected as the ultraviolet mutagenesis treatment time of the MX1strain. The lethality of the MX1 strain after microwave-UV irradiation is shown in Figure 4b. After irradiating the strain with microwave for 50 s, the lethality rate also increased with the increase of ultraviolet irradiation time. When the irradiation time was 4 min, the lethality rate was 84.38%. Accordingly, the UV mutagenesis treatment time of the strain was set to 4 min. After 3 min of UV irradiation, with the continuous increase of microwave irradiation time, the lethality of the strain also increased continuously. When the irradiation time was 30 s, the lethality rate was 87.5%, and all the bacteria were killed by continuing microwave irradiation. Therefore, 30 s was selected as the microwave mutagenesis treatment time of the MX1 strain.

3.5. Screening of Mutant Strains by UV and Microwave

Fourteen colonies with strong growth after UV mutagenesis were selected from the plate for transfer, and four strains were obtained after continuous transfer for three times. These samples were subjected to shake flask experiments, and the content of petroleum hydrocarbons in the fermentation broth was determined. The results are shown in Figure 5a. The effect of the MXU2 strain in degrading petroleum hydrocarbons was significantly better than that of the other three strains, and the degradation proportion of petroleum hydrocarbons in 7 days was 45.18%, which was 28.24% higher than that of the starting strain. The mechanism of UV mutagenesis of strains is mainly caused by the production of pyrimidine dimers, 6-4 photoproducts, and Dewar covalent isomers after UV rays act on bacterial DNA, resulting in the production of a large number of mutant strains.

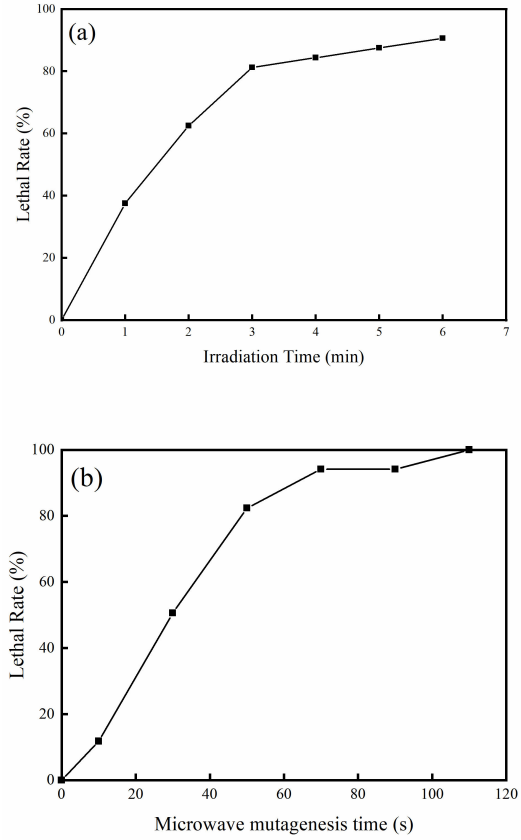


Figure 4. Lethal rate curve for UV (**a**) and microwave (**b**) mutagenesis.

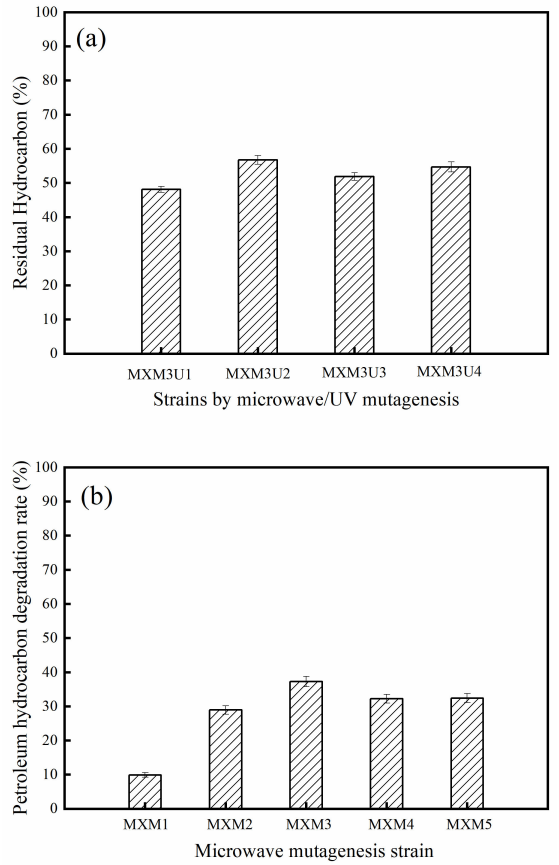


Figure 5. TPHs removal efficiency of strains by UV (**a**) and microwave (**b**) mutagenesis.

As shown in Figure 5b, eight colonies with vigorous growth after microwave-UV mutagenesis were selected from the plate for transfer, and five strains were obtained after three replicates of continuous transfer. The samples were subjected to shake flask experiments, and petroleum hydrocarbon degradation proportion was measured. Based on the comparison of Figure 5a,b, the average degradation proportion of the former to petroleum hydrocarbons was 11.78%, higher than that of the latter. The microwave-UV mutagenized strains had a better degradation effect on petroleum hydrocarbons, and the highest degradation proportion of petroleum hydrocarbon by d was 56.72%, which was 51.66% higher than that of the starting strain. Therefore, the mutant strain MXM3U2 was selected for further study. The mutant strain MXM3U2 was enriched and cultured for five consecutive passages, and the degradation proportion of petroleum hydrocarbons by each generation strain was determined by shaking flask fermentation. The results are shown in Figure 6. No significant difference was observed in the degradation proportion of petroleum hydrocarbons between different generations of the MXM3U2strain, indicating that the mutant MXM3U2 strain had good genetic stability to the degradation of petroleum hydrocarbons.

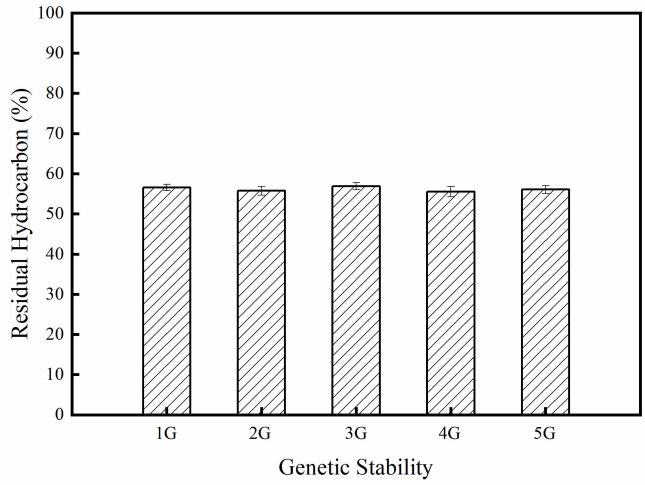


Figure 6. TPH removal efficiency by analysis of strain genetic stability.

3.6. Degradation of Alkanes by Mutant Strain MXM3U2

Figure 7 shows the growth and degradation of the MXM3U2 strain when C16, C24, C32, and C40 were the only carbon sources. The figure shows that the CFU of the MXM3U2 strain initially showed slowly increasing trend, followed by a gradually decreasing trend with the increase of culture time, and the CFU reached the maximum after 12–18 days of culture time. At the same time, the residual petroleum hydrocarbon content in the fermentation broth decreased with the increase of culture time. In addition, the CFU of the strain and the ability of the strain to degrade alkane decreased gradually with the increase of carbon number. The strains degraded alkanes significantly from day 7 to day 14, and the degradation proportions of C16, C24, C32, and C40 were 78.52%, 54.17%, 42.55%, and 28.69%, respectively, after 21 days of culture. This finding is consistent with the utilization of saturated linear alkanes by most petroleum hydrocarbon-degrading bacteria, that is, linear alkanes with high carbon numbers are more difficult to utilize than those with low carbon numbers.

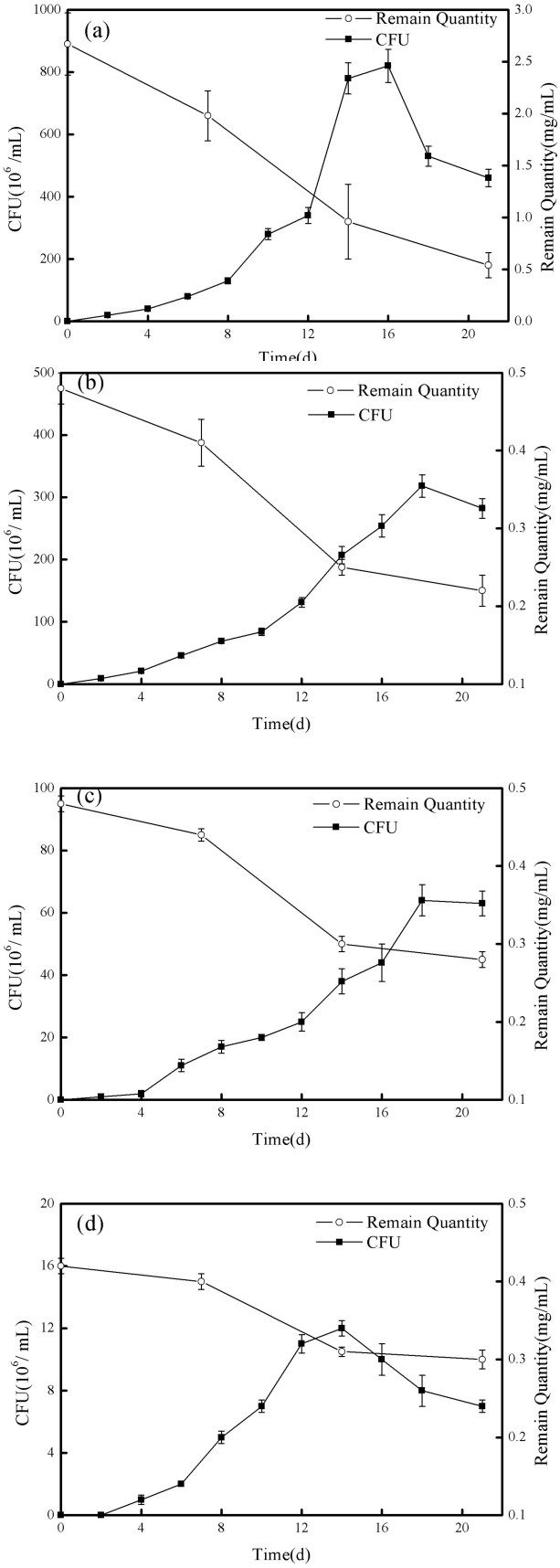
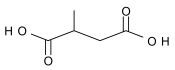
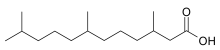
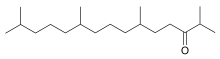
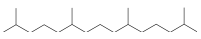


Figure 7. Growth of the strain on different n-alkanes as the sole carbon sources and its degradation of the alkanes (**a**–**d**) represented the case of n-alkanes C16, C24, C32, and C40, respectively.

3.7. Degradation Pathway of Pristane, Naphthalene, and Phenanthrene by Mutant Strains

Pristane is a branched alkane composed of 19 carbons, and its special structure makes its biodegradation difficult. Therefore, pristane is often used as an internal standard for the study of alkane degradation. However, a few strains such as *Corynebacterium* sp. can degrade pristane as the sole carbon source. At present, the analysis of the biodegradation characteristics of pristane mainly focuses on the single-end and double-end oxidation, while the degradation mechanism is rarely studied. As shown in Table 2, the degradation products of pristane are 2,6,10,14-tetramethyl-3-one, 3,7,11-trimethyldodecanoic acid, and 2-methylbutane diacid. Therefore, in the degradation pathway of pristane by the MXM3U2 strain, it is first oxidized to alcohol under the action of subterminal oxidase, then further oxidized to ketone, and oxidized to 3, 7, 11-trimethyldodecanoic acid; finally, yt undergoes single-end oxidation to 2-methylsuccinic acid (Figure S5).

Table 2. Mass spectrometric data of compounds isolated during growth on pristane by strain MXM3U2.

Serial Number	*t*/min	Degradation Products	m/e (%)
1	11.2		129 (18.12), 128 (13.43), 101 (12.13), 100 (11.57), 87 (8.19), 69 (12.92), 59 (100.00), 41 (21.08), 39 (10.87) 129 (29.35), 100 (16.58), 59 (100.00), 58 (60.96), 42 (12.87), 41 (21.93)
2	22.6		169 (11.54), 141 (7.09), 129 (9.33), 113 (12.74), 99 (72.95), 88 (100.00), 71 (33.18), 59 (35.37), 43 (41.56) 169, 141, 129, 113, 99, 88, 71, 59, 43 standard from the library NIST
3	25.7		238 (11.35), 226 (7.16), 212 (12.84), 197 (6.39), 185 (24.74), 167 (26.26), 155 (12.54), 125 (4.78), 113 (12.25), 85 (34.73), 71 (11.25), 69 (20.04), 58 (35.79), 43 (100.00) 238, 226, 212, 197, 185, 167, 155, 125, 113, 85, 71, 69, 59, 43 standard from the library NIST
4	25.4		113 (12.43), 99 (10.77), 85 (28.27), 71 (77.91), 69 (11.25), 57 (100.00) 56 (20.62), 55 (20.65), 43 (65.33), 41 (30.34)

Considering that branched-chain hydrocarbons are difficult to be degraded by enzymes in the β-oxidation pathway, few microorganisms have been found in nature that can degrade branched-chain alkanes. The aerobic degradation of isoprenoid alkanes in the natural environment must meet three conditions: 1. Alkane monooxygenases or genes must be present; 2. these β-oxidases must also be able to interact with branched chain fatty acids; and 3. abnormal β-oxidation end products must be able to be integrated into the intermediate metabolic process. Bacteria with the three abovementioned characteristics can efficiently degrade branched-chain alkanes. These bacteria can degrade various organic substances because their β-oxidation pathway can serve as a metabolic pathway, as well as a source of fatty acids to produce a large amount of lipids with complex functions.

The degradation products of naphthalene were analyzed by GC/MS (Figure S3), and four metabolites of naphthalene were found. The peak at m/z = 109.1 was identified as catechol, the peak at m/z = 127.0 was identified as naphthalene, and the peak at m/z = 127.0 was identified as naphthalene. The peak at z = 137.0 was identified as salicylic acid, and the peak at m/z = 159.2 was identified as 1,2-dihydroxynaphthalene. Based on the analysis of the above degradation products, in the pathway of naphthalene degradation by the MXM3U2 strain, naphthalene is first converted into 1,2-dihydroxynaphthalene by dioxygenase and dehydrogenase, then into salicylic acid. Hydroxylase is converted to catechol and finally enters the tricarboxylic acid cycle until complete degradation (Figure S6).

As shown in Figure S4, the GC/MS results showed three metabolites of phenanthrene, in which the peak at m/z = 138.1 was identified as salicylic acid, the peak at m/z = 160.2 was identified as 1,2-dihydroxynaphthalene, and the peaks with m/z = 210.1 were identified as 1,2-dihydroxyphenanthrene or 3,4-dihydroxyphenanthrene. The abovementioned degradation products indicate that in the pathway of phenanthrene degradation by the MXM3U2 strain, phenanthrene is first hydroxylated to 1,2-dihydroxyphenanthrene or 3,4-

dihydroxyphenanthrene by dioxygenase, and then dehydrogenase and dioxyphenanthrene. It is converted into 1-hydroxy-2-naphthoic acid or 2-hydroxynaphthoic acid under the action of enzymes, isomerase, and aldolase, then enters the tricarboxylic acid cycle through the salicylic acid pathway, and finally degrades completely (Figure S7).

4. Conclusions

In the present study, a high-efficiency petroleum hydrocarbon-degrading strain was screened by microwave-ultraviolet composite mutagenesis technology [39], and the characteristics of the strain for degrading petroleum were investigated. The refractory PAHs in petroleum were degraded, thus providing a theoretical basis for petroleum degradation. The MX1 strain was identified, and its optimum growth conditions were determined. The results of physiological and biochemical tests and 16S rDNA identification showed that the MX1 strain belonged to *Enterobacter* sp. The single-factor test results showed that the optimal growth conditions of the MX1 strain were temperature of 30 °C, environmental pH of 7, and salinity of 2%. The degradation proportion of crude oil by the MX1 strain reached 61.08% when cultured under optimum conditions for 21 days. The mutant strain MXM3U2 was obtained by microwave mutagenesis for 30 s and then UV mutagenesis for 4 min. The degradation test of n-alkanes (C16, C24, C32, and C40) by mutant strains showed that with the increase of carbon number, the number of strains and the degradation proportion gradually decreased, and the alkanes with lower carbon chains were easy to degrade. GC/MS results showed that the carbon numbers of crude oil are complementary, that is, hydrocarbons with carbon number C < 24 are degraded first, and then hydrocarbons with carbon number C > 24 are degraded. The strains can degrade pristane, naphthalene, and phenanthrene with good degradation effects. The high-efficiency petroleum hydrocarbon-degrading bacteria screened in this study have a good prospect in the field of treating petroleum pollutants and degrading petroleum in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14162544/s1, Figure S1: The standard curve for detecting the concentration of crude oil; Figure S2: The morphology of strain MX1 under LB flat microscope; Figure S3: GC/MS data of the metabolite of naphthalene during the degradation by strain MXM3U2; Figure S4: GC/MS data of the metabolite of phenanthrene during the degradation by strain MXM3U2; Figure S5: Proposed pathway for the degradation of pristane by strain MXM3U2; Figure S6: Proposed pathway for the degradation of naphthalene by strain MXM3U2; Figure S7: Proposed pathway for the degradation of phenanthrene by strain MXM3U2.

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