

Microbial enhanced heavy oil recovery through biodegradation using fungal extracellular enzymes from *Aspergillus* spp.

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Research

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Abstract

Background: The progressive depletion of light crude oils has led to increased focus on efficient exploitation of heavy oil reserves to meet energy demand. Microbial enhanced oil recovery makes a substantial contribution to the recovery of heavy oils; however, most methods use bacteria, with less attention paid to the potential of fungi. In this study, we investigated the efficiency of fungal extracellular enzymes in biotransformation and biodegradation of heavy oil fractions into light compounds and the feasibility of the use of such enzyme preparations in enhanced oil recovery.

Results: Two fungal strains of *Aspergillus* spp., isolated from bitumen samples, showed good growth on plates of mineral salts medium with heavy oil as the sole carbon source. The fungal extracellular enzymes, with dehydrogenase and catechol 2,3-dioxygenase activities, exhibited the ability to biodegrade heavy oil. The biodegradation process was coupled with abundant production of gases, mainly CO₂ and H₂. Gas chromatography analysis revealed a significant redistribution of n-alkanes in the heavy oil after treatment with fungal enzyme preparations, which resulted in an increase in individual n-alkanes. The viscosity of the heavy oil was decreased 66.33% by fungal enzymatic degradation.

Conclusions: These results demonstrate the potential of fungal extracellular enzymes from *Aspergillus* spp. for applications in enhanced heavy oil recovery, including biotransformation of heavy to lighter crude oil and byproduct biogas formation.

Background

Crude oil is an important non-renewable natural resource to support economic and social development; it is the lifeblood of industry and the key source of energy. It has been estimated that fossil fuels will account for approximately 80–90% of global energy requirements in the next 20 years [1]. With the depletion of light crude oil and increasing global energy demand, the use of heavy crude oil is interesting for the economics of petroleum development. According to some estimates from the International Energy Agency, heavy oil represents at least half of the recoverable oil resources of the world [2]. Heavy crude oil is highly viscous and closely related to natural bitumen. It has density <20° API gravity (American Petroleum Institute) and viscosity >200 mPa·s in reservoir conditions [3]. The production, transportation, and refining of heavy oil presents operational difficulties and is costly compared to light crude oil because of the high viscosity and complex oil composition. Conventional heavy oil recovery technologies, such as thermal extraction techniques including steam flooding and *in situ* combustion, and cold production methods including alkaline drive, polymer injection, and miscible displacement processes, face great challenges in terms of complex operation and environmental costs [3, 4]. Therefore, significant and less costly recovery techniques are required for exploration and production of heavy oil.

Microbial enhanced oil recovery (MEOR) has been successfully applied in crude oil recovery [5, 6]. It is a cost-effective and environmentally-friendly approach with great potential and broad prospects compared to conventional petroleum extraction technologies [7]. MEOR is a biologically-based technology that uses

microorganisms or their metabolites to increase recovery of residual oil; it was first proposed in the 1920s and was developed in the early 1980s [8, 9]. The main mechanisms of MEOR are [10]: (1) degradation of high-molecular-weight hydrocarbons to improve the liquidity of residual oil; (2) production of chemicals, such as biosurfactants, acids, solvents, and biopolymers that increase the oil sweep efficiency through altering oil/water/rock interfacial properties; and (3) generation of gases (CO₂, N₂, H₂, and CH₄) that can dissolve in crude oil to reduce the oil viscosity and increase the reservoir pressure. MEOR technology has been actively pursued both in laboratory and field conditions. In 2010, various countries allocated one-third of their oil recovery programs to MEOR, and up to 50% of trapped oil can be extracted after the primary and secondary phases of production using this tertiary recovery technology [6].

The MEOR technology involving biotransformation and/or biodegradation of crude oil is a biocatalytic process for transformation of heavier hydrocarbons to lighter ones by various enzymes, such as oxygenases, hydroxylases, dehydrogenases, cytochrome P450 monooxygenases, and dioxygenases [11]. Due to their versatility, bacteria play a major role in biodegradation of petroleum hydrocarbons. However, fungi have been shown to have stronger hydrocarbon-metabolizing abilities than bacteria [12]. Additionally, fungal extracellular enzymes, such as oxidases and hydrolases, may degrade long-chain petroleum hydrocarbons and complex polycyclic aromatic hydrocarbons [13, 14]. Recent laboratory studies have shown that fungal extracellular enzymes can be used to degrade paraffin, aromatic, and resin fractions of crude oil; the enzymatic degradation is associated with dynamic production of gases and organic acids, thereby reducing oil viscosity [15, 16].

The use of enzymes to enhance oil recovery is one of the novel methods under consideration in recent research [17]. Nemati and Voordouw [18] have suggested that enzymes can be used to modify reservoir rock permeability by aiding in plugging using the products generated in catalyzed reactions. In China's Shengli oilfield, an enzyme-based reservoir blockage removing agent composed of proteinases and bacteria recovered dramatically the permeability of cores polluted by sandy crude oil and extended the validity of the production wells; this agent was applicable to releasing formation damage, removing wax, acidizing fluid, controlling sand, and water shutoff [19]. Enzymes have several advantages over whole-cell catalysts, such as robustness, ease of modulation, and multiple usage strategies. Thus, enzymatic cracking is probably the most interesting target of biotechnology for enhanced heavy oil recovery [20].

In this study, we used extracellular enzymes of fungi (*Aspergillus* spp.) to treat heavy crude oil. The aim of this study was to investigate the capacity of fungal enzymes for heavy oil degradation; to determine the effects of enzymatic degradation on oil physicochemical properties; and to demonstrate the potential of fungal enzymes for biotransformation and biodegradation of heavy oil. This study proposed a technically feasible and desirable procedure for transforming heavy oil into lighter components. The application of fungal extracellular enzymes in MEOR could open a window for improving heavy oil recovery.

Results And Discussion

Screening and identification of oil-degrading fungi

Four pure fungal strains with the ability to degrade heavy oil, designated HJ1 to HJ4, were isolated from bitumen enrichment cultures. Strains HJ2 and HJ4 exhibited good growth on oil plates of mineral salts medium (Fig. 1). Thus, we selected these two strains as potential candidates for analyses of heavy oil biodegradation, extracellular enzyme production, and enzymatic degradation of heavy oil. Based on morphological analysis (Fig. 2) and partial sequencing of the rDNA internal transcribed spacer (ITS) region (Fig. 3), strains HJ2 and HJ4 were identified as *Aspergillus terreus* and *A. nidulans*, respectively. The rDNA-ITS gene sequences of the two strains were deposited in the GenBank database with accession numbers MG 732935 and MG 732936. Several fungi of the genus *Aspergillus* isolated from petroleum-contaminate dtropical soils are known to be able to degrade petroleum hydrocarbons. They can use petroleum hydrocarbons as their sole carbon and energy source to produce simpler compounds such as CO₂ and H₂O [21].

Fungal degradation of heavy oil

The ability of *A. terreus* HJ2 and *A. nidulans* HJ4 to degrade heavy oil was initially tested on oil and bitumen agar plates of mineral salts medium (containing 20 g L⁻¹ heavy oil or bitumen; Fig. 1). The biodegradation of heavy oil was verified by observation of a decrease in the total weight of heavy oil and a redistribution of four fractions (alkans, aromatics, resins, and asphaltenes). After 15 d of fungal degradation, there was a significant difference in the total weight of heavy oil between the control group and fungi-treated samples ($P < 0.05$). The degradation efficiencies of heavy oil by strains HJ2 and HJ4 were similar, 25.29% and 27.36%, respectively. Among the four fractions, alkanes were decreased by 36.19–39.08%, while resins were increased by 16.00–18.00% ($P < 0.05$; Table 1). Gas chromatography (GC) provided a more detailed assessment of the heavy oil degradation and characterized the variation of saturates before and after fungal degradation. Both strains HJ2 and HJ4 were able to degrade the heavy oil efficiently; 33.33–66.67% of the *n*-alkanes were eliminated when compared to the control group. The degradation efficiencies of *n*-alkanes with different chain lengths were determined from the decreases in the peak areas of individual *n*-alkanes. Strain HJ4 exhibited a higher degradation capacity for *n*-alkanes and particularly showed higher degradation efficiencies of long-chain *n*-alkanes (retention time >37.00 min) compared to HJ2 (Table 2).

Generally, mesophilic microorganisms readily degrade alkanes with carbon chain lengths ranging from 10 to 16 to generate energy for their growth; however, polycyclic aromatic hydrocarbons, which are less bioavailable than alkanes, are not completely degraded because they are more effectively partitioned into heavier fractions (e.g., resins and asphaltenes) [22, 23]. Here, we found that *A. terreus* HJ2 and *A. nidulans* HJ4 had the ability to degrade long alkyl chains of heavy oil in aerobic conditions. Microbial degradation of heavy oil is a complex process involving various enzymes such as oxygenases, dehydrogenases, and hydroxylases; the enzymes can catalyze biochemical transformations including aliphatic and aromatic hydroxidation, oxidative deamination, and hydrolysis of substances in heavy oil [12]. A test of *in-vitro* oil degradation by crude enzymes demonstrated an accelerated biodegradation of

aliphatic, aromatic, resin, and asphaltene fractions due to enhanced enzyme activities in the co-culture [22]. Thus, we speculated that our *Aspergillus* isolates HJ2 and HJ4 produce multiple enzymes that can be directly used to degrade heavy oil.

Enzymatic degradation of heavy oil

Enzymatic hydrocarbon degradation

When grown on the enzyme-producing medium, both strains HJ2 and HJ4 produced extracellular enzymes with dehydrogenase and catechol 2,3-dioxygenase (C23O) activities. The enzyme mixtures of strains HJ2 and HJ4 were designated E2 and E4, respectively. Their average dehydrogenase activities were 66.76 ± 0.25 (E2) and 68.77 ± 0.18 (E4) $\mu\text{g g}^{-1} \text{min}^{-1}$; the average C23O activities were 3.65 ± 0.014 (E2) and 4.32 ± 0.028 (E4) U mg^{-1} . Dehydrogenase and C23O activities reflect the ability of microorganisms to degrade alkanes and aromatic hydrocarbons. The activities of the two fungal enzyme preparations suggested that they could be used directly for hydrocarbon degradation.

The chemical inertness of hydrocarbons poses an energetic and mechanistic challenge for microbial metabolism. This is particularly true for the initial activation and eventual cleavage of the apolar C–H bond, where high energy barriers must be overcome, and, therefore, the rate of conventional biodegradation of hydrocarbons is relatively slow [24]. In the present study, the enzyme preparations E2 and E4 were capable of degrading hydrocarbons and releasing biogas. The cumulative gas production after 15 d of enzymatic degradation with 2 g of hydrocarbons as the substrate is shown in Fig. 4. The maximum gas productions from bituminous crude oil (15% w/w), heavy oil, light oil, wax, and liquid paraffin were 40.0–67.5, 64.0–80.5, 75.0–80.0, 81.0–109.5, and 73.0–76.0 mL bottle^{-1} , respectively, for E2 and E4. E4 gave a higher gas production than E2 when applied in the same quantities; this implied the greater ability of E4 to degrade petroleum hydrocarbons compared to E2. Both enzyme preparations showed higher efficiency in biodegrading wax and light oil than bituminous crude oil. According to Al-Sayegh et al. [25], due to being rich in resins and asphaltenes, bituminous crude oil is relatively resistant to biodegradation and more slowly degraded than linear oils. The aliphatic fraction, which is more susceptible to degradation than the aromatic and asphaltene fractions, was probably more easily broken down by the preparations E2 and E4. Based on the gas production results, we inferred that the enzyme preparations E2 and E4 can be used to enhance oil mobilization by degrading heavy oil and producing bioproducts.

Effect of enzyme concentration on heavy oil degradation

Evaluation of heavy oil degradation by different concentrations of crude enzymes in oxygen-deprived conditions showed positive results (Table 3). The four fractions of heavy oil were redistributed after 15 d of incubation at 40 °C. As the crude enzyme concentration increased from 2% to 10%, the content of alkanes markedly decreased, with the percentages of degradation in the range of 16.28–27.61%. The content of aromatics and resins first increased and then decreased, both of which remained higher than the values of the control group, by 30.85–64.89% and 2.45–28.82%, respectively. By contrast, the content

of asphaltenes decreased slightly by 2.97–13.86% compared to the values of the control group. The observed trends in the four fractions of heavy oil indicate that enzyme concentration is a critical factor in determining the oil degradation efficiency.

The degradation efficiency of different heavy oil fractions by bacterial consortia was reported to be 10.57–23.68% for alkanes, 6.03–20.62% for aromatics, and 3.63–16.90% for resins and asphaltenes [10, 26, 27]. By comparison, the fungal enzyme preparations from our *Aspergillus* cultures had higher abilities to degrade saturates, while the aromatic and resin fractions of heavy oil were relatively increased after enzymatic degradation. According to Mohamed et al. [28], the primary consumption or depletion of the saturated fraction can result in high content of polyaromatics and asphaltenes, which are more recalcitrant to biodegradation. In MEOR, it is important to select appropriate strains that can survive and secrete specific metabolites under the reservoir conditions, yet it remains difficult to isolate functional microorganisms that can tolerate the harsh environment of oil reservoirs. Therefore, the fungal enzyme preparations, which exhibit unique biodegradation capabilities and require no use of live microorganisms, are a good choice for MEOR.

The process of heavy oil degradation by the two enzyme preparations at different concentration was associated with dynamic production of gas. The total gas production varied from 25.5 to 100.0 mL bottle⁻¹ for preparation E2 and 24.0 to 186.5 mL bottle⁻¹ for E4 (Table 3). GC analysis showed that these gases were mainly CO₂ and H₂ with small amounts of CH₄. Production of gas was significantly correlated with the concentration of enzyme preparation added ($P < 0.05$); the respective correlation coefficients were 0.93984 (E2) and 0.99569 (E4). The result indicates that when applied within the range 0–10%, a higher concentration of the enzyme preparation would give a higher production of gas from heavy oil. The gas produced by enzymatic degradation can contribute to enhanced heavy oil recovery by increasing the pressure of the reservoir and reducing the viscosity of the oil.

Effect of surfactant and biosurfactant on heavy oil degradation

Surfactants are amphiphilic compounds which partition at the interface between fluid phases with various polarities. Surfactants decrease interfacial and superficial tensions of solutions and facilitate the bioavailability of hydrophobic chemicals by solubilization and emulsification [29]. In the present study, we evaluated the effects of surfactants of both biological and chemical origin on the enzymatic degradation of heavy oil by crude enzymes (Table 4). In the control group (without crude enzyme solution), gravitational analysis of 2 g heavy oil showed a content of: saturates, 1567 mg; aromatics, 274 mg; resin, 157 mg; and asphaltene, 101 mg. After 15 d of enzymatic degradation, the content of alkanes was substantially decreased to 1327–1353 mg (E2 with or without bacterial biosurfactant and sodium dodecyl sulfate [SDS]) and 1272–1371 mg (E4 with or without biosurfactant and SDS); the corresponding content of aromatics was considerably increased to 330–417 mg and 371–440 mg; the content of asphaltenes was slightly decreased to 95–97 mg and 93–97 mg; and the content of resins was increased to 181–202 mg by the crude enzymes but decreased to 147–104 mg if (bio)surfactants were also added. For crude enzyme preparations E2 and E4, the biogas yield was reduced from 67.0 to

50.0 mL bottle⁻¹ and 86.5 to 52.0 mL bottle⁻¹, respectively, on addition of the (bio)surfactants. The results show that addition of surfactant and biosurfactant had an inhibitory effect on the enzymatic degradation of heavy oil. Many surfactants reportedly have antimicrobial properties or inhibitory effect on key enzymes, depending on the surfactant concentration and the microbial strain used for biodegradation [30, 31]. Specifically, the surfactants exhibit toxicity to microorganisms and enzymes at above the critical micelle concentration, which changes cell surface properties, alters native enzyme protein conformation, and influences metabolic pathways, resulting in a decrease in the biodegradation of crude oil [31–33].

Enzymatic degradation of gasifiable *n*-alkanes

Enzymatic degradation had a significant effect on the relative quantities of gasifiable *n*-alkanes compared to the control group. More detailed assessment of the variation of *n*-alkanes can be found in Table 2. The two enzyme preparations caused apparent increases in the total number of gasifiable *n*-alkanes; 11–13 new fractions appeared, while three fractions disappeared relative to the control group. Correspondingly, the overall total peak area of gasifiable *n*-alkanes was increased by 91.77% (E2) and 130.73% (E4). Both enzyme preparations showed higher degradation efficiency for long-chain alkanes. The increase in the peak area of individual *n*-alkanes ranged from 11.46 to 198.19% (E2) and 15.16 to 537.41% (E4). This variation of oil components, especially the decrease of long-chain alkanes, has positive effects on the physicochemical properties of the heavy oil, such as light component increase, viscosity reduction, and fluidity enhancement.

Degradation of heavy fractions is considered one of the main mechanisms of MEOR by which the oil's viscosity and freezing point are reduced, which in turn will increase the oil's mobility *in situ* [34]. Numerous functional microbes, such as members of *Petrobacter*, *Enterobacter*, *Bacillus*, and *Geobacillus*, can produce highly degradative enzymes, resulting in the breakdown of hydrocarbon chains, making them lighter and enhancing oil mobility [35, 36]. In the present study, the two fungal enzyme preparations from *Aspergillus* spp. showed strong abilities to degrade heavy oil into lighter fractions in oxygen-deprived conditions. Although the exact mechanism is still unknown, the fungal enzymes might specifically shorten long chain hydrocarbons, depolymerize asphaltenes, and increase the solvent fraction, which would facilitate heavy oil recovery.

Viscosity reduction of heavy oil

High viscosity is a crucial factor responsible for poor heavy oil recovery [37]. Conventional methods for reducing oil viscosity include heating, emulsification, and dilution with light crude oil, which have the disadvantages of high cost in application and potential damage to the formation [10]. Therefore, we examined the potential use of fungal enzyme preparations to reduce oil viscosity in the present study. The two fungal enzyme preparations with a final concentration of 4% (w/v) were able to reduce the viscosity of heavy oil from 29,700 to 13,700 (E2) and 10,000 (E4) mPa s. This result showed that E4 had greater viscosity reduction efficiency (66.33%) than E2 (53.87%). Other studies have shown that some bacterial strains (e.g., *Bacillus subtilis*, *B. licheniformis*, and *Geobacillus stearothermophilus*) reduce

heavy oil viscosity by 15.47–40.06% under aerobic and aerobic conditions [26, 27]. Compared to these bacteria, the fungal enzyme preparations tested in the current study exhibited markedly higher abilities to reduce heavy oil viscosity. Moreover, enzyme preparations have the advantage of short degradation time and they do not require the use of live microorganisms, which is conducive to application in heavy oil reservoirs with poor growth of oxygen-consuming microorganisms *in situ*. The high contents of long-chain hydrocarbons with a complex structure, heteroatoms (e.g., O, N, S, and metals), and asphaltenes in heavy oil lead to its high viscosity [38]. Moreover, these adverse conditions usually have negative effects on biodegradation of the oil. However, the fungal enzyme preparations used in our study exhibited the ability to decompose long-chain alkanes and heavy fractions (e.g., asphaltenes), leading to a decrease of average molecular weight and reduction of heavy oil viscosity.

Removal of heavy crude oil from sand

Table 5 lists the results of heavy oil removal from artificially contaminated sand after treatment with crude enzyme solution. The removal efficiencies of the two enzyme preparations (E2 and E4) ranged from 7.85 to 8.48%, that is, 2.29–2.47-fold that of the control (3.43%). Fig. 5 shows the removal efficiency of heavy oil adsorbed on sand by the crude enzyme solution. The fungal isolates in our study showed lower ability to produce biosurfactants than some bacteria (e.g., *B. subtilis* and *P. aeruginosa*). The diameter of oil spreading from the crude enzyme solutions reached only 12.0–13.0 cm (the oil spreading experiment was carried out according to the method described by Zhang et al. [16]). Biosurfactants are emerging as a promising agent for enhancing oil recovery and have been successfully applied in oilfield exploitation [39, 40]. França et al. [41] showed that the cell-free fermented broth of *B. subtilis* ICA56 containing biosurfactant removed 85% of crude oil from contaminated sand, which highlighted the crucial role of biosurfactant for the cleaning process. Thus, the crude enzyme solutions with relatively low content of biosurfactants might be insufficient to mobilize a significant amount of entrapped oil.

The removal of heavy oil by the two crude enzyme solutions was possibly due to heavy component degradation and gas production. The interactions of gas with oil, as well as the biotransformation of heavy oil fractions to lighter fractions, are mechanisms responsible for increasing the mobility and recovery of heavy oil [35]. Xia et al. [10] reported that one enriched methanogenic consortium increased by 14.70% of the tertiary enhanced oil recovery by oil degradation and methane production in core flooding tests, and the gas production made the inner pressure of the microbial core holder increase from 0.2 to 20.45 MPa; the viscosity of the heavy oil was reduced by 72.45%. Biodegradation in oil reservoirs affects the quantity and quality of the crude oil; crude oil will become lighter and more valuable through degradation of heavy oil fractions [37]. Our results suggest that supplementation with biosurfactants after enzyme solution flooding may be a more effective strategy to improve heavy oil recovery compared to flooding with enzyme solution only.

Conclusions

Two pure cultures *A. terreus* and *A. nidulans*, which were isolated from bitumen samples, exhibited high heavy oil biodegradation capacity. Crude enzyme preparations from the two fungal strains showed effective heavy oil degradation. Both enzyme preparations efficiently degraded different oil fractions, showed their best degradation activity toward high-molecular-weight alkanes, and reduced oil viscosity. The biodegradation process was associated with concurrent production of gases, mainly CO₂ and H₂. The fungal enzyme preparations exhibited great potential in the cracking of heavy oil, which could improve the crude oil quality. Additionally, the enzyme preparations facilitated removal of heavy oil from sand by heavy component degradation and gas production. This study demonstrates the potential feasibility of using fungal enzyme preparations to improve crude oil quality and enhanced heavy oil recovery. The application of fungal enzymes in oil recovery is a novel, environmentally-friendly, low-cost strategy.

Methods

Media, heavy oil, and bitumen

Mineral salts medium (pH 7.0) contained (g L⁻¹): KNO₃, 5.0; MgSO₄·7H₂O, 0.5; and KH₂PO₄, 1.0. Liquid enrichment medium was prepared by supplementing 20% (w/v) potato juice with 2% (v/v) heavy oil obtained from Yanchang Oilfield, Shaanxi Province, China. Potato dextrose agar (PDA) consisted of 20% (w/v) potato juice, 1% (w/v) cane sugar, and 1% (w/v) agar. Solid enzyme-producing medium was formulated with mineral salts solution, wheat bran, and heavy oil (80:100:2, mL g⁻¹ g⁻¹). The wheat bran was sieved through a fine screen with a pore size of 0.5 mm before use. Additionally, liquid fermentation medium was prepared by supplementing a solution of mineral salts (100 mL) with 1% (w/v) brown sugar as previously reported [45]. Bitumen samples were obtained from BAOLIRUS International Investment Co., Ltd. (Yangling, Shaanxi, China).

Fungal isolation and identification

Fungi were isolated through the enrichment method of Cho and Oh [42]. Approximately 10 g of bitumen sample was transferred to a 250-mL saline bottle containing 100 mL of enrichment medium and incubated at 30°C without shaking for 7 d; 10 mL of the broth enrichment culture was then transferred to fresh enrichment medium and cultured in the same conditions for another 7 d. After the third subculture, the enrichment culture was serially diluted to 10⁻³ dilution; 50 µL of sample from each dilution was spread on PDA plates and incubated at 30°C in aerobic conditions. Morphologically different colonies were picked and purified by using the dilution separation method on fresh PDA plates. Fungal cultures were identified by morphological examination and gene sequencing [12]. Spore structure of fungal cultures was observed using an S-4800 field emission scanning electron microscope (Hitachi, Tokyo, Japan). For molecular identification, DNA from isolated cultures was extracted and the rDNA-ITS gene was amplified by PCR using primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Purified cultures were directly sequenced and assembled and then preliminarily compared to sequence data from the GenBank database of NCBI

(<http://www.ncbi.nlm.nih.gov>) using the BLAST service to determine their approximate phylogenetic affiliations. A phylogenetic tree was constructed in MEGA v6.0 using rDNA-ITS sequences of the fungal isolates and their close relatives in the GenBank database.

Fungal degradation test of heavy oil

The heavy oil biodegradation ability of fungal isolates was tested initially by evaluating the growth of fungi on oil and bitumen agar plates. Briefly, the pure cultures were transferred from PDA slants into PDA liquid medium and incubated at 30°C with shaking (100 rpm) for 5 d; then with mycelial pellets. Roughly the same amounts of mycelial pellets were taken from the surface of the liquid medium and inoculated onto oil and bitumen agar plates containing the mineral salts medium (per liter mineral salts solution, with 20 g of heavy oil and bitumen, respectively). The inoculated plates were incubated at 30°C for 5–7 d, and the growth of each strain was observed and photographed. Strains with good growth on the agar plates showed excellent heavy oil biodegradation ability, and were chosen for verification of heavy oil biodegradation.

To verify the biodegradation of heavy oil, 2.00 g of heavy crude oil was mixed with 150 mL of potato juice (20%, w/v) in a 600-mL tissue culture vessel. One loop of pure fungal culture was taken from the PDA slant and transferred into the potato juice-crude oil mixture; the culture was incubated at 30°C for 15 d in a static mode. Control tests used sterile potato juice and heavy oil without fungal inoculation. After incubation, the residual oil was extracted from the 150 mL of culture solution with 100 mL of *n*-hexane. Then, 1.5 mL of the *n*-hexane containing residual oil was analyzed by GC coupled with gravimetric analysis to evaluate the degradation efficiency [43]. The heavy oil after degradation efficiency measurement was re-dissolved in 100 mL of *n*-hexane and left 24 h for asphaltene precipitation. The precipitate was collected by centrifugation at 5,000× *g* for 5 min, oven-dried at 40°C for 24 h, and quantified gravimetrically. Fractions of the organic phase were further distinguished into saturates, aromatics, and resins by loading onto an Al₂O₃ column and eluting with several different developing solvents (*n*-hexane, *n*-hexane/dichloromethane [3:1, v/v], and absolute ethyl alcohol), followed by gravimetric analysis. The degradation efficiency for heavy oil and four fractions ($VR_m\%$) was calculated using the following formula:

[Please see the supplementary files section to view the equation.]

where M_{Ctrl} and M_t are the masses of heavy oil and four fractions (alkanes, aromatics, resins, and asphaltenes) in the control and fungal-treated samples, respectively .

Enzymatic degradation test of heavy oil

Enzymatic activity assay

Crude enzyme preparations of fungi were formulated as previously reported [16]. All fungal spores formed in one PDA slant were inoculated into 50 g of enzyme-producing medium with an initial moisture

content of 44% and incubated by a solid-state fermentation process for 5 d at 30°C. Subsequently, the medium containing extracellular enzymes and fungal cells was placed in a ventilated place to obtain 30% moisture content and then oven-dried at 40°C for 48 h. The dried material was ground into powder and passed through a 0.10-mm sieve to obtain powdered enzyme preparations. Crude enzyme solutions were prepared by following the steps below: 4.000 g of solid enzyme preparation was dissolved in 100 mL of distilled water in a 250-mL conical flask, oscillated at 30°C with shaking at 100 rpm for 15 h, and then filtered through sterile fiberglass.

The dehydrogenase and C230 activities of fungal enzyme preparations were assayed using a UV-1800 UV-vis spectrophotometer (MAPADA, Shanghai, China) based on increases in absorbance at specific wavelengths. Dehydrogenase activity was measured by a colorimetric method using 2,3,5-triphenyltetrazolium chloride [44]. C230 activity was assayed based on the procedure reported by Yanto and Tachibana [12]. All enzymatic activity assays were performed in a water bath at 37°C. The dehydrogenase activity was calculated according to a calibration curve prepared using triphenyl formazan and was expressed in terms of the amount of triphenyl formazan produced per gram of enzyme preparation per min. The C230 activity was expressed in units per milligram of enzyme preparation (U mg^{-1}), where one enzyme unit (U) was defined as the amount forming 1.0 μmol of 2-hydroxymuconic semialdehyde per min in the assay conditions.

Enzymatic hydrocarbon degradation test

The hydrocarbon substrates included bituminous crude oil (15%, w/w), heavy oil, light oil, wax, and liquid paraffin. The experiment was conducted in a 100-mL saline bottle with 30 mL of crude enzyme solution with concentration of 4% (w/v) and the average dehydrogenase activities of 66.76 (E2) and 68.77 (E4) $\mu\text{g g}^{-1} \text{min}^{-1}$. After 2 g of each hydrocarbon substrate was added respectively, the saline bottles were sealed with rubber stoppers. Incubation was carried out at 40°C for 15 d with shaking once per day throughout the experimental period. Enzymatic hydrocarbon degradation was evaluated by measuring gas production. The gas produced was captured using a 100-mL glass syringe. Because the pressure inside the bottle had increased with gas production, the syringe filled spontaneously. The total amount of gas produced was recorded.

Evaluation of the effect of enzyme concentration on heavy oil degradation

Different amounts (2.0, 4.0, 5.0, 6.0, 8.0, and 10 g) of solid enzyme preparations were respectively weighed into 250-mL conical flasks containing 100 mL of distilled water. The mixtures were oscillated at 120 rpm at 30°C for 15 h and then filtered through sterile fiberglass to obtain 2, 4, 5, 6, 8, and 10% enzyme solutions. Enzymatic degradation of heavy oil was performed in 100-mL saline bottles containing 2 g of heavy oil and 30 mL of crude enzyme solution at different concentrations. The bottles were sealed with rubber stoppers. The reaction solutions were incubated at 40°C for 15 d with shaking once per day throughout the experimental period. Controls were prepared by adding 30 mL of distilled water instead of crude enzyme solution. After enzymatic degradation, the amount of gas produced was recorded and the

gas components were identified by GC [16]. The residual oil was extracted with *n*-hexane and analyzed by Al₂O₃ column chromatography.

Evaluation of the effect of surfactant and biosurfactant on enzyme activity

Chemically pure SDS was from Xilong Scientific Co., Ltd. (Shantou, Guangdong, China). Biosurfactant was produced by a pure culture of *B. amyloliquefaciens* previously isolated from an oil-contaminated soil [45]. The *B. amyloliquefaciens* culture was incubated in a 600-mL tissue culture vessel containing 100 mL of fermentation medium at 30°C with shaking (100 rpm) for 7 d. The culture was centrifuged at 10,000× *g* for 10 min at 4°C to obtain the cell-free supernatant containing biosurfactant. To evaluate the effects of the surfactant and biosurfactant on heavy oil degradation, three treatments were performed in sealed 100-mL saline bottles containing 2 g of heavy oil plus 30 mL of reaction solution. The reaction solutions included: (i) 30 mL crude enzyme solution (4% w/v final concentration); (ii) 15 mL crude enzyme solution (8%, w/v) + 15 mL bacterial fermentation broth containing biosurfactant; or (iii) 15 mL crude enzyme solution (4% w/v final concentration) + 15 mL SDS (1 mg mL⁻¹). Control samples were prepared in the same way without the addition of crude enzyme solution. The bottles were incubated at 40°C for 15 d. The degradation of heavy oil was evaluated by measuring gas production coupled with residual oil fraction analysis.

Gas chromatographic analysis of heavy oil

Residual oil was extracted from reaction solutions with 100 mL *n*-hexane. *n*-Hexane extract (1.0 μL) was analyzed using a Thermo Finnigan Trace GC Ultra (San Francisco, CA, USA) equipped with a flame ionization detector and a DB-Wax capillary column (30 m × 0.25 mm × 0.25 μm). The carrier gas was nitrogen. The injector and detector temperatures were maintained at 240 and 280°C, respectively. The column was programmed with an initial temperature of 40°C, held for 2 min, then heated to 240°C at 6°C min⁻¹, then held for 8 min. *n*-Alkane degradation was evaluated by comparing the difference in the chromatographic peak areas of the hydrocarbons recovered after treatment with those of the hydrocarbons recovered from abiotic control samples at the same retention time. Relative variations ($VR_a\%$) in the chromatographic peak area of each *n*-alkane fraction were calculated as follows:

[Please see the supplementary files section to view the equation.]

where A_{Ctrl} is the chromatographic peak area of the individual *n*-alkane fraction from the control and A_t is the chromatographic peak area of the individual *n*-alkane fraction after treatment.

Viscosity measurement of heavy oil

Changes in the viscosity of heavy oil after enzymatic degradation were measured using an NDJ79 rotating viscometer (Yutong, Shanghai, China) at 50°C and atmospheric pressure (~0.1 MPa). Heavy oil viscosity was compared between enzymatically-degraded and control samples. The viscosity reduction rate ($VRR\%$) of heavy oil was calculated as follows:

[Please see the supplementary files section to view the equation.]

where V_{Ctrl} and V_t represent the viscosities of heavy oil recovered from the control and enzymatically-degraded samples, respectively.

Removal of heavy oil from sand

A custom-designed oil removal experiment using artificially contaminated sand was conducted to test the efficiency of the crude enzyme preparations in heavy oil recovery. The sand (0.25–0.50 mm fractions) was taken from the Weihe River in Yangling, China. The crude enzyme solution (150 mL, 4% w/v, average dehydrogenase activities of 66.76 (E2) and 68.77 (E4) $\mu\text{g g}^{-1} \text{min}^{-1}$) was added to a 600-mL tissue culture vessel containing 90 g of sand contaminated with 10% crude oil, and incubated statically in the dark at 40°C for 9 d. Control samples were prepared in the same way, with the addition of 150 mL distilled water. After incubation, the mixtures were filtered through sterile cotton wool to separate the sand and heavy oil. The heavy oil-covered sterile cotton wool was extracted with 150 mL of *n*-hexane, dried by vacuum-rotary evaporation at 40°C, cooled to ambient temperature, and then weighed (m). The heavy oil removal efficiency ($RE\%$) was calculated as follows:

[Please see the supplementary files section to view the equation.]

where M is the mass (g) of heavy oil removed from the artificially contaminated sand after treatment, and 10 (g) is the original mass of the heavy oil.

Statistical analysis

All experiments were performed in triplicate. Data are presented as mean \pm standard deviation ($n = 3$). Comparison of group mean values to obtain significant differences was conducted using Duncan's test. All statistical analyses were performed at $P < 0.05$ using one way analysis of variance (ANOVA) by the software SAS v9.2 (SAS Institute Inc., Cary, NC, USA).

Declarations

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Authors' contributions

JZ conducted the experiments, analyzed the data, and drafted the manuscript. QX and SH conceived the project, supervised the study, and reviewed the final manuscript. HG assisted in fungal isolation and identification experiments. HL collected oil-contaminated soils and heavy oil samples, and participated in the design and coordination of the study. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors consented on the publication of this work.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 The four fractions of heavy oil before and after fungal treatment

Sample	Heavy oil		Alkanes		Aromatics		Resins		Asphaltenes	
	(mg)	$VR_m\%$	(mg 2 g ⁻¹)	$VR_m\%$						
Ctrl	1977 ± 4 ^a	-	1589 ± 25 ^a	-	259 ± 11 ^a	-	150 ± 1 ^b	-	101 ± 2 ^a	-
HJ2	1477 ± 13 ^b	-25.29	1014 ± 17 ^b	-36.19	266 ± 4 ^a	2.70	174 ± 1 ^a	16.00	93 ± 4 ^a	-7.92
HJ4	1436 ± 22 ^b	-27.36	968 ± 27 ^b	-39.08	272 ± 4 ^a	5.02	177 ± 4 ^a	18.00	90 ± 4 ^a	-10.89

The data are presented as the mean ± standard deviation ($n = 3$). Different letters within the same column indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. $VR_m\%$ represents the degradation efficiency of heavy oil and four fractions. The same applies in the Tables below, unless otherwise specified.

Table 2 Relative variations in chromatographic peak area of individual gasifiable *n*-alkanes in heavy oil after degradation by *Aspergillus* spp. (HJ2 and HJ4) and their enzyme preparations (E2 and E4)

Retention time (min)	Ctrl			HJ2			HJ4			Retention time (min)	Ctrl			E2			E4		
	Peak area	Peak area	VR _a %	Peak area	Peak area	VR _a %	Peak area	Peak area	VR _a %		Peak area	Peak area	VR _a %	Peak area	Peak area	VR _a %	Peak area	Peak area	VR _a %
33.705	273782.2	87830.9	-67.9	132902.9	-51.5	21.953	0	53582.2	-	43534.5	-								
34.505	121444.3	103415.8	-14.9	77981.9	-35.8	23.804	42393.1	64895.8	53.1	50868.6	20.0								
34.725	66673.4	57271.0	-14.1	0	-100.0	25.651	53999.7	73266.6	35.7	62185.2	15.2								
35.15	242523.5	128486.8	-47.0	87237.1	-64.0	27.359	60772.0	83923.2	38.1	72783.1	19.8								
35.666	102722.8	186629.8	81.7	137882.6	34.2	29.035	69266.3	93490.0	35.0	85099.5	22.9								
35.932	119966.9	90799.5	-24.3	60473.8	-49.6	30.645	84026.5	131741.2	56.8	115991.3	38.0								
36.143	88483.7	67070.4	-24.2	57834.8	-34.6	32.209	98793.5	185020.4	87.3	175945.0	78.1								
36.659	247365.5	142455.3	-42.4	106912.7	-56.8	33.692	132998.9	372787.8	180.3	383790.2	188.6								
37.013	123071.4	63092.1	-48.7	42298.9	-65.6	34.472	56953.7	144010.8	152.9	184841.5	224.6								
37.544	77791.0	50395.3	-35.2	0	-100.0	34.736	42429.7	80588.0	89.9	99382.7	134.2								
38.099	284365.4	173945.7	-38.8	0	-100.0	35.139	168243.4	245320.7	45.8	282016.5	67.6								
38.16	0	55890.1	-	133879.2	-	35.465	70479.6	210165.8	198.2	323388.2	358.8								
38.422	187753.3	120771.3	-35.7	0	-100.0	36.137	48979.0	92257.7	88.4	312197.2	537.4								
38.847	128370.4	54638.4	-57.4	0	-100.0	36.648	183498.5	253092.2	37.9	286903.5	56.4								
39.502	107157.9	92600.1	-13.6	0	-100.0	36.919	41772.1	54143.1	29.6	70375.4	68.5								
40.553	165227.1	92845.0	-47.0	0	-100.0	37.003	54394.7	130283.5	139.5	172205.9	216.6								
41.007	82206.9	0	-100.0	0	-100.0	37.529	57113.2	91951.0	61.0	87954.1	54.0								
43.166	51843.7	0	-100.0	0	-100.0	37.816	41167.0	61650.3	49.8	136030.3	230.4								
30.67	65449.4	0	-100.0	0	-100.0	38.42	159099.6	177326.7	11.5	199632.6	25.5								
32.213	92606.9	0	-100.0	0	-100.0	38.814	60825.7	123250.6	102.6	168363.6	176.8								
33.027	56095.1	0	-100.0	0	-100.0	39.487	80050.9	122579.2	53.1	143343.8	79.1								
34.069	55144.5	0	-100.0	0	-100.0	39.812	42308.5	53926.7	27.5	62644.3	48.1								
35.548	128622.2	0	-100.0	0	-100.0	40.539	146760.8	186414.5	27.0	187064.7	27.5								
37.331	60972.8	0	-100.0	0	-100.0	43.133	71462.8	60389.7	-15.5	54728.5	-23.4								
39.201	54394.2	0	-100.0	0	-100.0	31.498	0	58638.5	-	55256.5	-								
						31.741	0	73926.8	-	65842.9	-								
						32.637	0	72031.1	-	79603.4	-								
						32.891	0	76788.8	-	69762.9	-								

33.016	0	62569.1	-	53845.4	
34.052	0	87139.3	-	86419.1	-
34.208	0	54497.7	-	50920.7	-
38.085	0	77826.5	-	222749.5	-
41.014	0	58639.1	-	117149.1	-
36.015	0	130574.6	-	0	-
38.183	0	64394.9	-	0	-
33.24	0	0	-	42921.1	-
33.463	0	0	-	42921.1	-
39.174	0	0	-	53702.9	-
40.274	0	0	-	67565.7	-
35.704	49180.3	62681.8	27.5	0	-100.0
35.923	74185.8	0		0	-100.0
36.379	61162.9	0		0	-100.0
37.365	46919.9	0		73572.8	56.8

Table 3 The four fractions of heavy oil and gas production after treatment with fungal enzyme preparations at different concentrations

Enzyme conc.	Alkanes		Aromatics		Resins		Asphaltenes		Gas production	
	(mg 2g ⁻¹)		(mg 2g ⁻¹)		(mg 2g ⁻¹)		(mg 2g ⁻¹)		(mL bottle ⁻¹)	
	E2	E4	E2	E4	E2	E4	E2	E4	E2	E4
0%	1579 ± 4 ^a	1579 ± 4 ^a	282 ± 4 ^d	282 ± 4 ^e	163 ± 10 ^d	163 ± 10 ^c	101 ± 3 ^a	101 ± 3 ^a	0.0 ± 0.0 ^g	0.0 ± 0.0 ^g
2%	1322 ± 31 ^b	1291 ± 11 ^b	369 ± 7 ^c	372 ± 8 ^d	167 ± 3 ^d	178 ± 21 ^{bc}	98 ± 2 ^{ab}	98 ± 3 ^{ab}	25.5 ± 2.1 ^f	47.0 ± 1.4 ^f
4%	1289 ± 13 ^{bc}	1277 ± 21 ^{bc}	411 ± 3 ^b	419 ± 4 ^c	188 ± 1 ^{bc}	190 ± 5 ^b	95 ± 3 ^{abc}	94 ± 4 ^{bc}	65.5 ± 2.1 ^e	89.5 ± 2.1 ^e
5%	1250 ± 2 ^{cd}	1252 ± 9 ^c	416 ± 3 ^{ab}	465 ± 1 ^a	214 ± 15 ^a	229 ± 4 ^a	93 ± 7 ^{abc}	93 ± 1 ^{bc}	79.0 ± 1.4 ^d	101.0 ± 2.8 ^d
6%	1228 ± 27 ^d	1183 ± 16 ^d	425 ± 11 ^{ab}	445 ± 5 ^b	202 ± 1 ^{ab}	197 ± 3 ^b	91 ± 2 ^{bc}	92 ± 1 ^{bc}	86.0 ± 1.4 ^c	118.5 ± 3.5 ^c
8%	1195 ± 12 ^{de}	1175 ± 16 ^d	417 ± 8 ^{ab}	435 ± 4 ^b	174 ± 2 ^{cd}	190 ± 4 ^b	87 ± 1 ^c	90 ± 3 ^c	91.0 ± 1.4 ^b	163.0 ± 4.2 ^b
10%	1155 ± 52 ^e	1143 ± 5 ^e	427 ± 3 ^a	423 ± 1 ^c	158 ± 11 ^d	188 ± 4 ^b	88 ± 3 ^c	89 ± 1 ^c	100.0 ± 2.8 ^a	186.5 ± 2.1 ^a

Table 4 Effect of addition of enzyme preparations and surfactants on the four fractions of heavy oil

Sample	Alkanes (mg 2g ⁻¹)	Aromatics (mg 2g ⁻¹)	Resins (mg 2g ⁻¹)	Asphaltenes (mg 2g ⁻¹)	Gas production (mL bottle ⁻¹)
Ctrl	1567 ± 17 ^a	274 ± 2 ^d	157 ± 3 ^{bc}	101 ± 1 ^a	0.0 ± 0.0 ^f
E2	1313 ± 19 ^{cde}	417 ± 13 ^a	181 ± 8 ^{ab}	95 ± 4 ^a	67.0 ± 1.4 ^c
EB2	1353 ± 3 ^{bc}	352 ± 2 ^b	147 ± 7 ^{cd}	97 ± 1 ^a	56.5 ± 2.1 ^d
EC2	1321 ± 6 ^{cd}	330 ± 13 ^c	104 ± 11 ^e	96 ± 3 ^a	50.0 ± 2.8 ^e
E4	1289 ± 7 ^{de}	428 ± 11 ^a	202 ± 21 ^a	93 ± 4 ^a	86.5 ± 2.1 ^a
EB4	1371 ± 40 ^b	372 ± 5 ^b	173 ± 9 ^b	97 ± 3 ^a	75.5 ± 2.1 ^b
EC4	1272 ± 9 ^e	371 ± 4 ^b	132 ± 2 ^d	96 ± 4 ^a	52.0 ± 4.2 ^{de}

E2 and E4 are crude enzyme solutions; EB2 and EB4 indicate the use of crude enzyme solutions + bacterial fermentation broth containing biosurfactant; EC2 and EC4 indicate the use of crude enzyme solutions + sodium dodecyl sulfate.

Table 5 Heavy oil viscosity reduction and removal efficiencies of fungal enzyme preparations from *Aspergillus* spp.

Sample	Viscosity (mPa·s)	Reduction efficiency (%)	Removal efficiency (<i>RE</i> , %)	<i>RE/RE_{Ctrl}</i>
Ctrl	29,700 ± 1500	-	3.43 ± 0.13 ^b	-
a				
E2	13,700 ± 1200	53.87%	7.85 ± 0.16 ^a	2.29
b				
E4	10,000 ± 1000	66.33%	8.48 ± 0.13 ^a	2.47
c				

Figures

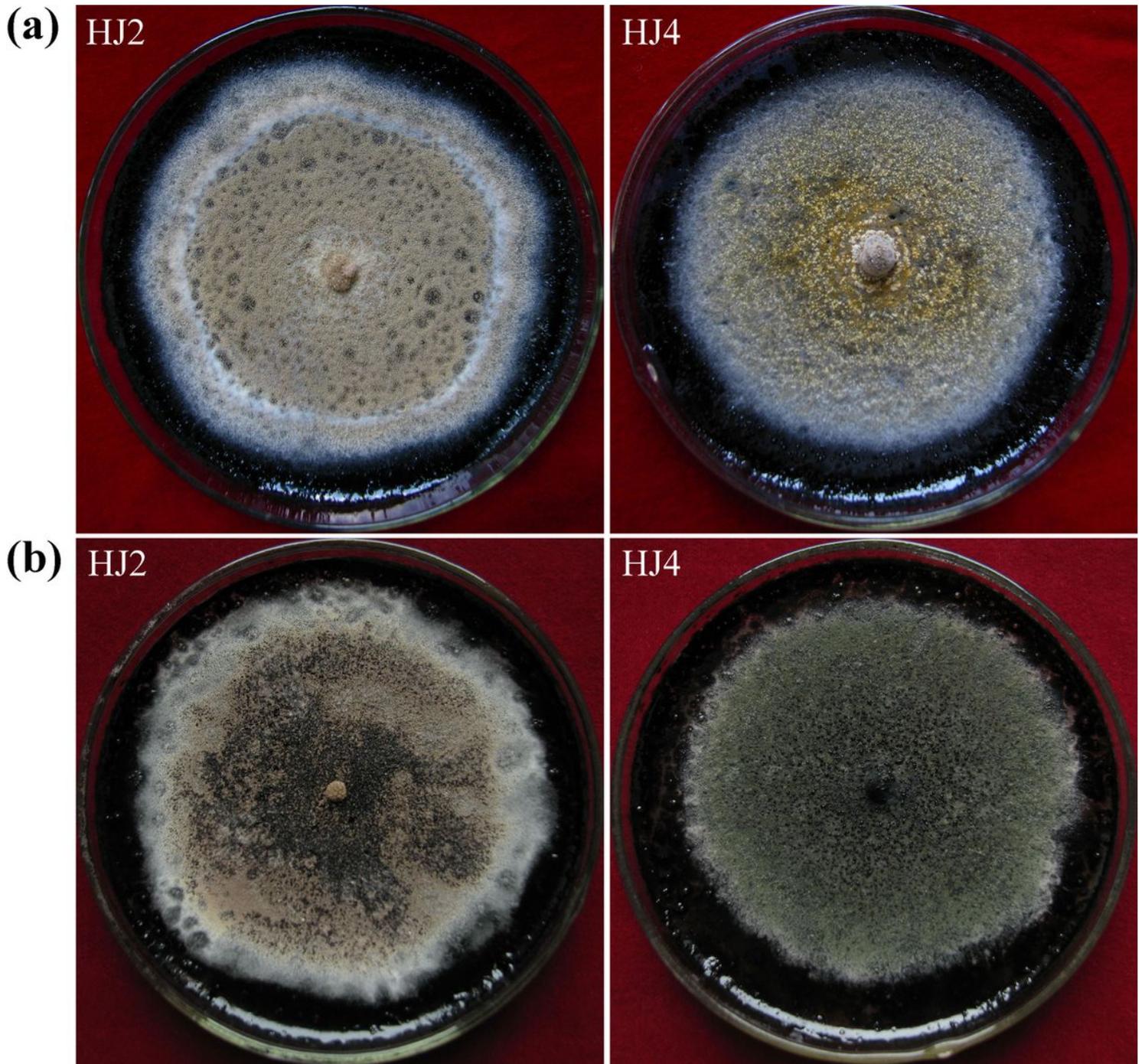


Figure 1

Photographs showing luxuriant growth of two fungal cultures on heavy oil (a) and bitumen (b) agar plates (9 cm in diameter) of mineral salts medium

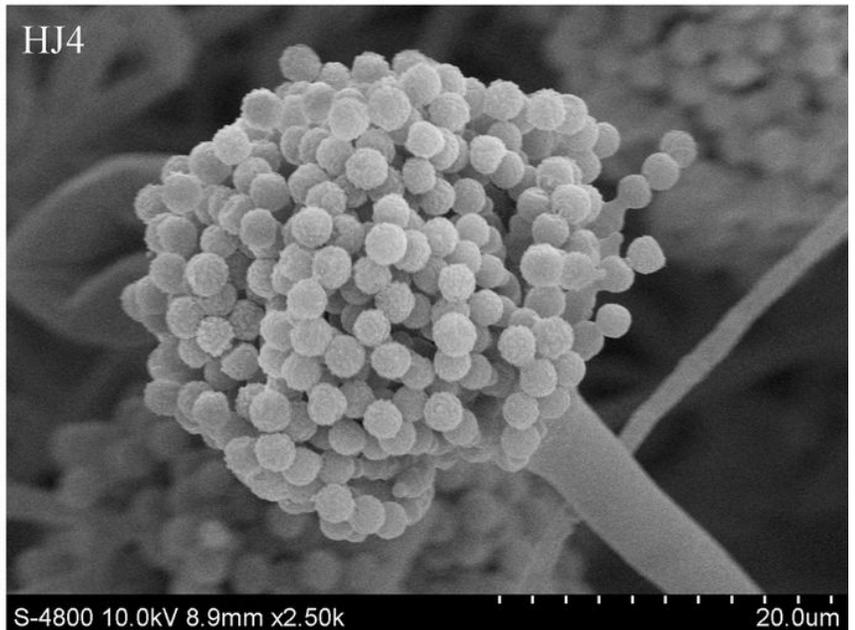
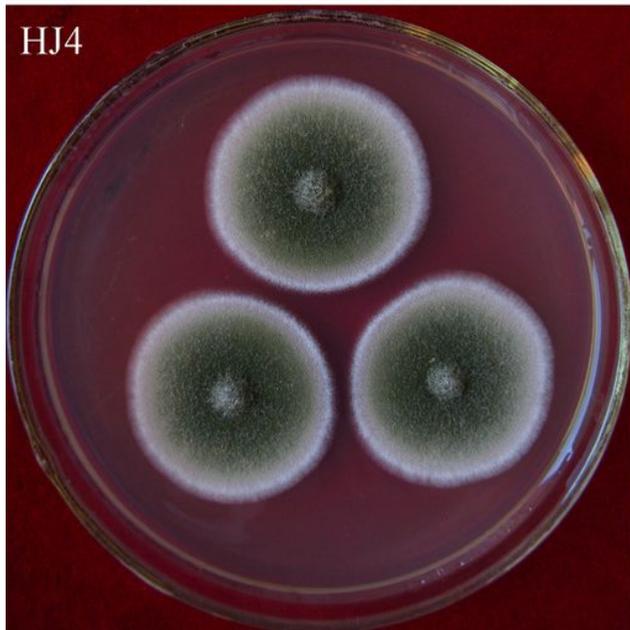
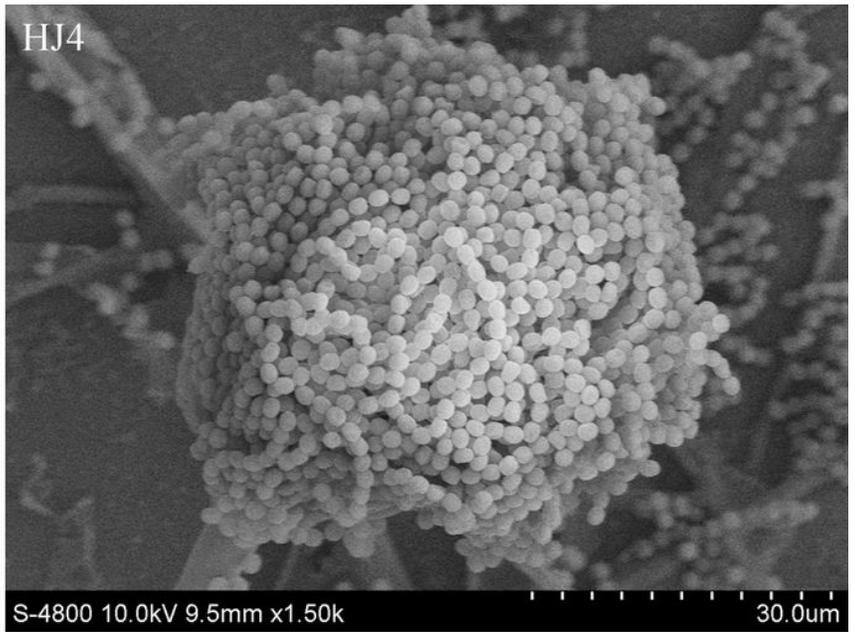
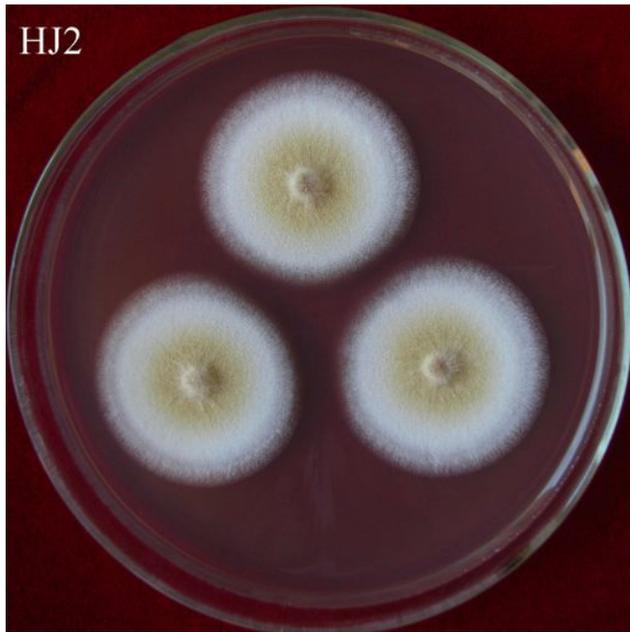


Figure 2

Distinctive colony morphologies and spore structures of two fungal cultures isolated in this study, *Aspergillus terreus* HJ2 and *A. nidulans* HJ4

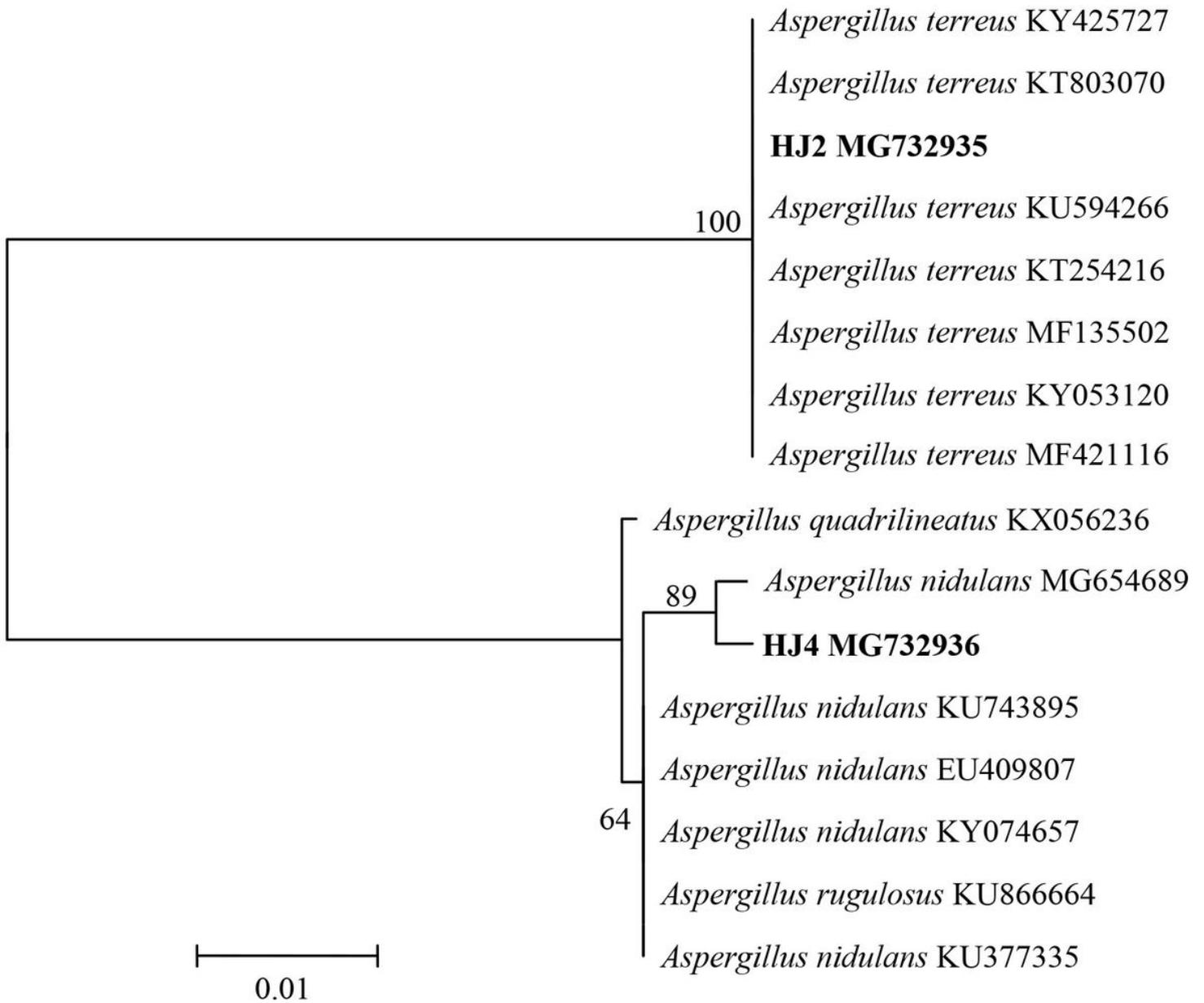


Figure 3

Phylogenetic tree constructed by the neighbor-joining method from rDNA-ITS sequences of two fungal cultures obtained in this study (HJ2 and HJ4) and their close relatives retrieved from the GenBank database. Bootstrap values are shown at nodes for frequencies $\geq 50\%$ (1000 bootstrap resamplings). Bar indicates 1% sequence variance

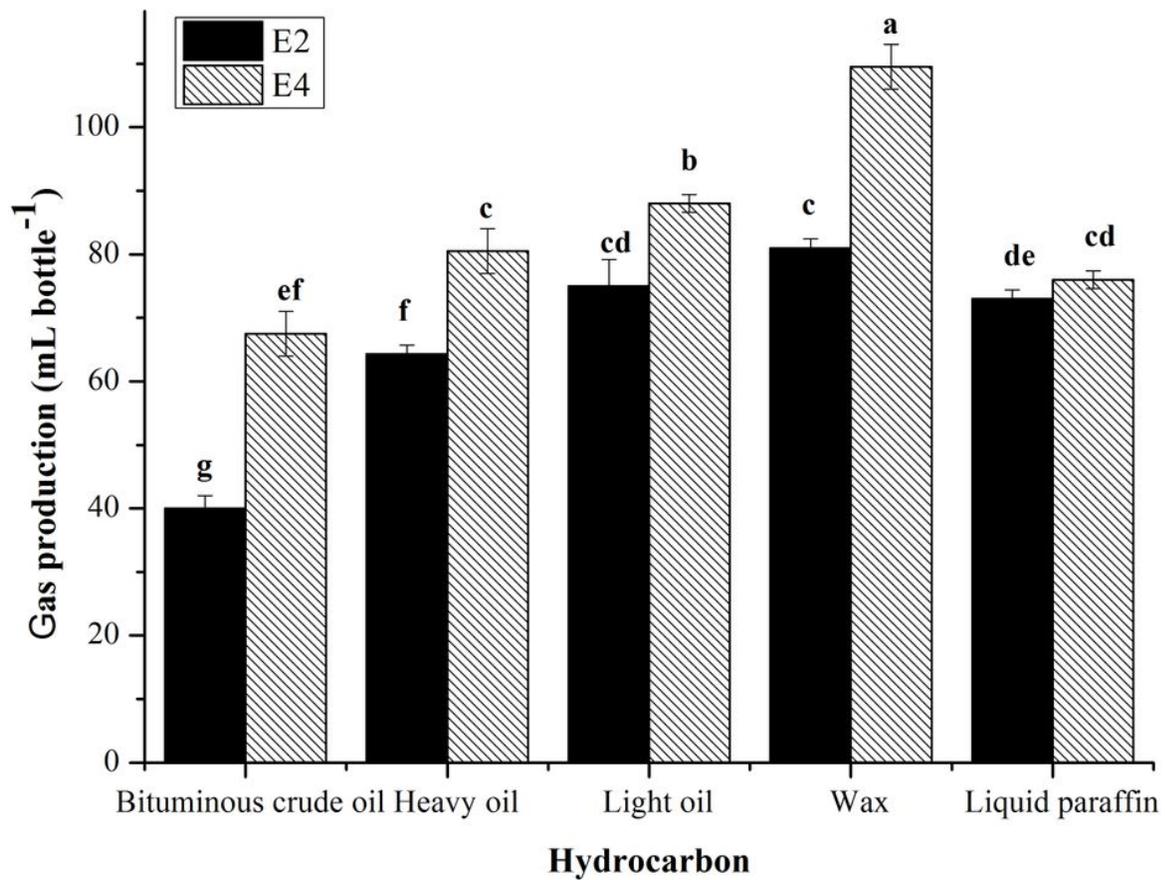


Figure 4

Gas production from hydrocarbon degradation by two fungal enzyme preparations after 15 d of incubation. The data are presented as the mean \pm standard deviation ($n = 3$) and error bars are standard deviations (means \pm SD). Different letters on top indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.



Figure 5

Photographs showing the removal efficiency of heavy oil adsorbed on artificially contaminated sand by crude enzyme solutions from E2 and E4

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