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Bacterial degradation of crude oil using solid formulations of *Bacillus* strains isolated from oil-contaminated soil towards microbial enhanced oil recovery application

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Running Title: Application of *Bacillus* strains in microbial enhanced oil recovery

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Abstract

Microbial enhanced oil recovery has played a major role in enhancing crude oil recovery from depleted oil reservoirs to solve stagnant petroleum production. Most studies have focused on the use of bacteria by liquid-state fermentation, with less attention paid to solid-state fermentation. Here, we examined the efficiency of crude oil degradation using solid formulations of *Bacillus* strains and evaluated their feasibility for application in enhanced oil recovery. Three *Bacillus* strains, namely *B. atrophaeus* 5-2a, *B. aryabhattai* 6-2a and *B. amyloliquefaciens* 6-2c, were isolated from oil-contaminated soil samples. Two strains, 5-2a and 6-2c, secreted extracellular biosurfactants with excellent oil-displacing and emulsifying activity; their diameter of clear zone and emulsification index were 19.7–20.1 cm and 56.6–61.1%, respectively. Three bacterial formulations demonstrated high efficiency to degrade resins (max 24.18%) and asphaltene (max 56.17%), and they decreased the viscosity of crude oil to varying degrees at 40 °C (max 26.47%). The associated CO_2 and H_2 production was 33.0–36.2 mmol L^{-1} and 61.8–68.0 mmol L^{-1}, respectively, and acid production was 1410–1560 mg L^{-1}. Bacterial formulations removed 82.32–94.50% of crude oil adsorbed on filter paper in 4 d at 40 ℃. The results indicate that the three bacterial formulations are efficient in degrading and removing crude oil.

Keywords: Microbial enhanced oil recovery, solid formulation, *Bacillus* strain, Bacterial degradation, Crude oil removal

Introduction

It is urgent to improve crude oil production to meet the global energy demand. Conventional oil extraction can recover less than half of an oil reservoir, and nearly two-thirds of crude oil still is trapped in the rock pores.[1-3] As the petroleum reserves directly available shrinks, it is necessary to exploit profoundly the oil resources from existing and marginal reservoirs. Therefore, increasing attention has been focused on chemical enhanced oil recovery to mobilize entrapped oil from the existing and abandoned oilfields.[3-4] Chemical methods are claimed to have significant potential based on successful laboratory testing; however, the results in field trials have not been encouraging. Furthermore, chemical processes are expensive and complex operational, leaving non-biodegradable residues potentially toxic to the environment.[5-6]

Microbial enhanced oil recovery (MEOR) is emerging as a cost-effective, environmental-friendly, and potentially efficient approach to improve oil production.[7-9] In the process of MEOR, microbial formulations are inoculated into a reservoir, allowing microbial population and/or associated metabolic products to pose certain beneficial effects, such as formation of oil-water emulsions, decrease of interfacial tension between oil and water, increase of permeability, and mobilization of residual oil.[10-11] *Bacillus* strains inhabiting oil-contaminated soils presumably are able to utilize hydrocarbon and tolerate the reservoir conditions. For instance, the genus *Bacillus* can survive and produce desirable MEOR formulations when grown on glucose mineral salts medium.[12] *B. subtilis* PTCC 1365,[13] *B. subtilis* PT2[14] and *B. licheniformis* ACO1[15] isolated from oil-contaminated soils recovered 22–60% of the residual oil entrapped in sand-packed columns.

Microorganisms can enhance the recovery of residual oil by excreting metabolic byproducts including biosurfactants, emulsifiers, biopolymers, solvents and acids.[1,16] Biosurfactants was able to alter oil/water/rock interfacial properties, thereby leading to enhanced oil recovery.[3,11] Biosurfactant flooding as a green and promising methods has been extensively studied.[17-18].The production of acids (e.g., acetic, propionic acids, and butyric acid) helps dissolve carbonates, thereby increasing the permeability and porosity of limestone reservoirs.[3] Additionally, gas production has been mentioned as an important mechanism for oil recovery, since gas products (e.g., CO_2, H_2, and CH_4) can improve the mobility of oil by reducing its viscosity and re-pressurizing the reservoir.[19] Moreover, bacteria can selectively plug high permeability channels and thereby improve volumetric sweep efficiency, as has been studied by nutrient resuscitation and growth of starved cells in sandstone cores.[20-21]

There are three strategies commonly used to implement MEOR: (1) injection of selected nutrients to stimulate the growth of indigenous microorganisms in a reservoir, (2) injection of exogenous microorganisms and nutrients, allowing for colonization of microorganisms in-situ, and (3) injection of *ex-situ* produced products.[4,22] The first two approaches are more favorable from an economic point of view; however, they require specific microorganisms that can survive and produce large amounts of metabolic byproducts in a reservoir. The third strategy is the simplest and thus most likely to succeed at the field-scale. However, the use of liquid formulations has added the difficulty in transporting and storing microbial agents for a long term. To address this problem, we proposed to prepare bacterial formulations through solid-state fermentation. Such advanced MEOR based on the use of solid formulations of bacterial agents may prove effective and successful in oil production.

The aim of this study was to assess the efficiency of solid formulations for bacterial degradation of crude oil. Three *Bacillus* strains were obtained from oil-contaminated surface soils in an oilfield. Bacterial formulations were prepared through solid-state fermentation. After bacterial degradation, changes in the physicochemical properties of crude oil were assessed in terms of crude oil fraction degradation, oil viscosity, oil removal, and gas-acid production. This study provides evidence for the feasibility of using solid formulations in MEOR.

Results and discussion

Identification of bacterial cultures

Three pure bacterial cultures, designated as 5-2a, 6-2a, and 6-2c, were isolated from oil-contaminated soil samples, all of which could utilize crude oil as the sole carbon source. Based on morphological (Figs. 1 and 2) and sequencing analysis (Fig. 3), these pure cultures were identified as *B. atrophaeus* (5-2a), *B. aryabhattai* (6-2a), and *B. amyloliquefaciens* (6-2c). GenBank accession numbers to their sequences are KP314029 (5-2a), KP314030 (6-2a), and KP314031 (6-2c). Solid formulations from these *Bacillus* strains were named B1 (*B. atrophaeus* 5-2a), B2 (*B. aryabhattai* 6-2a), and B3 (*B. amyloliquefaciens* 6-2c).

Bacterial degradation of crude oil fractions

The efficiency of bacterial formulations B1 to B3 to degrade various fractions of crude oil is shown in Table 1. Percentages of degradation after 4 d of incubation were 12.46–15.09% for saturates, 23.89–39.26% for aromatics, 19.78–24.18% for resins, and 53.09–56.17% for asphaltenes present in crude oil. The current results are higher than the efficiency of bacterial consortia to degrade aromatics (0–18%) and asphaltenes (4–20%), and close to the upper

limit for resins (6–29%) present in crude oil, as illustrated by Sugiura et al.,[8] Tehrani et al.,[29] and Zhang et al.[30] A number of compounds slightly degradable by microorganisms are contained in the fractions of resins and asphaltenes. Resins and asphaltenes are high-molecular-weight and strongest polar components of crude oil that are difficult to be degraded, which results in the difficulty of oil recovery.[30] The current bacterial formulations with high degradation efficiency for the resin and asphaltene fractions may be useful for improving the flow properties of crude oil.

The relative variations in total number and peak area of gasifiable *n*-alkanes at 240 °C are shown in Table 2. The three formulations caused progressive increases in the total number of gasifiable *n*-alkanes by 6.67% (B1) to 33.33% (B3). Correspondingly, the total peak area of gasifiable *n*-alkanes was increased by 62.64% (B1) to 107.99% (B3). These results demonstrate the strong ability of solid formulations B1 to B3 to degrade *n*-alkanes into lighter fractions under extreme oxygen-deprived conditions. Numerous bacterial genera have been identified as hydrocarbon-degrading microorganisms, including *Bacillus* species such as *B. thermoleovorans* and *B. subtilis*.[2,31] Felix and Cooney[32] reported that spore-forming bacteria generally have a major role in oil biodegradation. These bacteria can transform heavier fractions into lighter fractions to decrease the viscosity and enhance the flow characteristics of crude oil.

Biosurfactant production and crude oil removal efficiency

Biosurfactant production was assayed by using the oil spreading test and emulsifying activity determination. According to Youssef et al.,[25] the diameter of a clear zone is directly proportional to the concentration of a biosurfactant. In the current study, crude bacterial

biosurfactants from strains 5-2a and 6-2c exhibited strong oil-spreading activity. Their diameter of oil spreading from cell-free culture both reached 19.7–20.1 cm, that is, 3.0-fold that of the control; the emulsification indices were between 56.6% and 61.1%. The oil-spreading activity of strain 6-2a was weak, as indicated by a small diameter (7.9 cm) of clear zone and a lack of emulsifying activity.

Moreover, crude oil removal test was used to evaluate the removal efficiency of the bacterial formulations. All the three formulations removed the majority of crude oil adsorbed on filter paper (Fig. 4). The removal efficiency of bacterial formulations ranged from 82.32% (B2) to 94.50% (B3), that is, 7.32–8.40-fold that of the control. During the recycled use, crude oil removal efficiency relatively decreased but still reached 71.78–92.65%, that is, 6.38–8.24-fold that of the control (Table 3).

In MEOR, biosurfactants are able to reduce the interfacial tension and alter the wettability of reservoir rock for water-flood to displace more oil from the capillary network.[11] Our results show that formulations B1 and B3 exhibit markedly higher ability to produce biosurfactants and remove crude oil compared with *Bacillus* strains in sand pack column studies (30.22–34.19% oil recovery)[33] and a consortium of *Enterobacter cloacae* and *Pseudomonas* sp. (27.2% oil recovery).[34] Therefore, *B. atrophaeus* 5-2a (B1) and *B. amyloliquefaciens* 6-2c (B3) have promising potential for use in MEOR.

Changes in crude oil viscosity

The high viscosity of crude oil is one of the major factors responsible for poor oil recovery from producing wells and it prevents the migration of oil through the rock pores within the reservoir.[1,3] In the present study, all the three formulations decreased oil viscosity to varying

degrees at 40 °C due to bacterial degradation (Table 3). B3 showed higher efficiency (26.47%) to decrease oil viscosity than B1 (25.63%) and B2 (22.27%). The decreases in oil viscosity by *Bacillus* formulations are in agreement with previous findings about bacterial strains such as *Rhodococcus ruber* Z25[35] and *B. subtilis*.[2] The ability of three *B. subtilis* strains to degrade oil and decrease oil viscosity provides a mechanism to enhance the mobility and increase the production of oil in a reservoir.

Gas production from bacterial degradation of crude oil

During the process of crude oil degradation by bacterial formulations, a considerable amount of gases was produced. The gas products mainly included CO_2 (~35%) and H_2 (~65%), with the production of 33.0–36.2 and 61.8–68.0 mmol L^{-1}, respectively. The total gas production was 94.8–104.2 mmol L^{-1} and the gas production rate was 212.3–233.3% (Table 4).

Gas, as a product of microbial activity in MEOR, can improve the mobility of oil by reducing the oil viscosity and re-pressurizing the reservoir.[1,3,36] The current study shows that the solid formulations of three *Bacillus* strains are able to promote high levels of CO_2 and H_2 production, which can benefit oil recovery from depleted reservoirs. Oil recovery studies have detected comparable large amounts of gas in reservoir with favorable results where microorganisms were injected.[19] *Bacillus* species are the most common microorganisms used for gas production in MEOR processes. Spore production by these species is also beneficial because spores survive harsh conditions and penetrate deep into the petroleum reservoir.[36] Therefore, the three biogas-producing strains obtained in the present study would be promising microorganisms for use in MEOR.

Acid production from bacterial degradation of crude oil

Laboratory experiments have shown that the efficiency of MEOR depends on the ability of microorganisms to grow and produce metabolites such as solvents, acids, gases, and biosurfactants.[3,8] Acids (e.g., acetic, propionic acids, and 1-butanoic acid) can dissolve carbonates and thereby increase the permeability and porosity of oil reservoir.[3] In the present study, substantial acids were produced during bacterial degradation of crude oil. The total acid number was within the range of 1410–1560 mg L^{-1}, while the pH of reaction solutions decreased by 26.47–36.03% compared to the control (Table 5).

The total acid numbers for crude oil degradation by B1 to B3 were nearly 2.6-fold that reported for a mixed culture of *Thermoanaerobacter* (540 mg L^{-1}) grown at 70 °C with molasses as carbon source.[37] Additionally, certain bacterial cultures, such as a bacterium consortium of *Enterobacter Cloacae* and *Enterobacter hormaechei*, can barely cause a real and significant change in the pH during fermentation.[38,39] Together these results highlight that bacterial formulations obtained in the present study can promote high levels of acid production when breaking down crude oil.

Liquid chromatography showed that the organic acids produced by bacterial degradation of crude oil mainly comprised oxalate, formate, and propionate. Since oxalate (a dicarboxylic acid), formate and propionate (a carboxylic acid) have strong acid strength, all of these acids have the potential of dissolving carbonate rocks to increase permeability and porosity. This mechanism would contribute to increasing crude oil fluidity and thereby enhancing crude oil recovery.

Conclusions

Three *Bacillus* strains were isolated from oil-contaminated surface soil. Two isolates were

selected as high biosurfactant producers, both of which exhibited desirable oil-spreading activity and emulsifying activity. Solid formulations from these bacterial cultures exhibited excellent activities for crude oil degradation, including degradation of resin and asphaltene fractions, reduction of oil viscosity, and production of gases and organic acids. Given their convenient storage, transportation and use, these solid formulations have the potential for application in MEOR. This study establishes a new approach for the development of solid formulations of bacterial agents towards MEOR application. Future investigations should test the feasibility of B1 to B3 for field application, for example, using laboratory-scale sand-pack columns.

Materials and Methods

Media, crude oil, and soil

Crude oil and soil samples were collected in the Ansai oilfield, Shaanxi Province, Northwest China. An oil sample was obtained from a low permeability reservoir (Hua-20-4). Oil-contaminated soil samples were obtained in the vicinity of kowtow machines and oil tanks near wells Hua-119 and Hao-129. Details on the source of oil and soil samples are available in our recent publication.[23]

The basal mineral salt medium (MSM) contained (g L^{-1}): $NaNO_3$ 2.0, $(NH_4)_2SO_4$ 1.0, $MgSO_4 \cdot 7H_2O$ 0.3, KH_2PO_4 5.0, $K_2HPO_4 \cdot 3H_2O$ 5.0, and NaCl 5.0, with pH adjusted to 7.0. The enrichment and isolation media were prepared by supplementing MSM with 2% (v/v) crude oil as the sole carbon source. The fermentation medium contained (g L^{-1}): beef extract 3.0, peptone 10.0, NaCl 5.0, and brown sugar 10.0, with pH adjusted to 7.0. The solid formulation-producing medium contained MSM, wheat bran, and crude oil (60:100:2, v/g/g).

All regents were of analytical grade.

Bacterial isolation and identification

Soil enrichment cultures were prepared in 250-mL glass bottles containing 100-mL of enrichment medium supplemented. Ten grams of soil samples were weighed into each bottles and then incubated at 30 °C in the dark for 5 d. The cultures (5 mL each) were subsequently passaged to fresh enrichment medium three times. Aliquots (50 μL) of the fourth-generation enrichment cultures were spread on oil plates of the isolation medium and incubated at 30 °C under aerobic conditions.

Morphologically distinct colonies were picked and purified by streaking on fresh fermentation medium plates at least three times. Three pure bacterial cultures, designated as 5-2a, 6-2a, and 6-2c, were obtained and identified by morphological examination and gene sequencing.[2] The purified cultures were maintained on beef extract peptone agar and deposited in the China Center for Type Culture Collection (CCTCC) under the accession numbers CCTCC M 2014673 (5-2a), CCTCC AB 2015004 (6-2a), and CCTCC M 2015740 (6-2c).

Preparation of solid formulations and bacterial suspensions

Single colonies of each bacterial culture were transferred to 100 mL of fermentation medium, incubated at 30 °C with shaking (120 rpm) for 3 d to obtain a cell density of 10^{10} colony-forming unit per milliliter (CFU mL^{-1}). The seed inoculum with 20% (v/v) was transferred into 50 g of solid formulation-producing medium and grown for 5 d at 30 °C. Thereafter, medium containing *Bacillus* strains was air-dried to 30% moisture content and then oven-dried at 40 °C for 48 h. The dry material was ground into powder and passed

238 through a 0.10-mm sieve to obtain a powder formulation. The solid formulations from
239 *Bacillus* strains 5-2a, 6-2a, and 6-2c were named B1 to B3 and their viable counts were
240 9.5×10^{10}, 12.0×10^{10} and 35.3×10^{10} CFU g^{-1}, respectively. The formulations were stored at
241 room temperature until use.

242 The prepared formulations and distilled water were blended 1:3 in glass bottles. The
243 suspensions were incubated at 30 °C with shaking (120 rpm) for 0.5 h and then filtered
244 through sterile cotton wool to obtain bacterial cell suspensions.

245 **Crude oil degradation**

246 Crude oil (2 g each) was prepared in 100-mL glass bottles containing 30 mL of bacterial
247 suspension. The bottles were sealed with rubber stoppers and incubated statically at 40 °C for
248 4 d, with regular shaking. Abiotic controls were prepared following the same procedure
249 without addition of bacterial suspension. After bacterial degradation, liquid and gaseous
250 samples were taken from the reaction system in triplicate for further tests.

251 **Saturate, aromatic, resin, and asphaltene analysis of crude oil**

252 The degrading potential of bacterial formulations for crude oil was examined by quantifying
253 the remaining total petroleum hydrocarbons (TPH) in batch cultures. TPH was extracted from
254 30 mL of reaction solutions with 60 mL of hexane and further fractionated into soluble and
255 insoluble fractions using Al_2O_3 column chromatography.[24]

256 Precipitated asphaltenes were collected by centrifugation at 4,000 rpm for 5 min, and the
257 precipitate was oven-dried at 40 °C for 24 h, cooled in a vacuum desiccator, and quantified
258 gravimetrically.[23] The soluble fraction was loaded at the top of a neutral Al_2O_3 column (300
259 mm × 10 mm ID, with a polytetrafluoroethylene stopcock at bottom) and the column was

successively eluted with 80 mL of hexane, 60 mL of hexane/dichloromethane (v/v, 3:1), and 40 mL of methanol. The fractions eluted with these solvents were called saturates, aromatics, and resins. Each procedure was independently repeated three times. Bacterial degradation-associated variations ($VR_m\%$) in the mass of four crude oil fractions were described as follows:

$$VR_m\% = \frac{M_{ck} - M_s}{M_{ck}} \times 100 \quad (1)$$

where M_{ck} and M_s are the masses of specific crude oil component (saturates, aromatics, resins, or asphaltenes) in the control and degraded samples, respectively .

Gas chromatography analysis

Following bacterial degradation, the crude oil was separated and diluted to 50 mg mL^{-1} in *n*-hexane. Gas chromatography (GC) analysis was performed using Thermo Finnigan Trace GC Ultra (San Francisco, CA, USA) equipped with an on-column injector, an FID detector, and a DB-Wax capillary column (30 m × 0.25 mm × 0.25 μm). The operating temperature was set to 240 ℃ for the FID detector and 230 °C for the injector. The column temperature was set at 40 °C for 2 min before raised to 240 °C by 6 °C min^{-1} and then held at 240 °C for 8 min. The sample volume was 1 μL. Nitrogen was used as a gas carrier at a constant flow rate of 1 mL min^{-1}. The quantities of alkanes in the extracts were determined from the peak areas corresponding to these compounds on the gas chromatogram. Bacterial degradation-associated variations ($VR_a\%$) in chromatographic peak area of each *n*-alkane at different retention times were described as follows:

$$VR_a\% = \frac{A_{ck} - A_s}{A_{ck}} \times 100 \quad (2)$$

where A_{ck} and A_s are chromatographic peak areas of individual *n*-alkane at different retention times in the control and degraded samples, respectively.

Surface activity determination

Surface activity was measured by the oil displacement test according to the method of Youssef et al.[25] with slight modifications. Five milliliters of pre-seed inoculum with a cell density of 10^{10} CFU mL^{-1} was inoculated into 100 mL of fermentation medium and grown for 5 d at 30 °C to obtain culture broth. The oil displacement test was performed in a 25-cm diameter plastic tub containing 3000 mL of tap water. Two drops of paraffin oil (Tianli, Tianjin, China) were added to form a thin oil layer on the surface of the water. Then, cell-free culture broth obtained by centrifugation (10,000× g, 10 min, 4 °C) and one drop of it was added onto the surface of paraffin oil. The diameter of the clear zone formed on the paraffin oil surface was measured to indicate the surface activity of culture broth.

Emulsification index determination

Emulsifying activity was determined by adding 5 ml of paraffin oil to an equal volume of cell-free culture broth in glass tubes, followed by vortexing at high speed for 2 min and incubation at ambient temperature for 24 h. All emulsification indexes were performed in triplicate. The emulsification index (E_{24}) was described as follows:[26]

$$E_{24}\% = \frac{HE}{HT} \times 100 \quad (4)$$

where *HE* and *HT* are the height of emulsion layer (mm) and total height of liquid column (mm), respectively.

Crude oil removal test

A custom-designed oil removal test was performed at 40 °C under atmospheric pressure (~0.1

303 MPa). Filter papers (Wohua, Hangzhou, Zhejiang, China) were cut into small pieces (60 mm
304 × 60 mm) and weighed (m) before covered with crude oil (m_1). The oil-covered filter papers
305 were transferred to 100-mL conical flasks followed by addition of 80 mL of bacterial
306 suspensions and incubation at 40 °C for 4 d. The filter papers were then washed with water to
307 remove surplus bacterial suspensions and dried in the air to constant weight, before weighed
308 (m_2) and photographed. Abiotic controls were prepared following the same procedure without
309 addition of bacterial suspensions. The recyclability of bacterial suspensions was tested by
310 repeating the test once again. Crude oil removal efficiency ($RE\%$) was described as follows:

311 $$RE\% = \frac{m_1 - m_2}{m_1 - m} \times 100 \quad (3)$$

312 where m is the mass of filter paper, m_1 is the mass of filter paper covered with crude oil, and
313 m_2 is the mass of oil-covered filter paper after bacterial treatment.

314 **Viscosity reduction test**

315 To measure viscosity changes, crude oil was collected from the reaction solutions by
316 centrifugation at 8000 rpm for 10 min. Viscosity measurement was carried out using a NDJ79
317 rotating viscometer (Yutong, Shanghai, China) at 40 °C under atmospheric pressure (~0.1
318 MPa). Crude oil viscosity recovered from degraded samples was compared with that
319 recovered from the control. Viscosity reduction rate ($VRR\%$) was described as follows:

320 $$VRR\% = \frac{V_s - V_{ck}}{V_{ck}} \times 100 \quad (5)$$

321 where V_s and V_{ck} are the viscosity of crude oil recovered from degraded samples and the
322 control, respectively.

323 **Gas production test**

Gas samples were withdrawn from the headspace of each reaction system using a 60-mL syringe. Prior to the sampling procedure, sealed glass bottles containing the reaction solutions were shaken gently to disperse gas products. Since the pressure inside the bottle had increased due to gas production, the syringe would be filled spontaneously to a certain volume. Total gas production (V_a) was recorded and CO_2 was quantified by the alkali-absorption test.[27] Gas production rate (*GPR%*) was described as follows:

$$GPR\% = \frac{V_a}{30} \times 100 \qquad (6)$$

Gaseous components were identified by GC analysis using the 663-30 Limited gas chromatograph, equipped with an on-column injector, an FID detector, and a Porapak column (1 m × 0.05 m). Nitrogen was used as a gas carrier at the constant flow rate of 60 mL min^{-1}. The column and injector temperatures were set to 130 °C and 160 °C, respectively. The sample volume was 5 μL.

Acid production test

For acid analysis, the reaction solutions were filtered through sterile cotton wool. The filtrate (30 mL each) was then centrifuged at 8,000 rpm for 10 min and passed through a 0.22-μm Millipore filter to remove impurities. The pH of the purified filtrate was assayed with a PHS-3D pH meter (Jiapeng, Shanghai, China). Acid yield was determined by acid-base titration.

The pH reduction rate (*RR%*), total acidity (*TA*), and total acid number (*TAN*) of purified filtrate were described using Formulae (7) to (9), respectively.

$$RR\% = \frac{pH_{ck} - pH_s}{pH_{ck}} \times 100 \quad (7);$$

$$TA\left(mmol/L\right)=\frac{M_{NaOH}\times V_{NaOH}}{V_t} \quad (8);$$

$$TAN\left(mg/L\right)=\frac{M_{NaOH}\times V_{NaOH}}{V_t}\times 60 \quad (9)$$

where pH_{ck} is the pH of control filtrate, pH_s is the pH of degraded filtrate (Formula 7), M_{NaOH} and V_{NaOH} are respectively the concentration (0.1 M) and volume of standard NaOH solution used for acid-base titration, and V_t is the total volume of purified filtrate tested (Formulae 8 and 9).

Organic acids were identified by liquid chromatography analysis using WATERS.600, equipped with an on-column injector, a UV-vis detector (wavelength 215 nm), and a C_{18} column. Methanol and 0.01 mol L^{-1} $(NH_4)_2HPO_4$ (3:97, v/v, pH 2.8) was used as the mobile phase at a constant flow rate of 0.7 mL min^{-1}. Oven temperature was set to 25 °C. The sample volume was 10 μL. Standard organic acid solutions were prepared as 5.0 mg mL^{-1} oxalate and 10 mg mL^{-1} formate and propionate in ultrapure water.

Date analysis

Data are mean ± standard deviation (n = 3). Differences between group means were identified using Duncan's multiple range test. The analysis was performed at $P < 0.05$ using SAS 9.2 (SAS Institute Inc, Cary, NC, USA).

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459 **TABLES**

460 **Table 1**

461 Degradation of crude oil fractions by solid formulations from *Bacillus atrophaeus* 5-2a (B1),

462 *B. aryabhattai* 6-2a (B2), and *B. amyloliquefaciens* 6-2c (B3)

Formulations	Saturates		Aromatics		Resins		Asphaltenes	
	(mg $2g^{-1}$)	VR_m%	(mg $2g^{-1}$)	VR_m%	(mg $2g^{-1}$)	VR_m%	(mg $2g^{-1}$)	VR_m%
Ctrl	1027 ± 5^{a}	-	540 ± 6^{a}	-	91 ± 3^{a}	-	162 ± 1^{a}	-
B1	899 ± 6^{b}	12.46	336 ± 4^{c}	37.78	71 ± 1^{b}	21.98	76 ± 2^{b}	53.09
B2	889 ± 4^{b}	13.44	328 ± 6^{c}	39.26	73 ± 1^{b}	19.78	72 ± 2^{b}	55.56
B3	872 ± 6^{c}	15.09	411 ± 3^{b}	23.89	69 ± 5^{b}	24.18	71 ± 3^{b}	56.17

463 Oil fractions are mean ± standard deviation (n = 3). Different letters within a column indicate

464 significant differences ($P < 0.05$) according to Duncan's multiple range test. Ctrl for control;

465 VR_m% for bacterial degradation-associated variations in the mass of four crude oil fractions.

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475 **Table 2**

476 Relative variations in the peak area of individual gasifiable *n*-alkanes in crude oil before and
477 after bacterial degradation by solid formulations from *Bacillus atrophaeus* 5-2a (B1), *B.*
478 *aryabhattai* 6-2a (B2), and *B. amyloliquefaciens* 6-2c (B3)

No.	Retention time (min)	Ctrl	B1		B2		B3	
			Peak area	VR_a%	Peak area	VR_a%	Peak area	VR_a%
1	15.813	10525	19863	88.72	36989	251.44	42218	301.12
2	18.113	20859	31249	49.81	46363	122.27	51271	145.80
3	20.275	24158	34366	42.26	40363	67.08	47917	98.35
4	22.338	26328	36593	38.99	39164	48.75	46748	77.56
5	24.275	23045	32714	41.96	32605	41.48	39608	71.87
6	26.138	19523	33829	73.28	31288	60.26	39361	101.61
7	27.900	20116	33394	66.01	30103	49.65	36807	88.53
8	29.575	24824	37147	49.64	32759	31.97	39377	58.62
9	31.188	23649	38531	62.93	32886	39.06	38699	63.64
10	32.725	25795	38162	47.94	33963	31.67	41402	60.50
11	34.213	24129	36583	51.61	31570	30.84	39996	65.76
12	35.638	25833	38539	49.19	32761	26.82	41612	61.08
13	37.275	28055	43649	55.58	32026	14.15	42999	53.27
14	39.300	19010	34404	80.98	27578	45.07	32361	70.23
15	41.863	15355	24725	61.02	20558	33.88	26475	71.77
16	21.575	0	13267		14734		18451	-

17	13.388	0	0		26068		27871	-
18	26.238	0	0		10596		13065	-
19	10.850	0	0		11651		10677	-
20	17.500	-	-		-		12001	-
∑		331200	538665	62.64	564024	70.30	688868	107.99

Note: $VR_a\%$ for bacterial degradation-associated variations in chromatographic peak area of each *n*-alkane at different retention times.

Table 3

Crude oil viscosity reduction and removal efficiency by solid formulations of *Bacillus atrophaeus* 5-2a (B1), *B. aryabhattai* 6-2a (B2) and *B. amyloliquefaciens* 6-2c (B3)

Formulations	Viscosity (Pa·s)	*VRR%*	Fresh formulation suspension		Recycled formulation suspension	
			RE%	RE_s/RE_{ck}	*RE%*	RE_s/RE_{ck}
Ctrl	23.8±0.3[a]	-	11.25±0.19[c]	-	11.25±0.19[c]	-
B1	17.7±0.8[b]	25.63	94.50±0.21[a]	8.40	92.65±0.042[a]	8.24
B2	18.5±0.5[b]	22.27	82.32±0.13[b]	7.32	71.78±0.092[b]	6.38
B3	17.5±0.5[b]	26.47	94.20±0.21[a]	8. 37	92.47±0.14[a]	8.22

Values are mean ± standard deviation (n = 3). Different letters within a column indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. *VRR%* for viscosity reduction rate; *RE%* for crude oil removal efficiency.

Table 4

Gas production during crude oil degradation by solid formulations of *Bacillus atrophaeus* 5-2a (B1), *B. aryabhattai* 6-2a (B2) and *B. amyloliquefaciens* 6-2c (B3)

Formulations	Gas production (mmol L^{-1})			Gas production rate
	Total	CO_2	H_2	*GPR%*
Ctrl	0±0.0[d]	0±0.0[d]	0±0.0[d]	0
B1	94.8±1.5[c]	33.0±0.5[c]	61.8±1.0[c]	212.3
B2	99.3±1.5[b]	34.5±0.6[b]	64.7±1.0[b]	222.3
B3	104.2±1.0[a]	36.2±0.4[a]	68.0±0.7[a]	233.3

Gas production is mean ± standard deviation (n = 3). Different letters within a column indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

528 **Table 5**

529 Acid production during crude oil degradation by solid formulations from *Bacillus atrophaeus*

530 5-2a (B1), *B. aryabhattai* 6-2a (B2) and *B. amyloliquefaciens* 6-2c (B3)

Formulations	pH		Acid production	
	Measurements	pH reduction rate *RR*%	Total acidity *TA* (mmol L^{-1})	Total acid number *TAN* (mg L^{-1})
Ctrl	6.80±0.025[a]	-	0.0±0.0[a]	0
B1	5.00±0.029[b]	26.47	23.5±0.7[b]	1410
B2	4.49±0.016[c]	33.97	25.5±0.7[c]	1530
B3	4.35±0.016[d]	36.03	26.0±0.0[d]	1560

531 Values are mean ± standard deviation (n = 3). Different letters within a column indicate

532 significant differences ($P < 0.05$) according to Duncan's multiple range test.

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FIGURES

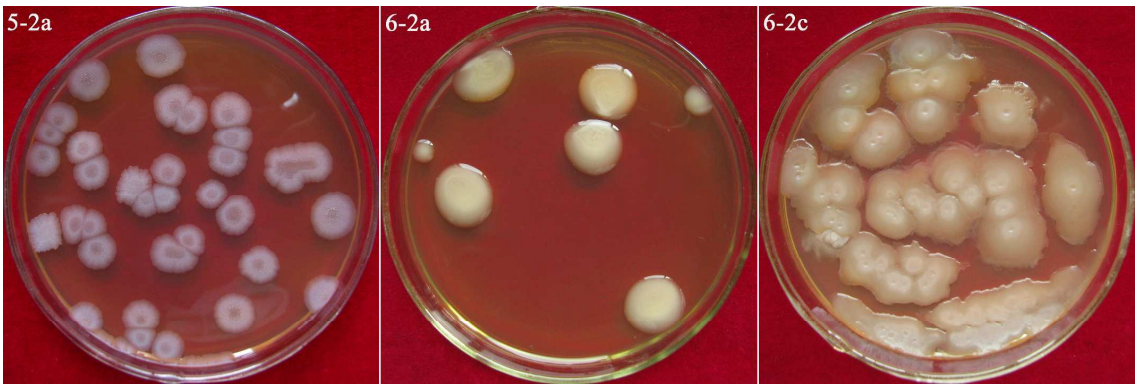


Fig. 1 Photos showing distinctive colonial morphologies of three soil bacterial cultures grown on beef extract peptone agar plates 9 cm in diameter, including *Bacillus atrophaeus* (5-2a), *B. aryabhattai* (6-2a) and *B. amyloliquefaciens* (6-2c).

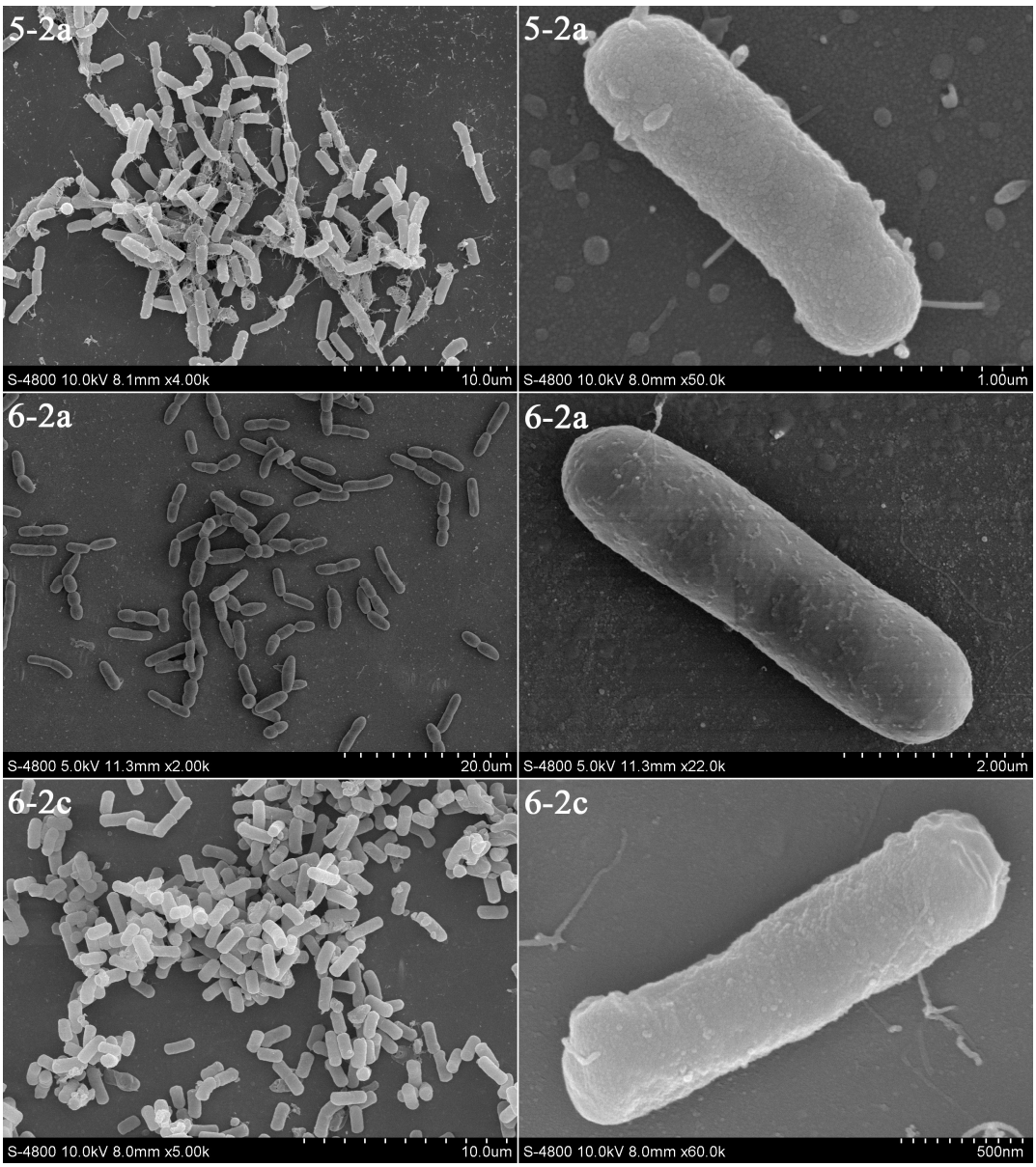
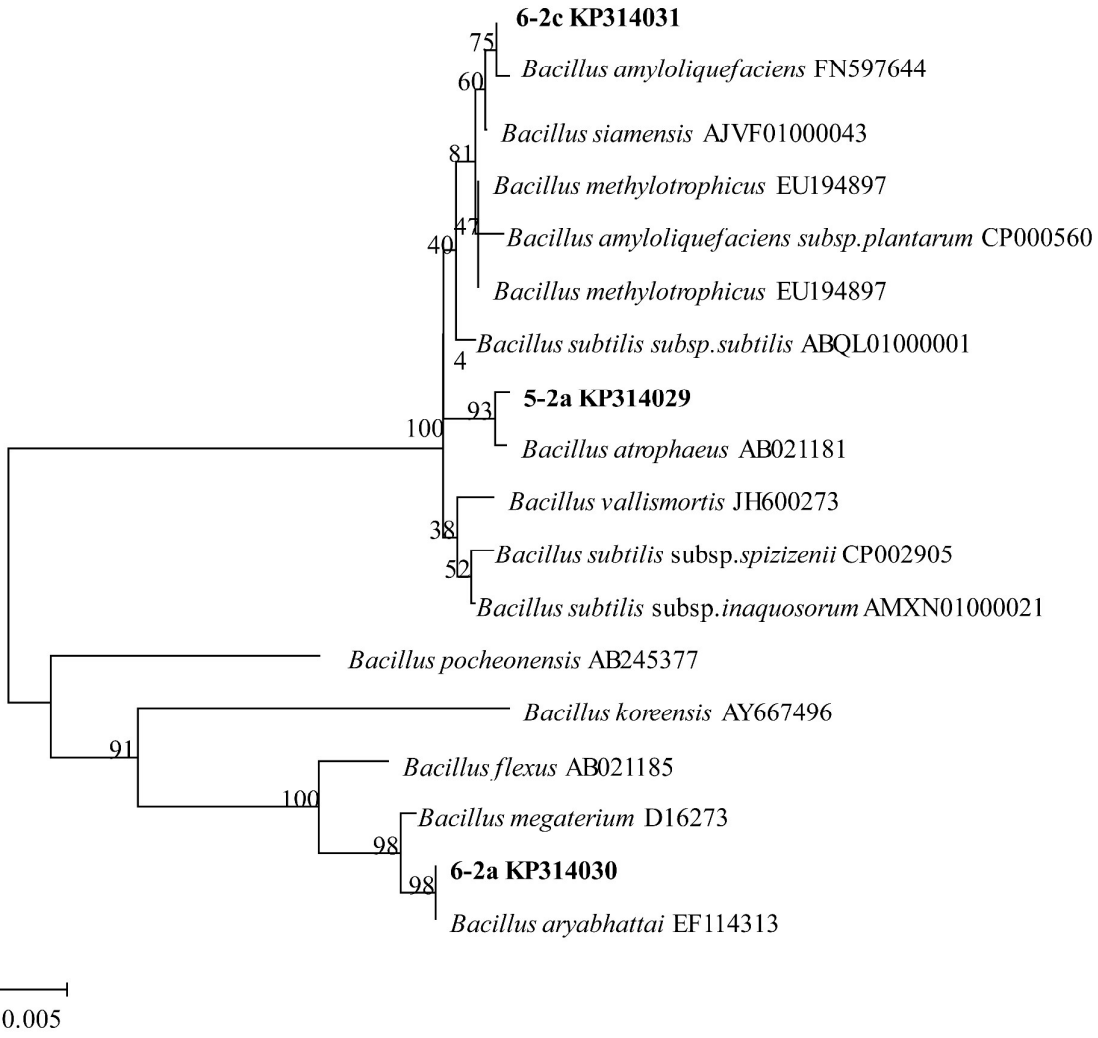


Fig. 2 Scanning electron images showing the morphological structure of three bacterial cultures isolated from oil-contaminated soil, including *Bacillus atrophaeus* (5-2a), *B. aryabhattai* (6-2a) and *B. amyloliquefaciens* (6-2c).



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562 **Fig. 3** Phylogenetic tree constructed by the neighbor-joining method using 16S rDNA
563 sequences of three bacterial cultures obtained in this study (in bold) and their close relatives
564 retrieved from the GenBank database. Bootstrap values shown at nodes (1000 bootstrap
565 resampling). Bar indicates 0.5% sequence variance.

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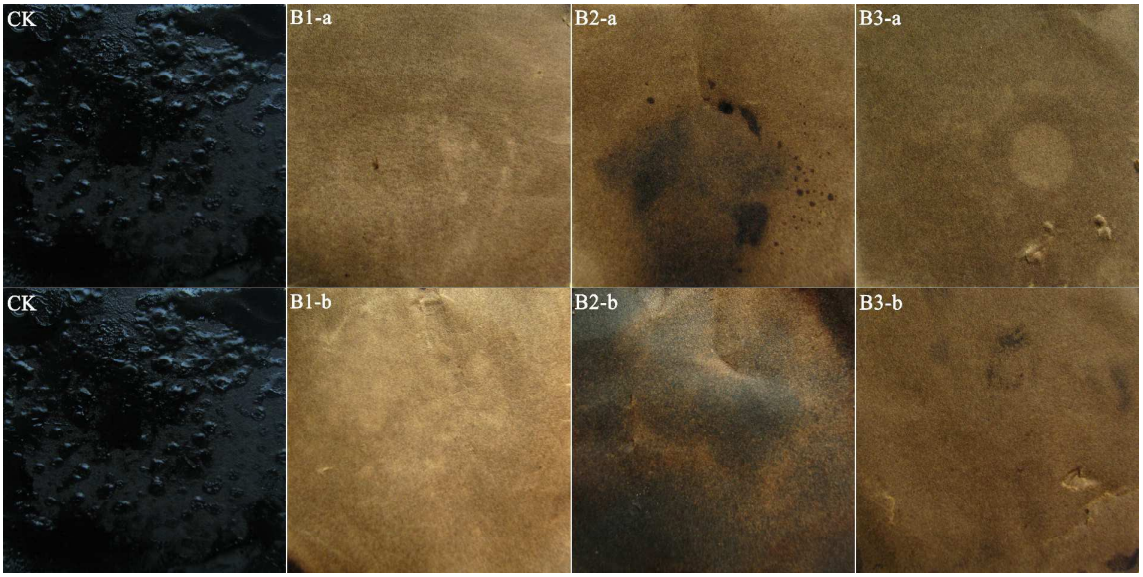

Fig. 4 Photos showing the removal efficiency of crude oil adsorbed on qualitative filter paper by bacterial formulation suspensions of *Bacillus atrophaeus* 5-2a (B1), *B. aryabhattai* 6-2a (B2) and *B. amyloliquefaciens* 6-2c (B3): (a) oil-covered filter paper treated with fresh formulation suspensions for 0.5 h at 30 °C with shaking (120 rpm); and (b) oil-covered filter paper treated with recycled formulation suspensions after the completion of the first trial.