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Crude Oil Biodegradation by a New Isolate Strain *Bacillus* sp. DG24

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A new strain, Bacillus sp. DG24, isolated from crude oil polluted soils, showed high biodegradability of oil under low temperature conditions. In the present work, effect of environmental factors including, crude oil concentration, temperature, salinity, pH, and nutrient on the biodegradation of crude oil as well as the biodegradability of different alkanes by *Bacillus* sp. DG24 were investigated. GC-MS results showed that these oil components were mainly C10-C28 alkanes, and the biodegradability of different alkanes decreased along with the increase of molecular weight. Moreover, the results also showed that the optimal substrate concentration, temerature, pH, salinity and nutrients ratio for *Bacillus* sp. DG24 was 100 mg·L-1, 25°C, 7-8, 1% and 20:1:1, respectively. The biodegradability of crude oil under tested conditions ranged from 0-69.12%.

Key words: *Bacillus*, Crude oil, Biodegradation, Environmental factors, Alkane.

Bioremediation, a non-destructive, cost- and treatment-effective and sometimes logistically favorable cleanup technology, attempts to accelerate the naturally occurring biodegradation of contaminants through the optimization of limiting conditions (Allard and Neilson, 1997). Many environments are characterized by low or elevated temperatures, acidic or alkaline pH, high salt concentrations, or high pressure (Baptista *et al*., 2005; Chaillan *et al*., 2006). Extremophilic microorganisms are adapted to grow and thrive under these adverse conditions. Hydrocarbon degrading extremophiles are thus ideal candidates for the biological treatment of polluted extreme habitats.

A large number of bacterial genera have been identified as being involved in crude oil degradation, including some *Bacillus* species (Wentzel *et al*., 2007), and oil degrading *Bacillus* strains have been isolated from a number of oil polluted sites (Calvo *et al*., 2004; da Cunha *et al*., 2006; Cubitto *et al*., 2004). The aim of this study was to identify a newly isolated *Bacillus* strain DG24 and investigate the crude oil biodegradation properties of this culture under low temperature condition. The results of this study could help to moderate the bioremediation conditions of oil polluted sites by supplementing *Bacillus* sp. DG24 in the low temperature regions.

MATERIALS AND METHODS

Culture and growth conditions

Bacillus sp. DG24 (CGMCC: NO. 5051; NCBI accession NO.: JN 216879), used in this study, was isolated from petroleum contaminated soil (Hua *et al*., 2013). Culture of *Bacillus* sp. DG24 was maintained at 4 °C on crude oil solid medium. Cells of *Bacillus* sp. DG24 were inoculated in mineral salt medium (MSM) containing (per liter at pH 7.0) 0.4g Na_2HPO_4, 0.15g KH_2PO_4, 0.1gNH_4Cl, 0.05g $MgSO_4 \cdot 7H_2O$, 0.0015g $CaCl_2$, 0.1g $NaNO_3$, 0.001g

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$FeSO_4 \cdot 7H_2O$, and 1 mL trace medium (per 100mL solution containing 0.5mg $CuSO_4 \cdot 5H_2O$, 1.0mg H_3BO_3, 1.0mg $MnSO_4 \cdot 5H_2O$, 7.0mg $ZnSO_4$).

For cell proliferation, *Bacillus* sp. DG24 was inoculated into LB medium (5 % yeast extract, 10 % peptone and 5 % sodium chloride, pH 7.0) at 10 ºC for 48 hours. Cells were centrifuged, washed with sterilized MSM and transferred to 50 mL centrifuge tube containing MSM. The final cell density (OD_{600}) was 1.6.

Environment factors that influence biodegradability of crude oil

To study salinity, pH, and nutrients on the biodegradation of crude oil, stock solution of crude oil (20000 $mg \cdot L^{-1}$) dissolved in dichloromethane was filtered by 0.2 ?m filter membrane into the flasks containing 150 mL of MSM (pH 7.0-7.2), and the final crude oil concentration was 2000 $mg \cdot L^{-1}$. After dichloromethane in the medium was removed through volatile, cells of DG24 were inoculated into the flasks, and the flasks were incubated at 150 rpm and 10 °C for 35 days.

Different crude oil concentrations

Different amount of crude oil stock solution were supplemented into the flasks and the final crude oil concentrations were 100, 200, 500, 800, 1000, 2000 $mg \cdot L^{-1}$, respectively. The final cell density (OD_{600}) was 0.13.

Different incubation temperature

Stock solution of crude oil (20000 $mg \cdot L^{-1}$) was added into the flasks, and the final crude oil concentration was 2000 $mg \cdot L^{-1}$. The final cell density (OD_{600}) was 0.15. The flasks were incubated at 150 rpm and 5°C, 10°C, 15°C, 25°C, 30°C, and 40°C, respectively.

Salinity and pH

Salinity of culture medium was adjusted to 0.5%, 1%, 2%, 3%, 5% by using NaCl. Stock solution of crude oil (20000 $mg \cdot L^{-1}$) was added into the flasks, and the final cell density (OD_{600}) was 0.13. To test effect of pH on the biodegradation of crude oil, culture medium was adjusted to 4, 5, 6, 7, 9, 10 using 2 $mol \cdot L^{-1}$ of HCl or 2 $mol \cdot L^{-1}$ of NaOH, and the final cell density (OD_{600}) was 0.15.

Preparation of nutrient addition

Nutrient amendment consisting of nitrogen and phosphorus addition was added to MSM to give a C: N: P soil ratio of 200:1:1, 100:1:1, 40:1:1, 20:1:1, 200:1:1, 10:1:1, respectively (calculated on mass of crude oil, nitrigen and phosphorus). Nitrogen was given in the form of ammonium chloride solution (38.98 $mg \cdot mL^{-1}$, pH 7) prepared using deionised water, and phosphorus via monopotassium phosphate solution (21.83 $mg \cdot mL^{-1}$, pH 7) prepared using deionised water. Different amount of ammonium chloride solution and monopotassium phosphate solution were added in the MSM (pH 7.0) which also contain sodium nitrate and sodium hydrogen phosphate. However, the massed of nitrogen and phosphorus in sodium nitrate and sodium hydrogen phosphate were not calculated into total massed of nitrogen and phosphorus. The final cell density (OD_{600}) was 0.14.

Crude oil components utilized preferentially by DG24

The liquid culture medium was sterilized by autoclaving at 121 °C for 20 min. Then, stock solution of crude oil (20000 $mg \cdot L^{-1}$) dissolved in dichloromethane was filtered by 0.2 μm filter membrane into the flasks containing 150 mL of MSM (pH 7.0-7.2), and the final crude oil concentration was 2000 $mg \cdot L^{-1}$. The flask was put in the table concentrator in order to remove dichloromethane. Cells of DG24 re-suspended in MSM were inoculated into the flasks, and the final cell density was 0.13. The flask was incubated at 150 rpm and 10 °C for 35 days.

After incubation for 35 days, 50 mL of water phase in the flask which became brown was transferred into 250 mL separating funnel, and extracted by equal volume of solvent dichloromethane and acetone (V:V was 3:1) for 3 times in order to extract the whole crude oil components in the water phase. The organic phase was ultrasonic- extracted for 3 times and 10 min each time, and transferred into a beaker (treated by nitric acid) in order to volatilize dichloromethane and acetone. Then, 2 mL of dichloromethane was supplemented into amber gas chromatotraphy vials possessing Teflon lined crimp-top seals and analyzed by GC-MS (450GC-320MS, Bruker Daltonics, Massachusetts, USA) according to Hua *et al.* (in press).

Biodegradability analysis of different alkanes

Biodegradation of saturable alkanes, including dodecane ($C_{12}H_{26}$), tetradecane ($C_{14}H_{30}$), hexadecane ($C_{16}H_{34}$), octadecane ($C_{18}H_{38}$), nonadecane ($C_{19}H_{40}$), docosane($C_{22}H_{46}$),

hexacosane ($C_{26}H_{54}$), and octacosane ($C_{28}H_{58}$) by *Bacillus* sp. DG24 as the sole carbon and energy source were studied. Stock solution of alkanes (1000 mg·L^{-1}) that dissolved in hexane (C_{12}-C_{19}) and diethyl ether (C_{22}-C_{28}) were injected into 250-mL Erlenmeyer flasks filtered by 0.2 ¼m filter membrane. The final alkane concentration in the MSM was 400 mg·L^{-1}. After hexane ordiethyl ether in the medium was removed through volatile, cells of DG24 re-suspended in the MSM were inoculated into the flasks. The inoculum of DG24 was 5% (v/v), and cell density at OD_{600} was about 0.145. Then, the flasks were incubated at 150 rpm and 10°C. After incubation for 8 days, residual alkanes in the medium was extracted and analyzed. The control group that not supplemented with cells was used for abiotic loss analysis of alkanes as described by Hua and Wang (2013).

Ultrastructure observation

Cellular hydrocarbons were observed by TEM (Transmission Electron Microscopy). After incubation for 8 days, cells grown on 400 mg·L^{-1} of *n*-hexadecane and *n*-octadecane were collected and used for the ultrastructural studies. Meanwhile, stock solution of naphthalene and phenanthrene (800 mg·L^{-1}) that dissolved in acetone were also injected into 250-mL Erlenmeyer flasks filtered by 0.2 ¼m filter membrane. The final alkane concentration in the MSM was 200 mg·L^{-1}. The inoculum of DG24 was 5% (v/v), and cell density at OD_{600} was about 0.14. Then, the flasks were incubated at 150 rpm and 10°C. After incubation for 8 days, cells of DG24 were collected and used for the ultrastructural studies. The pre-treatment method for TEM observation was as described by Hua *et al*. (in press).

RESULTS

Preferentially utilized crude oil components

The preferentially utilized crude oil components by *Bacillus* sp.DG24 were list in Table 1. The results showed that medium or long chain n-alkanes C_{12}-C_{29} were the major components, such as decane (6.431 min), tetradecane (12.411 min), hexadecane (14.901 min), octadecane (17.138 min), eicosane (19.166 min), docosane (21.011min), and hexacosane (24.281 min). Furthermore, some low ring aromatic hydrocarbons were also the major preferentially utilized crude oil components by *Bacillus* sp.DG24. For instance, Phenol (6.124 min); 7 Benzene, 1,2,4,5-tetramethyl (8.475 min), and Naphthalene, 1-methyl (11.456 min). Similarly, Acinetobacter sp. was found to be capable of utilizing n-alkanes of chain length C_{10} –C_{40} as a sole source of carbon (Throne-Holst *et al*., 2007).

Effect of substrate concentration, temperature, salinity, pH and nutrients on the biodegradation of crude oil

Effects of environmental factors on the biodegradation of crude oil by *Bacillus* sp. DG24 were presented in Fig. 1. Under the condition test of different initial oil concentrations, the crude oil removal percentage ranged from 61.47±5.14% to 36.37±4.18%, and the removal percentage decreased along with the increase of the initial oil concentration. Meanwhile, it was found that temperature also had an obvious influence on the biodegradation of crude oil. When the incubation temperature was 25°C, crude oil removal percentage achieved the highest vale with 48.49±4.11%. Moreover, under the condition of 10°C, biodegradability of crude oil was 33.48±4.173%, which indicated that *Bacillus* sp. DG24 could be used to the bioremediation of oil polluted soil under low-temperature regions. In the studies, it was also found thatstrong acid or alkali conditions and high salinity had negative effect on the removal of crude oil. The optimal pH and salinity values for crude oil removal percentage of crude oil by *Bacillus* sp. DG24 were 7 and 1%, respectively. Accordingly, the highest removal percentages of crude oil were 53.58 ±4.36% and 45.55 ± 2.58%, respectively. Meanwhile, nutrients had positive effect on the biodegradation of crude oil. The result suggested that the optimal nitrogen and phosphorus addition was 10:1:1, and the corresponding removal percentage was 62.58 ± 2.54%.

Biodegradability of different alkanes

As shown in Figure 2, the results showed that the biodegradability of alkanes decreased along with the increase of molecular weight. For example, the biodegradability of dodecane, octadecane, and hexacosane was 53.27±3.15%, 34.28±3.81%, and 22.48±3.67%, respectively. Meanwhile, it was also found that cell density of *Bacillus* sp.DG24 when growing on alkanes decreased along with the increase of carbon length, which inferred that the isolate utilized low molecular weight alkanes easily. The final cell density of DG24

when growing on tetradecane was 1.66, while, this value declined to 0.63 when growing on octacosane.

Transmission Electron Microscopy studies of inclusions

Intracellular inclusion bodies were observed by TEM in cells of *Bacillus* sp. DG24 when growing on hydrocarbons (Fig.3). Results showed that cells grown on *n*-hexadecane or *n*-octadecane displayed irregular type inclusions, while, when grown on naphthalene or phenanthrene, elliptic clear-vesicle type inclusions were observed (Fig. 3C-D). Meanwhile, some cells had one inclusion, and some cells contained more than one inclusion.

DISCUSSION

Some studies have reported a negative effect of the initial oil concentration on crude oil removal. For example, for *Yarrowia lipolytica*, increases in the initial oil concentration have a negative effect on oil removal (Ferreira *et al*., 2012). However, low crude oil concentration also limited the biodegradation of oil since indigenous microbial did not have sufficient carbon source and biomass was limited (Wiggins and Alexander, 1988). There is little information about the effect of salinity on bioremediation of soils, but studies have shown that high salinity had negative effect on the biodegradation of hydrocarbons. The effect of salinity on microbial cells varies from disrupted

Table 1. Preferentially utilized crude oil components by *Bacillus* sp. DG24

No	Retention Time (min)	Name	Molecular formula	Molecule weight
1	6.124	Phenol	C_6H_6O	94
2	6.431	Decane	$C_{10}H_{22}$	142
3	8.056	Undecaneÿ	$C_{11}H_{24}$	156
4	8.475	Benzene, 1,2,4,5-tetramethyl	$C_{10}H_{14}$	134
5	8.418	Benzene, 1-ethyl-2,3-dimethyl	$C_{10}H_{14}$	134
6	8.978	Benzene, 1,2,3,4-tetramethyl	$C_{10}H_{14}$	134
7	9.604	Dodecane	$C_{12}H_{26}$	170
8	9.781	Decane, 2,5,9-trimethyl	$C_{13}H_{28}$	184
9	10.638	Undecane, 4,7-dimethyl	$C_{13}H_{28}$	184
10	11.232	Tridecane	$C_{13}H_{28}$	184
11	11.456	Naphthalene, 1-methyl	$C_{11}H_{10}$	142
12	12.076	Dodecane, 2,6,10-trimethyl	$C_{15}H_{32}$	212
13	12.411	Tetradecane	$C_{14}H_{30}$	198
14	13.695	Pentadecane	$C_{15}H_{32}$	212
15	14.317	Naphthalene, 2,3,6-trimethyl	$C_{13}H_{14}$	170
16	14.901	Hexadecane	$C_{16}H_{34}$	226
17	16.053	Heptadecane	$C_{17}H_{36}$	240
18	16.503	1-Decanol, 2-hexyl	$C_{16}H_{34}O$	242
19	17.138	Octadecane	$C_{18}H_{38}$	254
20	18.178	Nonadecane	$C_{19}H_{40}$	268
21	18.797	Phytol	$C_{20}H_{40}O$	296
22	19.166	Eicosane	$C_{20}H_{42}$	282
23	20.112	Heneicosane	$C_{21}H_{44}$	296
24	21.011	Docosane	$C_{22}H_{46}$	310
25	21.877	Tricosane	$C_{23}H_{48}$	324
26	22.711	Tetracosane	$C_{24}H_{50}$	338
27	23.511	Pentacosane	$C_{25}H_{52}$	352
28	24.281	Hexacosane	$C_{26}H_{54}$	366
29	25.021	Heptacosane	$C_{27}H_{56}$	380
30	25.821	Octacosane	$C_{28}H_{58}$	394
31	26.744	Nontacosane	$C_{29}H_{60}$	408

tertiary protein structures and denatured enzymes to cell dehydration, with different species having different sensitivities to salt (Ulrich *et al*., 2009). For example, for the culture *Rhodococcus erythropolis* DCL14, in the presence of 1.0, 2.0 or 2.5% (w/v) NaCl, the lag phase of the cultures increased and growth rates decreased with increasing NaCl concentrations during the biodegradation process of C_5 to C_{16} hydrocarbons (de Carvalho and da Fonseca, 2005). Hydrocarbon mineralization is favored by near neutral pH values. It is common practice to add lime to bioremediate

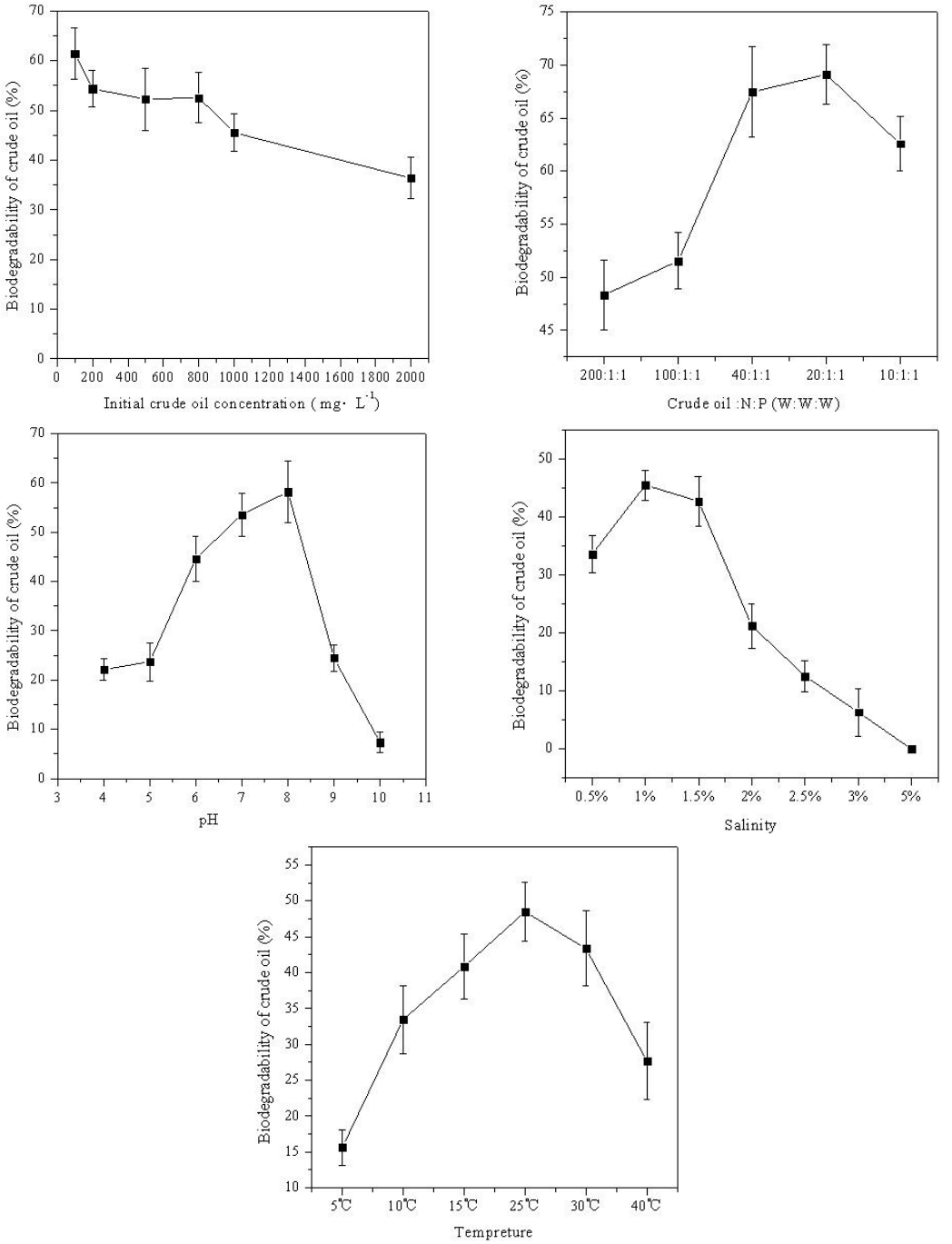

Fig. 1. Effect of environmental factors on crude oil biodegradation by *Bacillus* sp. DG24.

acid soils containing harmful organic compounds (Margesin and Schinner, 2001). Among the environmental influences known to affect biodegradation of soil petroleum hydrocarbons, temperature and nutrient availability are two most important ones, particularly in cold-region soils (Walworth *et al*., 2001; Ruberto *et al*., 2009). The low concentrations of N and P available for bacterial growth and the imbalance in the C:N:P ratio are other relevant factors that can successfully influence the biodegradation of hydrocarbon pollutants. Biostimulation provides adequate nutrient levels to increase degradation activity by the natural soil microflora during the bioremediation process. For instance, soil from Ft. Wainwright, AK responded positively as temperatures were increased from 1°C to 21°C, but microbial respiration decreased when temperatures were raised to 41°C. Microbial activity increased when 100 or 200 mg/kg of supplemental nitrogen was applied (Walworth *et al*., 2001). Similarly, when biostimulation was applied at a C:N:P ratio of 100:12.9:2.6, the indigenous microflora suggested a high adaptation to the presence of the added level of nutrients (Ruberto *et al*., 2009). While, when the environmental temperature is above 40 °C, the membrane becomes more susceptible to hydrocarbon toxicity and causes a reduction in microorganism enzymatic activity and therefore reduces the hydrocarbon biodegradation rate (Ferreira *et al*., 2012). On the other hand, improper supplement of nutrients can also inhibit the biodegradation activity of petroleum hydrocarbons (Chaillan *et al*., 2006). No significant difference in biodegradation rates was observed between fertilised and non-fertilised plots, which was attributed to the high background level of N and P in the site under study (Oudot *et al*., 1998). Moreover, a permanent inhibition of hydrocarbons assimilation was recorded with a high input of nutrients. The biodegradation of saturates, aromatics and polars was respectively, permanently, temporally and not reduced by excessive fertilization in soil (Chaineau *et al*., 2005).

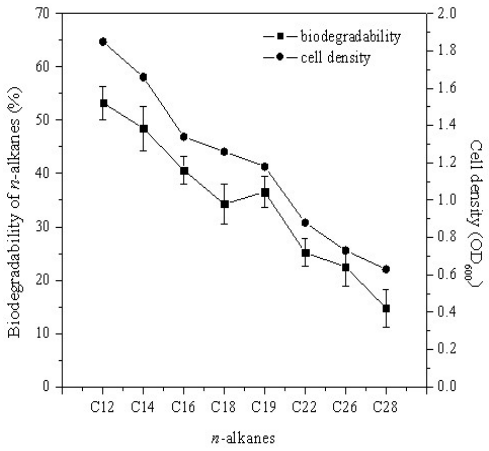

Fig. 2. Biodegradability of different alkanes by *Bacillus* sp. DG24

Alkanes are major constituents of petroleum hydrocarbons. Degradation of alkanes is a wide spread phenomenon in nature, and numerous microorganisms, both prokaryotic and eukaryotic, capable of utilizing these substrate s as a carbon and energy source have been isolated and characterized (Wentzel *et al*., 2007). *Cladosporium resinae* growing on alkane mixtures removed n-alkanes sequentially in order of increasing molecular weight since the uptake of shorter alkanes is competitively favoured by the uptake mechanism (Lindley and Heydeman, 1986). Similarly, *Bacillus thermoleovorans* B23 and H41 was reported to be able to utilize C_9-C_{30} alkanes (Kato *et al*., 2001). In the review of van Beilen and Funhoff (2007), two unrelated classes of enzymes for long-chain n-alkane oxidation were proposed:

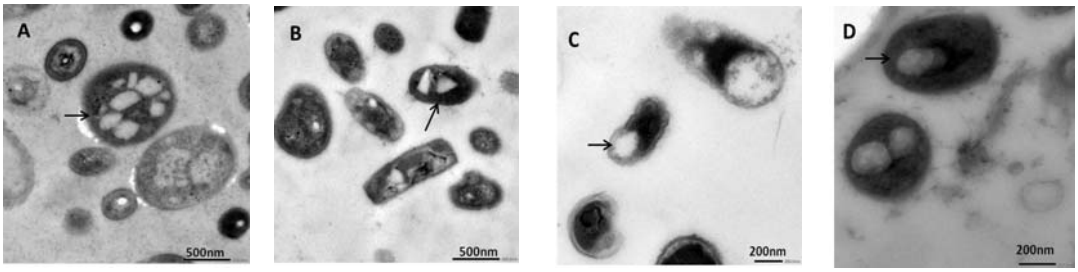

Fig. 3. Transmission Electron Microscopy of *Bacillus* sp. DG24 grown on *n*-hexadecane (A), *n*-octadecane (B), naphthalene (C), and phenanthrene (D). Arrows show inclusions of hydrocarbons

(1) the class of cytochrome-P450-related enzymes in both yeasts and bacteria, e.g., bacterial CYP153 enzymes, and (2) the class of bacterial particulate alkane hydroxylases (pAHs).

In our studies, inclusion bodies were obviously observed in the cell of DG24. Some studies have reported the same results. For example, sphere-shaped structure and disc-shaped inclusions were found in the cells of *Rhodococcus opacus* strain PD630 during cultivation on gluconate and phenyldecane (Alvarez *et al*., 1996). For *Streptomyces* strain, an increased proportion of *n*-hexadecanoic acid suggested that the alkane-utilizing microorganism tended to accumulate fatty acids with chains equivalent in length to those of the alkane substrate (Barabas *et al*., 2001). Similarly, the principal fatty acids of cellular lipids drived from *Pseudomonas aeruyinosa* 44T1 varied with the carbon source employed (Deandres *et al*., 1991).

CONCLUSIONS

Microorganisms useful for bioremediation must survive and be active under in situ conditions. The results showed that initial crude oil concentration, salinity, and pH had negative effect. In acidic or alkaline pH, or high salt concentrations conditions, could inhibit the biodegradation ability of microorganisms. For example, when salinity was 5%, no biodegradation occurred which inferred that *Bacillus* sp.DG24 was sensitive to salinity. The optimal pH ranged from 6 to 8, and the biodegradability of crude oil ranged from 44.64±4.54% to 58.17±6.21%. Nutrients could obviously promote the biodegradation of crude oil. Meanwhile, temperature above 30°C could inhibit the biodegradation of crude oil. However, at low temperature condition, *Bacillus* sp. DG24 could utilize crude oil well, and the preferentially utilized oil components were mainly C_{10}-C_{28} alkanes.

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