JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. 7(4), p. 3105-3109

Bioaugmentation of Oil-degradation Bacteria Isolated from the Petroleum-contaminated Soil

Lina Qu[1,2], Mingming Tu[1], Hongbai Jia[1] and Qiuyu Wang[1]*

[1]Department of Lifescience, Northeast Forestry University, 150040,Harbin, Heilongjiang, China.
[2]Department of Lifescience, Daqing Normal University, 163712, Daqing, Heilongjiang, China.

(Received: 23 September 2013; accepted: 06 November 2013)

Five bacterial strains isolated from the long-term petroleum-contaminated soil in the Daqing oilfield were used to bioremediated the petrol-oil pollution in soil. They were identified as *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Acinetobacter junii*(1), *Acinetobacter*[1] and *Microbacterium oxydans*. The results of oil-degrading efficiency showed that under both liquid and solid (i.e. soil) culture conditions, the five bacterial strains showed high petroleum hydrocarbon degradation ability. By regulating the concentration of initial salt, nitrogen and phosphorus in the medium, we explored the optimal conditions for growth and crude oil-degradation of the bacterial strains. The strain X3 showed higher petroleum degradation rate in both of 1% and 3% salt concentration, more than 60%. And the highest oil degradation rate required NH_4NO_3 and $K_2HPO_4{\cdot}3H_2O$ /KH_2PO_4 in mineral medium. This study provides new bacterial resources and information for the autochthonous bioaugmentation of oil contaminated soil.

Key words: Autochthonous bioaugmentation, Oil-contaminated soil, Petroleum hydrocarbon, Bacteria, Degradation rate.

Autochthonous bioaugmentation is a new technology by which autochthonous microorganisms isolated from petroleum-contaminated soil, and are enriched and reused for the bioaugmentation of the petroleum-contaminated soil first used in 2007 (Ueno 2007). Bento *et al*. (2005) isolated a bacterial strain J from the biostimulation soil that could efficiently degrade the contaminated soil. Following enrichment, they put the strain J back into the contaminated soil and tested the efficiency of the bioaugmentation. It was found that strain J induced a TPH (Total Petroleum Hydrocarbons) degrading rate of about 50%, which was higher than that induced by the exogenous strain. Ren XJ *et al*. (2012) isolated three bacterial strains from petroleum-contaminated soil for a bioaugmentation test, and found that these strains not only significantly reduced the TPH content of the soil, but also promoted the bacterial growth and the production of biosurfactants (De 2005; Coulon 2012). The pollutants degradation rates were obviously improved in the role of native or exogenous microorganism (Mahjoubi; Kim 2013; Zhang Jian 2012; Zedelius 2011; Fernando Rojo 2009). The above observations indicate that autochthonous microorganisms are advantageous in bioaugmentation compared to the exogenous bacterial strains(Timothy 1996,Das 2007). In the present study, we isolated some bacterial strains from crude oil contaminated soil, which can efficiently utilize the petroleum hydrocarbons. In addition, the bioaugmentation conditions of the strains by modulating the concentrations of N, P, saltions, and pH of the medium

Samples and medium

The crude oil was collected from the Daqing oilfield in Heilongjiang Province. Five oil-degrading bacterial strains were isolated from the

* To whom all correspondence should be addressed.
Tel.:+86 18745052420;
E-mail: wqy11@sina.com (Qiuyu Wang)

long-term petroleum-contaminated soil in the Daqing oilfield. They were identified as *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Acinetobacter junii*[1], *Acinetobacter junii*[2] and *Microbacterium oxydans* respectively and were named as X3, X5, X8, X11 and X12 in the later research.

Liquid enrichment medium consisted of some chemical groups (in g/L): NaCl 1.0, $K_2HPO_4 \cdot 3H_2O$ 1.0, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, NH_4NO_3 1.0, $CaCl_2$ 0.02, trace volume $FeCl_3$, beef extract 0.3%, and peptone 1%, pH7.0. Isolation culture medium consisted of some chemical groups (in g/L): beef extract 3, peptone 10, NaCl 5, pH 7.0-7.2; For the solid medium, 20g/L agar was supplemented.

Inorganic salt culture medium consisted of some chemical groups (in g/L): NaCl 1.0, $K_2HPO_4 \cdot 3H_2O$ 1.0, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, NH_4NO_3 1.0, $CaCl_2$ 0.02, and a trace amount of $FeCl_3$, pH7.0.

Oil-degrading experiment of the strains

The bacteria were cultured overnight on a shaker and then transferred into the inorganic salt medium containing 1% (w/v) petroleum and also into the soil extract liquid at a volume ratio of 1% (v/v), cultured at 30! on a shaker at 160 r/min. In the control sample sterile water was added into the same medium instead of strain cultured. On day 5, 10 and 15, three replications for each culture were used for extraction (Holt J G 1994) to determine the petroleum degradation rate. The degradation rate was calculated as follows:

$$\text{Degradation rate} = [1 - (W_1 - W_2)] / W_0 \times 100\%$$

W_0 is the initial petroleum mass (g), W_1 is the mass of the flask and the residual petroleum in the sample (g), and W_2 is the mass of the flask (g). The bacterial liquid cultured overnight was transferred into the bioclean contaminated soil containing 1% (w/v) petroleum at a volume ratio of 3% (v/v), and well mixed. The bacteria-soil mixture was cultured at 30!. On day 5, 10 and 15, three replications for each culture were used for extraction. The degraded soil was sequentially rinsed with hexane, methylene chloride and chloroform, and the supernatants of the three washes were mixed and placed at 60! in a fume hood to make the organic solvents fully volatilizing. Then the supernatants were cooled down in a desiccator and weighed to determine the petroleum degradation rate.

In the inorganic salt medium, the salt concentration was changed to 1%, 3%, 5%, and 7%. Nitrogen source and phosphate source was changed from NH_4NO_3 to $NaNO_3$, $(NH4)_2SO_4$, or NH_4Cl, and from KH_2PO_4/K_2HPO_4 to KH_2PO_4, K_2HPO_4, or NaH_2PO_4, respectively, but keeping a constant nitrogen and phosphate content in the whole operational process. The bacterial liquid cultured overnight was transferred into the modulated inorganic salt medium at a volume ratio of 1% (v/v), and cultured at 30°C on a shaker at 160 rpm for seven days. OD value and petroleum degradation rate of the bacteria were measured.

RESULTS AND DISCUSSION

Oil degradation of the strains in the inorganic salt medium

The X3 and X5 strains in the inorganic salt medium had a high oil degradation of ~50% for the early 15 days and eventually reached a peak of 60%. Compared to the control, all 5 strains could make good use of petroleum hydrocarbons when petroleum is the only carbon source (Fig. 1).

Oil degradation of the strains in soil-containing culture medium

The soil-containing culture medium provides an environment which is close to the natural environment for the petroleum-degrading bacteria. After 15 days' treatment, X3 and X5 showed the highest degradation of 75% and 72%, respectively, followed by X11 (68%), X8 (64.67%), and X12 (59%). These observations indicated a slight difference in degradation efficiency of the strains; moreover, their petroleum degradation efficiencies were much higher than that of the control (Fig. 2). It is confirmed that these strains can well degrade the petroleum under solid culture conditions. Hence, all 5 strains are very efficient petroleum-degrading strains, and can be used for *in situ* and ectopic bioremediation of soil.

All the five bacterial strains isolated from Daqing oilfield showed strong tolerance to salt and alkali soil condition. The isolated bacterial strains were all autochthonous microorganisms in the contaminated region and had restrict environmental requirements for growth and nutrients. Hence, it is necessary to explore the effects of nutrient type, matrix amount, and mixed inocula on pollutant degradation during the

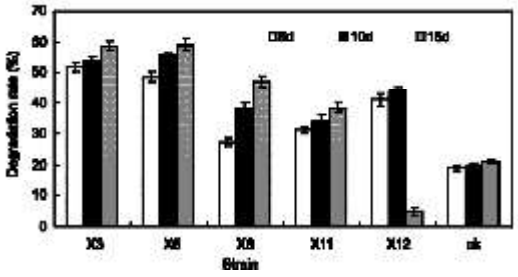

Fig. 1. Oil degradation rate of five strains in the inorganic salt medium. All five strains inoculated into 25ml inorganic salt medium containing 1%(w/v) crude oil were cultured under condition of 30! and 160rpm in 50ml conical flask. The ability to degrade crude oil of these isolates was compared with a control sample named ck in which no strains were inoculated. The oil degradation was determined by soxhlet extraction method on day 5, 10 and 15

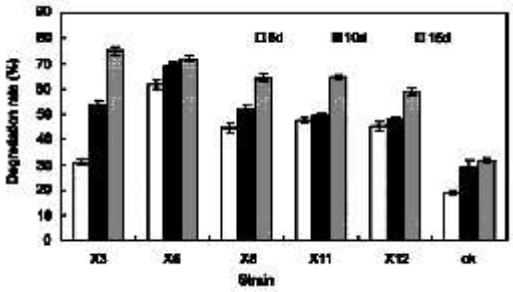

Fig. 2. Oil degradation rate of five strains in the soil solid culture medium. All five strains inoculated into 25ml soil solid culture medium with 1%(w/v) crude oil were cultured under condition of 30°C and 160rpm in 50ml conical flask. The ability to degrade crude oil of these isolates was compared with a control sample named ck in which no strains were inoculated. The oil degradation rate were determined by soxhlet extraction method at the days of 5,10 and 15

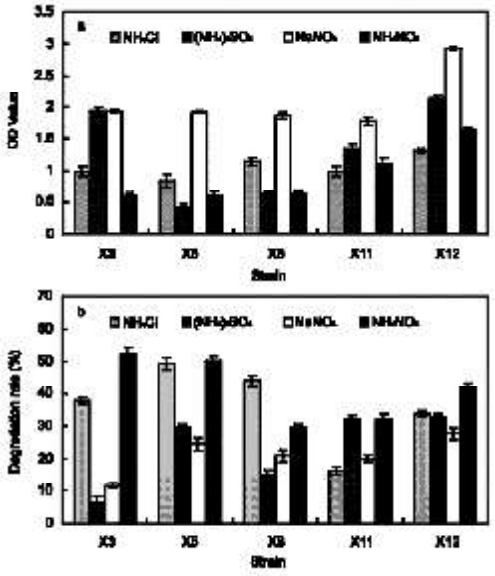

Fig. 3. Effects of different nitrogen sources on the growth and oil degradation rate of all strains. Equal nitrogen of $NaNO_3$0$(NH4)_2SO_4$ and NH_4Cl was added into inorganic salt medium individually as nitrogen source instead of NH_4NO_3. All strains were cultured 7 days in the four kinds of nitrogen source inorganic salt culture under 30°C and 160rpm. 25ml cluture with strains was added into each 50ml conical flask. a: Growth of five strains under different nitrogen sources.The OD values of X3,X5 and X8 were measured under 320nm, while X11 and X12 were 300nm. b: Oil degradation rate of all strains under different nitrogen sources. All five strains inoculated into 25ml four kinds of nitrogen source inorganic salt medium with 1%(w/v) crude oil were cultured 7 days under condition of 30°C and 160rpm in 50ml conical flask. The oil degradation rate were determined by soxhlet extraction method

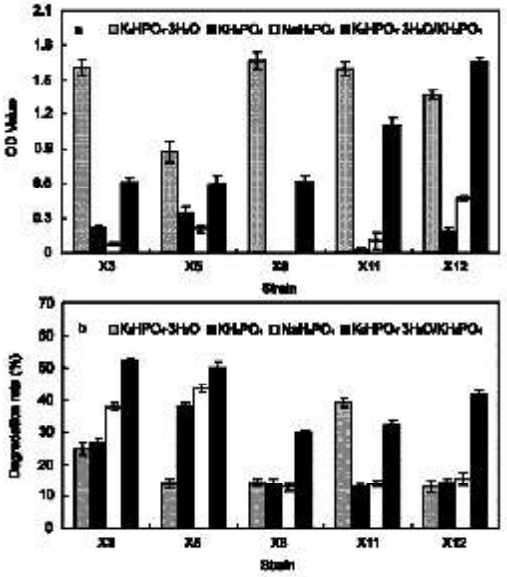

Fig. 4. Effects of different phosphate sources on the growth and oil degradation rate of all strains. Growth of all strains under different phosphate sources. Equal phosphorus of $KH_2PO_4$0$K_2HPO_4$0NaH_2PO_4 was added into inorganic salt medium individually as phosphate source instead of KH_2PO_4/K_2HPO_4. The ratio of KH_2PO_4 and K_2HPO_4 was 1:1 in inorganic salt medium. All strains were cultured in inorganic salt culture under 30! and 160rpm. 25ml cluture with strains was added into each 50ml conical flask. a:The OD values were measured at the seventh day. The five strains' OD value was the same as nitrogen sources experiment.b: Oil degradation rate of all strains under different phosphate sources. All five strains inoculated into 25ml four kinds of phosphate source inorganic salt medium with 1%(w/v) crude oil were cultured 7 days under condition of 30°C and 160rpm in 50ml conical flask. The oil degradation rate were determined by soxhlet extraction method

microbial remediation process based on the geographical features. These features include geography, climate, soil characteristics, contamination intensity, and groundwater depth. By this means, we should be able to establish a bioaugmentation plan suitable for autochthonous microorganisms to maximize bioremediation effect of the indigenous organisms.

Oil degradation of the strains in different nitrogen source

When $NaNO_3$ was the nitrogen source, all 5 strains showed the best growth but the lowest petroleum degradation. However, when $(NH_4)_2SO_4$ was used as the nitrogen source, the strains showed moderate growth but a high petroleum degradation (Fig. 3). Overall, the high petroleum degradation rate of the strains seems to show inverse correlation with $NaNO_3$ as nitrogen source, a possible reason may be the activity of bacterial enzymes related in petroleum hydrocarbon degradation varied under different nitrogen source conditions. The oil degradation indicated that it is good to choose NH_4NO_3 as nitrogen source when the strains are used to bioremediate oil pollution.

Oil degradation of the strains in different phosphate source

All the five strains grew well when $K_2HPO_4 \cdot 3H_2O$ or $K_2HPO_4 \cdot 3H_2O$ /KH_2PO_4 (1: 1) were used as phosphate sources. In contrast, the strains showed poor growth when KH_2PO_4 and NaH_2PO_4 as the phosphate sources, especially for X8 which did not grow (Fig.4). Overall, all the strains showed both good growth and an ideal petroleum degradation rate when $K_2HPO_4 \cdot 3H_2O$ /KH_2PO_4 (1: 1) were as the P sources.

All strains expect X11 showed the highest petroleum degradation rate when $K_2HPO_4 \cdot 3H_2O$ was used as the phosphate source, but they showed best growth in the medium with $K_2HPO_4 \cdot 3H_2O$ /KH_2PO_4 (1: 1) as phosphate source. This was probably because the expression of bacterial enzymes required for petroleum degradation was regulated by different nitrogen and phosphate sources, which was some correlated to gene regulation involved in bacterial growth. Audrew *et al*.(1992) found that when fertilizer with a C: N: P ratio of 100:5:1.7, where the N source was NH_4^+ and the P source was PO_4^{3-}, was added to the oil-contaminated soil and slowly released, the biodegradability was the optimal, which is consistent with our results. Therefore, to carry out microbial remediation by bioaugmentation, it is essential to first determine the N and P sources, and then to optimize the concentration of different supplement to maximize the degrading efficiency. Our future work will be focused on demonstrating the molecular mechanisms underlying the microbial degradation of petroleum hydrocarbons and identifying the degradation relevant genes using molecular biological techniques based on the selection of oil-degradation bacteria. Van Beilen (2001)reported that in *Pseudomonas putida* GPO1, the operon encoding the enzyme involved in alkanes degradation is located in the OCT plasmidsuggested that the alkB operon that controls alkanes degradation in *Burkholderia cepacia* can be easily negatively regulated by the repressive metabolites compared to the operon in *P.putida* GPO1. Demonstrating the type, activity, sequence, and regulation mechanism of petroleum hydrocarbon-degrading enzymes will greatly help the development of petroleum hydrocarbon-degrading bacterial strains for very efficient bioremediation.

ACKNOWLEDGMENTS

Foundation item: "948"Project from National Forestry Bureau in China (2008-4-34).

REFERENCES

1. Akio Ueno, Yukiya Ito, Isao Yumoto, Hidetoshi Okuyama., Isolation and Characterization of Bacteria from Soil Contaminated with Diesel Oil and the Possible Use of these in Autochthonous Bioaugmentation. *World J Microbiol Biotechnol* 2007; **23**: 1739-1745. doi:10.1007/s11274-007-9423-6
2. Al-Mailem DM, Eliyas M, Radwan SS., Oil-bioremediation potential of two hydrocarbonoclastic, diazotrophic Marinobacter strains from hypersaline areas along the Arabian Gulf coasts. Extremophiles 3:Epub ahead of print, 2013.
3. Andrew R Autry, Gary M., Ellis. Bioremediation: An effective semedial alternative for petroleum hydrocarbon-contamition. *Environment Progress* 1992; **11**: 318-322. doi: 10.1002/ep.670110420
4. B. Lal, S. Khanna., Degradation of crude oil by

Acinetobacter calcoaceticus and *Alcaligenes odorans*.*J App Microbio* 1996; **81**: 355-362. doi: 10.1111/j.1365-2672.1996.tb03519.x

5. Baoming Li., The Microbiology remediation of petroleum polluted soil. *Doctor Dissertation of Agricultural Sciences*, 2007.
6. Bergey, David Hendricks, Breed, Robert S, Everitt George Dunne,Smith Nathan R., Bergey's Manual of Determinative Bacteriology. Williams & Wilkins Baltimore, US, 1957.
7. Coulon F, Brassington KJ, Bazinb R, Linnetb P E, ThomasbK A, Mitchellb T R, Lethbridgeb G J, Smithb W N,Pollardet S J T., Effect of fertilizer formulation and bioaugmentation on biodegradation and leaching of crude oils and refined products in soils. *Environ Technol*,2012; **33**: 1879-1893. doi:10.1080/09593330.2011. 650221.
8. Fatima M. Bentoa, Flávio A.O. Camargoa, Benedict C. Okekeb, William T. Frankenbergerb., Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation & bioaugmentation. *Bioresource Technology*,2005; **6**:1049-1055. doi:10.1016/j.biortech. 2004.09.008,
9. Fernando Rojo., Degradation of alkanes by bacteria. *Environ Microbiol*,2009; **11**: 2477-2490. doi: 10.1111/j.1462-2920.2009.01948.x
10. Jan B. van Beilen, Marcel G. Wubbolts, Bernard Witholt., Genetics of alkane oxidation by *Pseudomonas oleovorans*. *Biodegra*,1994; **5**:161-174. doi:10.1007/BF00696457
11. Jan B. van Beilen, Sven Pankea, Sacha Lucchinib, Alessandro G. Franchini, Martina Röthlisberger and Bernard Witholt., Analysis of *Pseudomonas putida* alkane degradation gene clusters and flanking insertion sequences: Evolution and regulation of the *alk* genes. *Microbio*,2001; **147**: 1621-1630.
12. Jian Zhang, Xu Zhang, Juan Liu, Rong Li, , Biao Shen., Isolation of a thermophilic bacterium, *Geobacillus* sp. SH-1, capable of degrading aliphatic hydrocarbons and naphthalene simultaneously, and identification of its naphthalene degrading pathway. *Bioresour Technol*. 2012; **124**: 83-89. doi:10.1016/ j.biortech.2012.08.044.