Australian Journal of Basic and Applied Sciences, 3(3): 1512-1516, 2009
ISSN 1991-8178
© 2009, INSInet Publication

Change in Cytochromes Contetn of *Pseudomonas* Sp in the Medium Contaning Petroleum

[1]Saeed Minoui, [2]Dariush Minai-Tehrani, [3]Gita Eslami [2]Zahra Sobhani-Damavandifar

[1]Institute of Environmental Sciences, Shahid Beheshti University,G.C.
[2]BioResearch lab, Faculty of Biological Sciences, Shahid Beheshti University,G.C.
[3]Microbiology Section, Shahid Beheshti University of Medical Sciences

Abstract: The respiratory chain of bacteria consists of different cytochromes and terminal oxidases, which allow their adaptation to various media. *Pseudomana*s is a useful bacterium for industries, it can aerobically biodegrade some type of toxic material in the soil and water. One of the most important organic pollutants in oil producing countries is crude oil. *Pseudomonas* is able to biodegrade crude oil and uses it as a carbon source. Our results show that *Pseudomonas* can degrade aliphatic fractions of crude oil more efficiently than aromatic fractions. *Pseudomonas* uses cytochrome *bo* complex as its main terminal oxidase in rich medium, while in medium containing crude oil, it uses both cytochrome *bo* and *bd* complexes as terminal oxidase.

Key words: Crude oil, cytochrome, *Pseudomonas*, respiratory chain.

INTRODUCTION

Pseudomonas belongs to the category of gram-negative bacteria and is useful for industries. In the presence of oxygen, the bacterium uses aerobic biochemical pathway to biodegrade the organic substances and uses oxygen as terminal electron acceptor (Matsushia *et al.,* 1980). The respiratory chain of bacteria contains various cytochromes and different kinds of terminal oxidases (Anraku, 1988; Bartsch, 1968). The important terminal oxidases that use oxygen as an electron acceptor are cytochrome aa_3, *bd* complex and *bo* complex (Poole, 1983; Jones and Poole, 1985). In the presence of a certain substrate, the bacteria may use one of these terminal oxidases as an electron acceptor (Reichmann and Gorish, 1993; Di Tomaso *et al.,* 2002). The respiratory chain of *Pseudomonas aeruginosa* grown aerobically was reported to contain *b*, c_1, *c*, and *o* cytochromes (Matsushia *et al.,* 1980) and effects of growth conditions on formation of cytochromes of a denitrifying bacterium, *Pseudomonas stutzeri* was studied (Kodama, 1970). Crude oil is one of the important organic pollutants of soil and water. Some types of aerobic and facultative anaerobic bacteria use oxygen for biodegradation of oil and change it to H_2O and CO_2 (Cerniglia, 1992). In the aerobic condition oxygen plays an important role in the biodegradation of oil and its components. Aliphatic and aromatic fractions of crude oil are biodegraded by oil-degrading bacteria efficiently (Minai-Tehrani and Herfatmanesh, 2007). *Pseudomonas* is able to biodegrade the crude oil or its components and use them as its sole carbon source in the presence of oxygen (Leahy and Colwell, 1990). In this report the pattern of respiratory chain of *Pseudomonas* in medium containing crude oil is studied and compared with rich medium. On the other hand the effect of bacterium on total crude oil biodegradation is also studied.

MATERIALS AND METHODS

Culture media preparation:

The sait medium was prepared by adding KH_2PO_4 2.5 g, Na_2HPO_4 2.5 g, NH_4NO_3 1 g, $MgSO_4$ 0.2 g, $CaCO_3$ 0.01 g to 1 liter of distilled water and the pH was adjusted to 7.

Heavy crude oil was added to this medium in final concentration of 1% (v/v).

Rich medium was prepared by adding yeast extract 7g, peptone 5g and NaCl 2g per liter of water and pH was adjusted for 7. All chemicals were obtained from Merck Company.

Heavy crude oil (API gravity = 20) (American petroleum Institute gravity) was obtained from Soroush oil field.

Corresponding Author: Dariush Minai-Tehrani (PhD), Shahid Beheshti University, Faculty of Biological Sciences, BioResearch Lab, Chamran Highway, Evin, Tehran, IRAN
Tel: +98-932-9069911; Fax: +98-21-22431664
E-mail: D_MTehrani@sbu.ac.ir

Isolation of Pseudomonas:

The oil-degrading *Pseudomonas* strain was isolated from soil. 1 g of oil-contaminated soil was added to minimal liquid medium containing 1% sterilized crude oil as only carbon source (oil-contaminated soil was obtain from the city refinery) . The medium was cultured for a week in reciprocal shaker in 150 rpm at 30ºC. After a week, 2 ml of cultured medium suspension was transferred to new medium with the same conditions. This transfer was repeated twice. The growing bacteria in the last medium culture were transferred to a plate with solid medium, containing agar-agar enriched with 0.5% hexadecane as carbon source and incubated 72 hours at 30ºC. The growing colonies were isolated and transferred to a new solid medium. One of the colonies that were suspected for the *Pseudomonas* strain was examined for biochemical test to identify the *Pseudomonas.*

Biochemical Tests:

The isolated bacterium was Gram-negative and motile rod-shaped (bacillus) that produced green pigment and showed positive response to catalase and oxidase test (oxidase test was done with tetramethyl-p-phenylene-diamine dihydrochloride) (Collee *et al.,* 1989). This non-fermentative bacterium was also identified after Bromothymol blue test in the presence of glucose (Hugh and Leifson, 1953).

Growth curve:

The growth curve was measured in two conditions including the medium containing 1% crude oil and rich medium, by sampling from liquid medium each four hours and the turbidity was measured in 600nm. For further experiments the stationary phase was selected for respiratory and oil extraction analysis. In this phase the oil dispersion reached to its highest value, but the analysis of respiratory chain showed no significant difference between the logarithmic and stationary phases.

Oil Extraction and Analysis:

The purified *Pseudomonas* was cultured in minimal medium containing 1% heavy crude oil as sole carbon source. The dispersion of oil was observed in the medium which suggested the ability of bacterium for oil degradation. The bacteria were cultured for 72 hours in a reciprocal shaker at 150 rpm at 30ºC. After 72 hours (in the stationary phase) the medium was centrifuged (5000 x g for 30 minutes) to precipitate the cells (the precipitated cell were collected for further experiment). The supernatant was separated for oil analysis. The supernatant was mixed with equal volume of chloroform and transferred to a separating funnel. The suspension was shaken firmly to dissolve the oil fraction of supernatant in solvent phase. The solvent phase was separated and evaporated in a vacuumed chamber for 24 hours. The residue was weighed to determine the amount of total crude oil. The total aliphatic and aromatic fractions of oil were extracted and analyzed according to the method done by Minai-Tehrani (Minai- Tehrani and Herfatmanesh, 2007). The residue was dissolved in n-hexane and filtered. 5 ml of filtered solution was loaded to a 1 x 25 cm glass column filled with Silica gel (20 cm) and sodium sulfate (5 cm as a moist capturing material). The column was pre-washed with n-hexane and 30 ml of n-hexane was used as mobile phase to release aliphatic fractions. The fraction was collected and the solvent was evaporated. The residue was weighed to determine the amount of total aliphatic fractions. To release aromatic fractions from the column, 30 ml of n-hexane/dichloromethane (1:1, v/v) was used as mobile phase and the aromatic fractions were collected and the solvent was evaporated. The residue was weighed to determine the amount of total aromatic fraction. The above procedures were also done for extraction of total aliphatic, aromatic and crude oil in control sample containing sterilized minimal medium with 1% crude oil but no bacteria. The control sample was also incubated in reciprocal shaker for 72 hours at 30ºC.

Cell free extract:

The precipitated cells (from oil extraction procedure) were washed twice with phosphate buffer 0.1 M, pH =7 and centrifuged (7000 × g, 30 minutes at 25ºC). The pellet was weighed to determine the yield of cells and dissolved in phosphate buffer (1g cells/10 ml buffer). To break the cells, cell suspension was sonicated 5 × 20 seconds with 20 seconds interval at 4ºC. The sonicated suspension was centrifuged (12000 × g for 30 minutes at 0-4ºC) to precipitate unbroken cells. The supernatant was used as cell free extract for further experiments.

The isolated *Pseudomonas* was also cultured in rich medium for 72 hours and the above procedures were done for preparation of cell free extract and yield of cells.

Absorption spectra:

Room temperature reduced-minus-oxidized difference spectra of cell free extract were recorded with Perkin Elmer DW2 UV-Visible spectrophotometer according to the method used by Keyhani (Keyhani and Minai, 2001), by adding a few grain of sodium dithionite as reducing agent and 30 ul of 10% H_2O_2 as oxidant to cell free extract. The amount of protein was determined by biuret method (Robyt and White, 1987).

RESULTS AND DISCUSSION

Growth curve:

Figure 1 shows the growth curve of bacteria. The shorter lag period was observed in rich medium while the longer lag period was seen in medium with crude oil. In rich medium the cells entered to stationary phase after 45 hours while in medium containing crude oil the cells entered to this phase after 64 hours. The longer lag and exponential periods for the cells grown in medium containing crude oil suggests that the cells adaptation in crude oil took longer time than rich medium. The comparison of yield of the cells (Fig 1 insert) showed that the growth of cells in rich medium was more efficient than in the crude oil.

Crude Oil Reduction:

The total aliphatic, aromatic and crude oil reduction after 72 hours of incubation has been shown in figure 2 and compared with control. For all the cases the difference between the samples with bacteria and the control (without bacteria) were significant.

Our results showed that the aliphatic fractions of crude oil were biodegraded with higher rate than aromatic fractions (Fig 2). Since the crude oil consists of aliphatics, aromatics, resins and asphaltins (Colwell and Wallker, 1977), the reduction of aliphatc and aromatic fractions occurred higher than asphaltins and resins (Jobson *et al.,* 1972; Walker *et al.,* 1976).

These results suggest that the bacteria have used the oil as their sole carbon source and the aliphatic fractions have been biodegraded more efficiently than the aromatic. Volatilization played the main role in the reduction of oil and its fractions in control sample.

Cytochromes pattern:

The pattern of respiratory chain in culture media was recorded at stationary phase of growth curve (Fig. 3). The pattern of respiratory chain was also studied in logarithmic phase. The pattern was the same in logarithmic and stationary phase (data not shown).

The stationary phase was chosen for further experiments, because the amount of the cells were high enough for cell breakage and the oil was efficiently degraded in this phase.

In alpha region, the peak at 560 nm belonged to *b* type cytochromes, and the peak at 550 nm was related to cytochrome *c*. In beta region, the peak at 530 nm belonged to cytochrome *c*, while the peak at 525 was due to cytochrome *b*. The respiratory chain in crude oil sample had a higher peak at 550 nm than 560 nm, while in rich medium the 560 nm peak was higher than 550 nm peak. The peak at 630 nm was due to cytochrome *bd* complex. Previuos reports indicated the 630 nm for the cytochrome *bd* complex (Keyhani and Minai *et al.,* 2001).

These results suggest that in the presence of rich medium the bacteria have used the cytochrome *bo* complex as their main terminal acceptor while in the oil containing medium, the bacteria may use other terminal acceptors. The presence of 630 nm peak suggests that the cytochrome *bd* complex could be used as another electron acceptor. The presence of cytochrome *bd* complex has been reported in *Pseudomonas aeruginosa* previously (Creezynski *et al.,* 2004). Under anaerobic or semi-aerobic conditions, nitrite induced cytochrome a_2–*c* synthesis in denitrifying bacterium, *Pseudomonas stutzeri.*

The cytochrome *bd* seems to play an important role in oxidative stress, although it is also able to create an electrochemical membrane gradient for energetic requirements (Castresana, 2001). Its role was studied in cyanide resistance of *Pseudomonas pseudoalcaligenes* in medium culture (Quesada *et al.,* 2007).

Conclusions:

Our results show that in the presence of crude oil, *Pseudomonas* can use the aliphatic fractions more efficiently than aromatic fractions. Furthermore it uses *bo* complex and an alternative electron acceptor such as *bd* complex. While in the absence of crude oil, the bacterium mainly uses *bo* complex as a terminal oxidase.

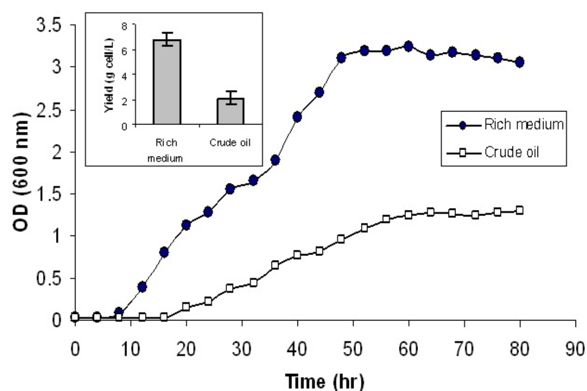

Fig. 1: Growth curve of *Pseudomonas* in rich medium and medium containing crude oil. Insert: the yield of cell in rich medium and in the presence of crude oil.

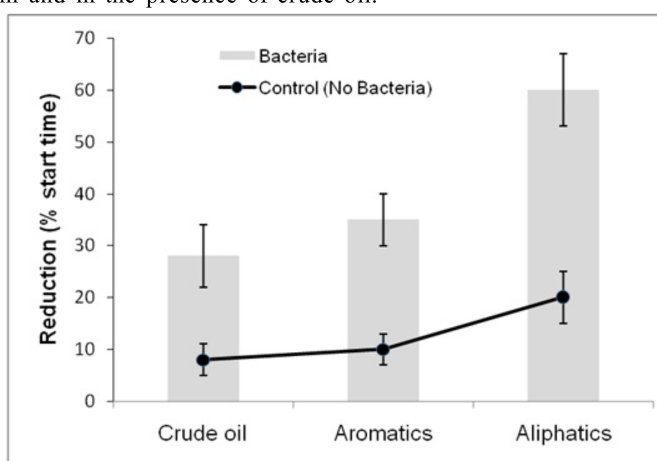

Fig. 2: Reduction of total crude oil, aliphatic and aromatic fractions in the medium with bacteria and without bacteria (control).

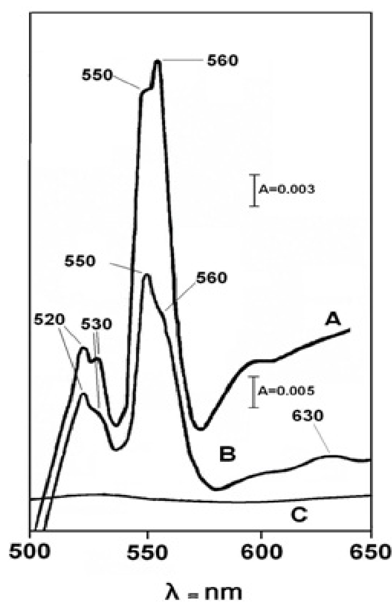

Fig. 3: Dithionite reduced-minus- H_2O_2 oxidized difference spectra of cell free extract of *Pseudomonas*, A= in rich medium (protein amount 6 mg/ml), B= in minimal medium containing crude oil (protein amount 11 mg/ml), C= base line.

ACKNOWLEDGEMENTS

This project was supported by the grant from Shahid Beheshti University.

REFERENCES

Anraku, Y., 1988. Bacterial electron transport chains. Ann.Rev.Biochem., 57: 101-132.

Bartsch, R.G., 1968. Bacterial cytochromes. Ann.Rev.Microbiol., 22: 181-201.

Castresana, J., 2001. Comparative genomics and bioenergetics. Biochim. Biophys. Acta, 1506: 147-162.

Cerniglia, C.E., 1992.Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation, 3: 354-368.

Collee, J.G., J.P. Duguid J.P., A.G. Fraser and B.P. Marmion, 1989. Mackie&McCartney Practical medical microbiology. Churchill Livingstone Pub, 13ed. P: 152.

Colwell, R.R. and J.D. Walker, 1977. Ecological aspects of microbial degradation of petroleum in the marine environment. Crit.Rev.Microbiol., 5: 423-445.

Creczynski-Pasa, T.B. and R.V. Antonio, 2004. Energetic metabolism of *Chromobacterium violaceum*. Genet. Mol. Res., 3: 162-166.

Di Tomaso, G., S. Fedi, M. Carnevali, M. Manegatti, C. Taddei and D. Zannoni, 2002. The membrane-bound respiratory chain of Pseudomonas pseudoalcaligenes KF707 cells grown in the presence or absence of potassium tellurite. Microbiology, 148: 1699-1708.

Hugh, R. and E. Leifson, 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. J.Bactriol., 66: 24-26.

Jones, C.W and R.K. Poole, 1985. The analysis of cytochromes. Meth.Microbiol., 18: 285-328.

Reichmann, P. and H. Gorisch, 1993. Cytochrome c550 from Pseudomonas aeruginosa. Biochem J. 289: 173–178

Jobson, A., F.D. Cook F.D and D.W.S. Westlake, 1972. Microbial utilization of crude oil. Appl. Microbiol, 23: 1082-1089.

Kodama, T., 1970. Effects of growth conditions on formation of cytochrome system of a denitrifying bacterium, Pseudomonas stutzeri. Plant.Cell Physiol., 11: 231-239.

Keyhani, E. and D. Minai-Tehrani, 2001.The binding of cyanide to cytochrome d in intact cells, spheroplasts, membrane fragments and solubilized enzyme from Salmonella typhimurium. Biochim.Biophys. Acta, 1506: 1-11.

Leahy, J.G. and R.R. Colwell, 1990. Microbial degradation of hydrocarbons in the environment. Microbiol. Rev., 54: 305-315

Matsushita, K., M.Yamada., E. Shinagawa, O. Adachi and M. Ameyama, 1980. Membrane-bound respiratory chain of Pseudomonas aeruginosa grown aerobically. J.Bactriol. 141: 389-392.

Minai-Tehrani, D. and A. Herfatmanesh, 2007. Biodegradation of aliphatic and aromatic fractions of heavy crude oil-contaminated soil, a pilot study. Bioremediation. J., 11: 71-76.

Poole, R.K., 1983. Bacterial cytochrome oxidases. A structurally and functionally diverse group of electron-transfer proteins. Biochim.Biophys. Acta, 726: 205-243.

Quesada, A., M.I. Guijo, F. Merchán, B. Bequez, M.I. Igeño and R. Blasco, 2007. Essential Role of Cytochrome bd-Related Oxidase in Cyanide Resistance of *Pseudomonas pseudoalcaligenes* CECT5344. App. Environ. Microbiol., 73: 5118-5124.

Robyt, J.F. and B.J. White, 1987. Biochemical techniques, Theory and practice. Brooke/Cole Pub Company, USA. P: 234.

Walker, J.D., R.R. Colwell and L. Petrakis, 1976. Biodegradation rates of componenets of petroleum. Can. J. Microbiol., 22: 1209-1213.