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Effect of Heavy Crude Oil on the Pattern of Respiratory Chain of *Pseudomonas* sp.

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ABSTRACT

Bacteria are versatile organisms which can grow in different mediums. The respiratory chain of bacteria consists of different cytochromes and terminal oxidases, which allow their adaptation to various media. *Pseudomona*s is a useful bacterium for industries, it can aerobically biodegrade some types of toxic materials in 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 use it as a carbon source. Our results show that *Pseudomonas* can degrade aliphatic fractions of crude oil more efficiently than aromatic fractions. *Pseudomonas* uses the cytochrome *bo* complex as its main terminal oxidase in nutrients rich medium and in medium containing ethanol as carbon source, while in medium containing crude oil, it uses both cytochrome *bo* and *bd* complexes as terminal oxidase.

Keywords: bacteria, biodegradation, cytochrome, pollution

INTRODUCTION

Pseudomonas is a Gram-negative bacterium useful in industries. In the presence of oxygen, the bacterium uses the aerobic biochemical pathway to biodegrade organic substances and uses oxygen as a terminal electron acceptor (Matsushita *et al.* 1980). The respiratory chain of the bacteria contains various cytochromes and different kinds of terminal oxidases (Bartsch 1968; Anraku 1988). The important terminal oxidases that use oxygen as an electron acceptor are cytochrome aa_3, and the *bd* and *bo* complexes (Poole 1983; Jones and Poole 1985). In the presence of certain substrates such as ethanol or succinate, the bacteria may use one of these terminal oxidases as an electron acceptor (Reichmann and Gorisch 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 (Matsushita *et al.* 1980) and also cyanide insensitive oxidase (CIO) which resembles to cytochrome *bd* (Cunningham and Williams 1995; Cunningham *et al.* 1997; Cooper *et al.* 2003). The effects of growth conditions on the formation of cytochromes of a denitrifying bacterium, *Pseudomonas stutzeri* showed that in the aerobic conditions, synthesis of all cytochrome components was repressed, while in the semi-aerobic conditions, both soluble and paniculate cytochromes c-552 and cytochrome b-558 contents increased even in the absence of nitrate and nitrite (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 biodegrade oil and change it to H_2O and CO_2 (Cerniglia 1992). In aerobic conditions oxygen plays an important role in the biodegradation of oil and its components. Aliphatic and aromatic fractions of crude oil are efficiently biodegraded by oil-degrading bacteria (Minai-Tehrani and Herfatmanesh 2007). *Pseudomonas* is able to biodegrade crude oil or its components, and uses them as its sole carbon source in the presence of oxygen (Leahy and Colwell 1990). In this report the pattern of the *Pseudomonas* respiratory chain in medium containing crude oil was studied and compared with nutrients rich medium and in medium containing ethanol. On the other hand, the effect of bacterium on total crude oil biodegradation is also studied.

MATERIALS AND METHODS

Culture media preparation

The minimal medium was prepared by adding 2.5 g KH_2PO_4, 2.5 g Na_2HPO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4$ and 0.01 g $CaCO_3$ to 1 liter of distilled water and the pH was adjusted to 7.0. Heavy crude oil was added to this medium to a final concentration of 1% (v/v). This concentration provided sufficient cells for further procedures and also it was below the toxic effect of crude oil on microorganisms. Ethanol was also used with minimal medium as an alternative carbon source to compare with crude oil. Rich medium was prepared by adding yeast extract (7 g), peptone (5 g) and NaCl (2 g) per liter of water and the pH was adjusted to 7. All chemicals were obtained from Merck. Heavy crude oil (API gravity = 20) (American petroleum Institute gravity) was obtained from the Soroush oil field in north of the Persian Gulf.

Isolation of *Pseudomonas*

The oil-degrading *Pseudomonas* strain was isolated from the oil-contaminated soil near the Tehran refinery. One g of oil-contaminated soil was added to minimal liquid medium containing 1% sterilized crude oil as the 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 30°C. After 1 week, 2 ml of cultured medium suspension was transferred to new medium under 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 the carbon source and incubated for 72 hours at 30°C. The growing colonies were isolated and transferred to a new solid medium. One of the colonies that were suspected as the *Pseudomonas* strain was examined by biochemical tests (explained next) for identification.

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Biochemical tests

The isolated bacterium was Gram-negative and a motile rod-shaped (bacillus) that produced green pigment. These morphological and phenotypic characteristics were examined for isolated bacterium according to the description of typical *Pseudomonads* in Bergey's manual of determinative Bacteriology (Bergey and Holt 1994) which describes the genus as being Gram-negative, non-spore forming and motile, catalase and oxidase positive. The bacterium was examined for catalase test, after adding a smear of bacteria onto the lid of the culture dish, a drop of H_2O_2 is then added to the smear. Formation of the bubbles showed that catalase test is positive for this bacterium (Chester 1979). The oxidase test was done by adding a drop of freshly prepared 1% tetramethyl-*p*-phenylene-diamine dihydrochloride solution on a piece of filter paper in a culture dish, and then a small amount of the test colony was added to the filter paper. The appearance of blue color indicated the positive oxidase test (Collee *et al*. 1989). The bacterium was determined to be non-fermentive after carbohydrate test in the presence of glucose and bromothymol blue (Hugh and Leifson 1953).

Growth curve and yield of cells

The growth curve was measured in all the growing conditions including the medium containing 1% crude oil, 1% ethanol and rich medium, by sampling from liquid medium every four hours and the turbidity was measured at 600 nm. For further experiments the stationary phase was selected for respiratory and oil extraction analysis. In this phase oil dispersion reached its highest value, but analysis of the 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 the sole carbon source. The dispersion of oil was observed in the medium which suggested the ability of the bacterium to degrade oil. The bacteria were cultured for 72 hours in a reciprocal shaker at 150 rpm at 30°C. After 72 h (in the stationary phase) the medium was centrifuged (5000 × *g* for 30 min) to precipitate cells (the precipitated cells were collected for further experiments). The supernatant was separated for oil analysis. The supernatant was mixed with an equal volume of chloroform and transferred to a separating funnel. The suspension was shaken firmly for about 5 min to dissolve the oil fraction of supernatant in solvent phase. The solvent phase was separated and evaporated in a vacuumed chamber for 24 h. 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 developed by Minai-Tehrani (2007). The residue was dissolved in *n*-hexane and filtered. Five ml of filtered solution was loaded to a 1 × 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 the 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 the 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 h at 30°C.

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 min at 25°C). The pellet was weighed to determine the yield of cells and dissolved in phosphate buffer (1 g cells/10 ml buffer). To break the cells, cell suspension was sonicated 5 × 20 sec with 20 sec intervals at 4°C. The sonicated suspension was centrifuged (12000 × *g* for 30 min at 0-4°C) to precipitate unbroken cells. The supernatant was used as the cell-free extract for further experiments.

The isolated *Pseudomonas* was also cultured in 1% ethanol and rich medium for 72 h 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 the cell-free extract were recorded with a Perkin Elmer DW2 UV-Visible spectrophotometer according to the method used by Keyhani and Minai-Tehrani (2001), by adding a few grains of sodium dithionite as the reducing agent and 30 µl of 10% H_2O_2 as oxidant to the cell-free extract.

The binding of cyanide to cytochrome *d* was determined by adding 10 mM KCN to oxidized form of cytochrome *d* and the oxidized+KCN-minus-oxidized difference absorption spectrum was recorded. The binding of cyanide to cytochrome *d* was monitored by the formation the trough at 650 nm.

The amount of protein was determined by the Biuret method (Robyte and White 1987).

RESULTS AND DISCUSSION

In oil-producing countries, the risk of contamination of surface or underground waters by oil is high. *Pseudomonas* is a potent bacterium mainly uses to biodegrade the organic contamination either in water or soil. The efficiency of oil biodegradation by this bacterium depends on the presence of oxygen (Leahy and Colwell 1990). There are no reports indicating the changes of the respiratory chain of this bacterium in the presence of oil which is important for its function in the oil degradation. This study was conducted in 1% crude oil, which had less toxic effect on the growth of bacterium. In most of studies the concentration of oil was considered about 1 to 2% and generally microorganisms can tolerate the crude oil concentration below 5% in their medium culture (Leahy and Colwell 1990; Diaz *et al.* 2002; Hamamura *et al.* 2006; Kelkarb *et al.* 2007).

Growth curve

Fig. 1 shows the growth curve of the bacteria. A shorter lag period was observed in rich medium while a longer lag period was seen in medium with ethanol. In the rich medium cells entered the stationary phase after 45 h while in medium containing crude oil the cells entered this phase after 64 h. 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 than in rich medium. The comparison of yield of the cells (**Fig. 1**, insert) shows that the growth of cells in rich medium was more efficient than in ethanol and crude oil-containing media.

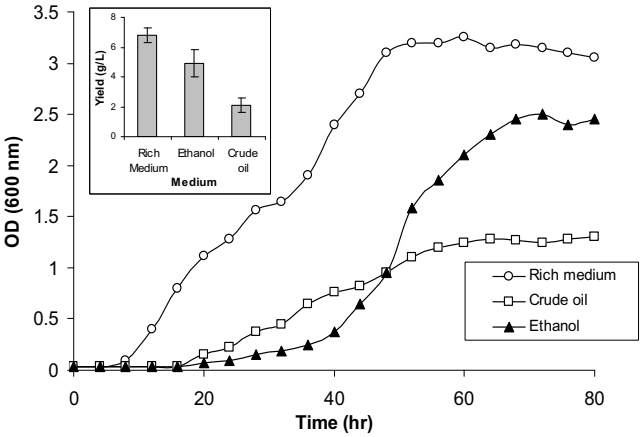

Fig. 1 Growth curve of *Pseudomonas* sp. in rich medium and media containing crude oil and ethanol. Insert: the yield of cell in rich medium and in the presence of crude oil and ethanol.

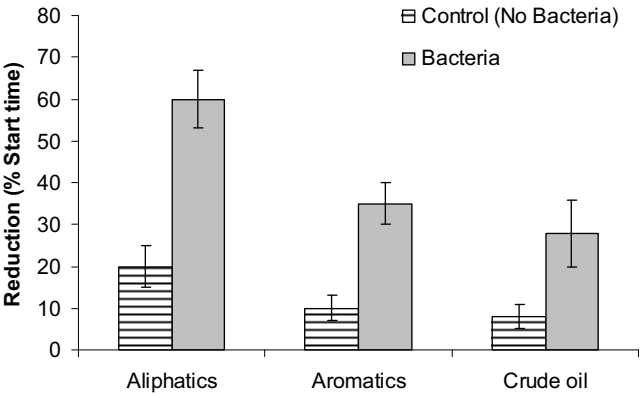

Fig. 2 Reduction of total crude oil, aliphatic and aromatic fractions in the medium with bacteria and without bacteria (control).

Oil reduction

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

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

These results suggest that the bacteria used the oil as their sole carbon source and that biodegradation of the aliphatic fractions was more efficient than the aromatic fractions. Because there were no bacteria in control sample to biodegrade the oil fractions, hence volatilization must be the main factor for the reduction of the oil and its fractions in control sample.

Respiratory chain pattern

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

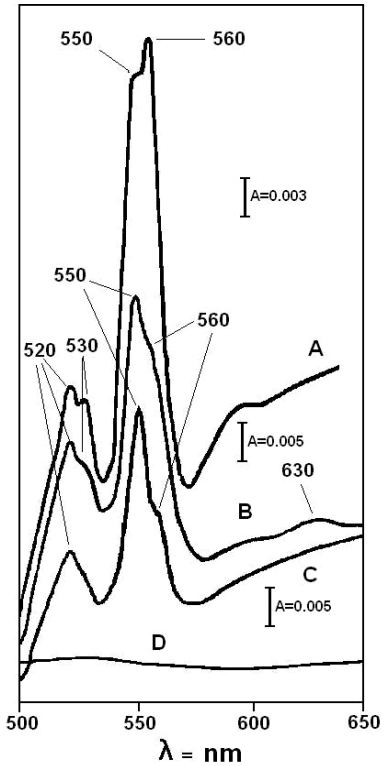

Fig. 3 Dithionite reduced-minus-H_2O_2 oxidized difference spectra of cell free extract of *Pseudomonas* sp. A = in rich medium (protein amount 6 mg/ml); B = in minimal medium containing crude oil (protein amount 11 mg/ml); C = in minimal medium containing ethanol (protein amount 7 mg/ml); D = base line.

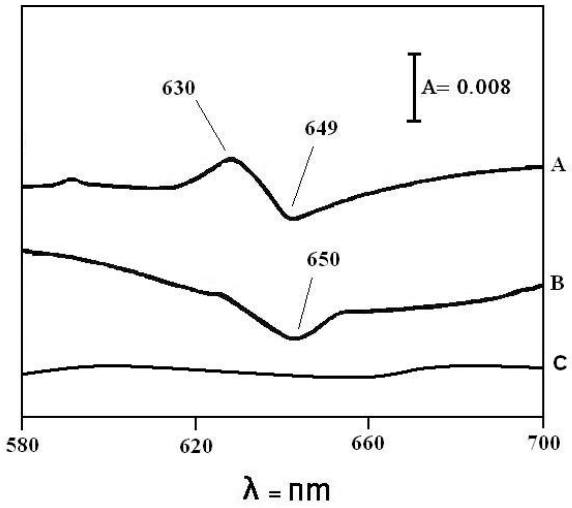

Fig. 4 Effect of KCN on the formation of cytochrome *d*-CN complex in cell free extract. A = Dithionite reduced-minus-H_2O_2 oxidized difference spectrum of cytochrome *d* induces a peak at 630 nm and a trough at 649 nm; B: H_2O_2 oxidized + KCN-minus-H_2O_2 oxidized difference spectrum. Cytochrome *d*-CN induces a trough at 650 nm (protein amount 18.5 mg/ml); C= baseline.

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

In the alpha region, the peak at 560 nm belongs to *b* type cytochromes, and the peak at 550 nm is related to cytochrome *c*. In the beta region, the peak at 530 nm belongs to cytochrome *c*, while the peak at 525 is due to cytochrome *b*. The respiratory chain in the crude oil and ethanol samples had a higher peak at 550 nm than at 560 nm, while in the rich medium the 560 nm peak was higher than the 550 nm peak. In medium containing ethanol, the peak at 560 nm was lower than the peak at crude oil medium suggesting that the expression of *bo* complex terminal oxidase in ethanol was lower than crude oil. In the respiratory chain of bacteria grown in crude oil the little peak at 630 nm was due to the cytochrome *bd* complex. No peak at 630 nm was observed in rich medium and the medium containing ethanol. Previous reports indicated 630 nm for the cytochrome *bd* complex (Keyhani and Minai-Tehrani 2001). Addition of KCN to oxidized form of respiratory chain obtained from crude oil containing medium induced a shallow trough at 650 nm suggesting the binding of KCN to oxidized form of cytochrome *d* (**Fig. 4**).

These results suggest that in the presence of rich medium and ethanol the bacteria 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 the 630 nm peak suggests that the cytochrome *bd* complex could be used as another electron acceptor in addition to *bo* complex. The existence of the cytochrome *bd* complex and cyanide insensitive oxidase (CIO) has previously been reported in *Pseudomonas aeruginosa* (Bryan and Kwan 1981; Cunningham and Williams 1995; Cunningham *et al.* 1997; Cooper *et al.* 2003) and also in *Pseudomonas putida* (Sweet and Peterson 1978). Under anaerobic or semi-aerobic conditions, nitrite induced cytochrome a_2–*c* synthesis in a denitrifying bacterium, *Pseudomonas stutzeri* (Kodama 1970).

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

Oil-degrading bacteria such as *Pseudomonas* can use aliphatic and aromatic fractions of crude oil as sole carbon source and biodegrade it to simple and non-toxic products such as CO_2 and H_2O (Van Hamme *et al.* 2003). This is done by metabolizing the crude oil through complex biochemical pathways to simpler organic metabolites. In this process oxygen was used as terminal electron acceptor, consequently the bacterium uses the oxygen linked terminal oxidases in its respiratory chain. Our results show that in the presence of crude oil, *Pseudomonas* uses the *bo* complex and an alternative terminal oxidase such as the *bd* complex which both of them use oxygen as electron acceptor. Cytochrome *d* was synthesized by *Pseudomonas putida* in response to restricted oxygen in medium culture (Sweet and Peterson 1978). In the absence of crude oil and in presence of the other carbon source such as ethanol, in contrast, the bacterium mainly uses the *bo* complex as a terminal oxidase.

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