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Full Length Research Paper

Bioremediation of crude oils / complex mixture of hydrocarbons (CMH) contaminants in seawater by a halotolerant bacterial under aerobic conditions: *Enterobacter cloacae, Pseudomonas spp. and Escherichia coli.*

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Complex mixture of hydrocarbons CMHs are a class of potentially hazardous chemicals of environmental and health concern. CMHs are one of the most prevalent groups of contaminants found in water and soil. Bioremediation of complex hydrocarbons usually requires the cooperation of more than single species. In this research bio-treatment of CMHs was studied in a liquid-phase reactor using halotolerant marine bacteria isolated from two petroleum contaminated sites in Oran, (i.e. industrial ports and fishing ports of Arzew, Oran Algeria). The potential of remediation the CMH was measuring the amount of oxygen consumed by microbial population, the measuring of COD = CO_2 released from the oxygen consumed during the reaction. This technique involves measuring COD oxidized mineralized to CO_2 and resulting oxygen consumption, cumulative consumption of oxygen associated with the degradation of organic matter. Between three bacterial species previously isolated from seawater was assessed in terms of ability or inability to grow in the presence of CMH and potential to degrade the CMH by *Enterobacter cloacae*, *Pseudomonas* spp. and *Escherichia coli*. The remaining eight: *Enterobacter cloacae* and *Pseudomonas spp.* showed tolerance to mineralize the crude oil. After 8 days, two representative bacterial strains *Enterobacter cloacae* and *Pseudomonas* spp. were chosen for mineralization and respirometry test, performed to evaluate biodegradability potential of CMH and thy capable of degrading petroleum components and utilize the oil as source of carbon and energy. We note that the higher rate of mineralization of CMH start after one week of incubation. The results of this study revealed that *Enterobacter cloacae* and *Pseudomonas* spp., are the most versatile species of bacteria that could utilized petroleum products. However, the most important specie remediation for petroleum in environments is *Enterobacter cloacae;* it's become predominant in oil-contaminated marine environment.

Keywords: bioremediation, seawater, Warburg, CO_2, CMHs, *Enterobacter cloacae*, *Pseudomonas* spp.

INTRODUCTION

Marine oil pollution has been receiving increasing attention since the middle of the 19th century with the intensification of tanker operations and oil use (Islam and Tanaka, 2004), marine tanker collisions (Owen, 1999), pollutant release from coastal refineries (Wake, 2005; Tolosa *et al*., 2005) and continuous operative discharges from ships (ESA, 1998; Carpenter and MacGill, 2001). Annually, 48% of the oil pollution in the oceans is due to fuels and 29% to crude oil. Tanker accidents contribute only 5% of all pollution entering into the sea (Brekke and Solberg, 2005). Despite this, an estimated 1.6 million tons of oil have spilled from tankers since 1965 (over 650,000 ton in Europe and Pacific Asia) (Wang and Fingas, 2003).

The discharge of oil in the environment is one of the phenomena of pollution of most concern in the sense that these hydrocarbons are toxic to humans, fauna and flora (Belhaj *et al*., 2000). The discharge of petroleum products in marine or terrestrial causes a proliferation of microorganisms able to grow on hydrocarbons and their degradation products. Their number is much higher in polluted areas of chronic and growing after an injection of oil into sites without contamination (Soltan, 2004). The studies of Chang *et al.* (2001) highlight the potential for anaerobic degradation of phenanthrene by sulfate-reducing bacteria in sediments. The removal of oil from the marine environment involves many different biotic and abiotic factors. Among these factors, biodegradation by microorganisms, particularly bacteria is the most important natural process in the cleanup of the marine environment (Soltani, 2004). But among the microorganisms able to grow on hydrocarbons, bacteria remain qualitatively and quantitatively predominant in metabolising these substrates (MacNaughton *et al*., 1999). Recent scientific work indicates that bacteria have developed mechanisms to degrade the organic substances (Spain, 1995, Freeman & Sutherland, 1998; Hawari *et al.* 2001; Ralebitso *et al*., 2002). A wide variety of bacteria can use hydrocarbons as sole source of carbone.Ce process is of great interest in preserving the natural environment by reducing the amount of oil-related contaminants (Pijanowski *et al*., 2007). Some bacterial strains can produce their own surfactant, which contributes to the effective assimilation and absorption of hydrocarbon (Phale and Prabhu, 2003). Some experiments have explained the mechanism of oxidation and also the co-metabolism of these materials (Gibson *et al.* 1975; Juhasz *et al*., 1997) and other reports have shown that bacteria isolated from contaminated soil could use some PAHs as sole carbon source (Mueller *et al*., 1989, Walter *et al.* 1991; Kastner *et al*., 1994). Metabolic processes of bacteria and other microorganisms that are naturally present in marine environments are commonly called biodegradation mechanisms.

The purpose of this study was to investigate the effect of mineralization rates of CMH and xylem using a selected halotolerant marine bacterium capable of removing petroleum substances in case of waste oil.

MATERIAL AND METHODS

Chemicals (CMHs)

In the present study, Complex mixture of hydrocarbons CMHs contaminants were purchased from Sonatrach (company for exploit the hydrocarbon) Arzew Oran, Algeria (purity 99 %), and xylem from chemical laboratory Abdelhamid Ibn Badis University (purity 99 %) as witness.

Strain sample collection

The halotolerant bacterial was isolated from a mixture of three different sampling sites which included petroleum and coal contaminated sites (ports) of Arzew, Oran, Algeria.

Studies on CMHs degradation by bacterial

The CMHs were added in the medium at a concentration of 3 mg/L. The bacterial consortium isolated from marine environment was grown and the bacterial count was checked every day.

Enrichment of halotolerant bacterial

It is natural sea water taken in a not polluted zone. A quantity of one liter is filtered on Whatman paper. At summer then added of ammonium chloride (2 g/l) as source of nitrogen and sodium phosphate (0.1 g/l) as source of phosphorus. The medium agitate magnetically to preserve at 4° C with the darkness for one month. The pH is adjusted to 8 (Boutefnoucht and *al*., 2009).

According to Boutefnoucht and *al.* (2009) the source of carbon added in the middle of culture is a derivative of the complex mixture of hydrocarbons CMHs (Arabian light) of Arzew “Oran”.

Respiration test

1. Determination the bioremediation by rate of mineralization (CO_2)

During the remediation study, the different kinds of metabolites formed were identified using thin layer respiration.

The remediation rate is determined every 4 days through analysis of the evolution rate of CO_2 in the medium relative to the amount of substrate consumed in the trials and that present in the abiotic controls during a period of 16 days of incubation temperature 25 ° C. The performance of mineralization is the ratio between the numbers of moles of carbon released as CO_2.

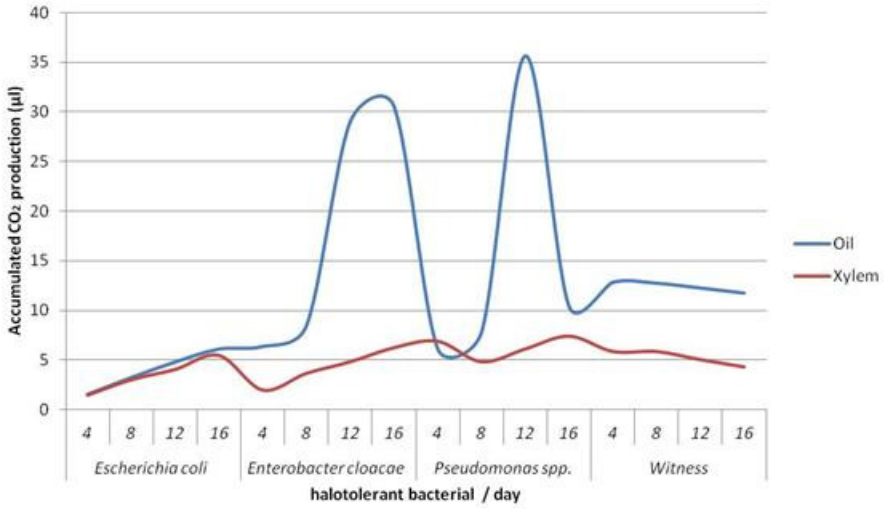

Figure 1. Time-course plot of accumulated CO_2 production halotolerant marine bacterium

2. Measurement

The measurement was performed every four days during a period of 16 days. We calculate the rate of CO_2 released, according to the formula (Waas, 1971):

$$x = h.KCO_2$$

$$KCO_2 = \frac{\frac{Vg\,273}{T} + Vf\alpha}{Po}$$

X: Representing the quantity of gas µl (0° C, 760 mHg)
h: Representing the change in millimeters of the open arms of the manometer
KCO₂: Representing the constant vial
Vf: Representing the quantity ul, liquid in the bottle
Vg: Representing the difference, ul, between the total volume of the gauge and the vial and the number of µl of liquid from the bottle
T: Representing 273+ operating temperature (27° C)
α: Representing the solubility of CO_2 in solutions, CO2/µl µl solution
P_0: Representative standard pressure expressed in terms of the solution gauge
The value used was the value of CO_2 in water, which is 0.759 to 25 ° C.
The solution was the key gauge known Brodie density 1.033, so that P_0:

$$Po = 760 \times \frac{13,6}{1,033} = 10000$$

RESULTS AND DISCUSSION

The produced CO_2 rate is deferred in the *Figure 1* .On notices after the incubation period, that the production of CO_2 is increasing according to time for each pure population of the microorganisms, the results showed an increase in the CO2 rate which is in direct contribution with the reduction in the rate of the CMHs and light arab oil.

The presence of CMHs at the beginning of fermentation will influence the growth of both strains. In the early phases 2 and 3 but xylem is weak in all incubation period. The concentrations of CO_2 have increased output levels were measured between 8.45 and 35.62 µl.

However, there clearly in graph, the concentrations increase progressively between Phases 2 and 3. Tow microorganisms capable of degrading petroleum components have been isolated. The results of this study revealed that *Enterobacter cloacae* and *Pseudomonas* spp., are the most versatile species of bacteria that could utilized petroleum products, which show activity biodegradation of oil and rapid elimination in the first two weeks of treatment (Namkoong *et al.* 2002). *E. cloacae* ATCC 43560 have shown the ability to degrade TNT and PETN (French *et al.*, 1998). Pudge *et al.* (2003) prove that E. cloacae ATCC 43560 for the first time. The volumetric rate of RDX degradation (22 mg l per day) appears to be the highest rate reported. In 2006, Iyer and coworkers (2006) found that the EPS excreted by marine

Enterobacter cloacae could emulsify various hydrocarbons, mineral oils, and vegetable oils.

This indicates that overall the selected molecules are very well disposed in the biological process, although this does not suggest what sort of process of elimination. In a period of severe inhibition of biomass (a measure of respiratory activity increased by 75% during Phases 3 and 4).

Stapleton *et al.* (1998) reported the biodegradation of aromatic hydrocarbons and PAHs that the indigenous microorganisms oxidized about 50% of the supplied naphthalene and toluene to CO_2 and water within 24 weeks. Harayama *et al.* (1999) most of the petroleum-degrading bacteria (bacteria capable of growing on Arabian crude oil as the sole source of carbon and energy) produce surfactants or emulsifiers. Nonetheless, there are several reports about microorganisms able to oxidize petroleum hydrocarbons even in the presence of 30% w/v NaCl. Among such microorganisms are crude oil-degrading (Kuznetsov *et al.* 1992).

Microorganisms are known to attack specific compounds present in crude oil that is a complex mixture of saturates, aromatics and polar compounds (Bharathi and Vasudevan, 2001). An effective degradation of crude oil would require simultaneous action of several metabolically versatile microorganisms with faourable environmental conditions such as pH, temperature and availability of nutrients (Venkateswaran and Harayama, 1995). An oil spill in the environment leads to an adaptive process and if metabolically active hydrocarbon utilising microorganisms could be added quickly, the long period before the indigenous population could respond would be reduced considerably. The necessity for seeding with complementary hydrocarbon degrading bacteria arises from the rationale that indigenous microbial populations may not be capable of degrading a wide range of potential substrates in a complex mixture such as crude oil (Chhatre et al., 1996).

In general, bacterial consortium showed maximum percentage (78%) of degradation of CMHs after 20 days of incubation. Chatre et al., (1996) reported about 60% of degradation of CMHs using a semi-continuous CMHs fed reactor using a four member consortium. Several other workers (Venkateswaran and Harayama, 1995; Lal and Khanna, 1996; Sugiura et al., 1997) showed that a bacterial consortium was able to degrade 28-51% of saturates and 0-18% of aromatics present in crude oil or up to 60% crude oil by mixed consortia. The percentage of biodegradation was significantly higher than that achieved by individual isolates. Among the four different pH tested, the bacterial growth and percentage degradation of crude oil were maximum at pH 7.5. Most of the heterotrophic bacteria favor a pH near neutrality (Atlas, 1981). Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (Atlas 1981). Our studies showed maximum biodegradation activity at 30°C.

According Vandecasteele (2001) shows that *Pseudomonas* degrading aromatic hydrocarbons via dioxygenases, bacterial germ that power exceptionneld'adaptation.He lives in soil, marshes and coastal environments, can degrade a variety of compounds (phenols, hydrocarbons, halodérivés) (Pelmont, 2005), eg n-alkanes as carbon source (Witholt et al., 1990; Pijanowski et al., 2007). An approach to extend the capacity of efficient hydrocarbon degraders for biodegradation in saline environments has been performed by impairing the phenotype of osmotolerance to a crude oil-degrading consortium, consisting of four *Pseudomonas* strains (Kapley et al., 1999).

Aono et al., (1994) observed a similar situation following the growth of *Escherichia coli* K-12 JA 300 in a medium containing hexane. However, with the addition of *p*-xylem, the cells were immediately killed. After addition of *p*-xylem, no viable cell was detected after five minutes.

We can therefore assume that the absence of the biodegradable strain *Escherichia coli* due to cell death when they are brought into contact with oil and xylem, that in growing bacterial strains thy using oil as the sole source of carbon and energy; reported that the biodegradation rate reached 90% after 50 days of incubation (Richard and Vogel, 1999). Other authors Suparna et al., (2004) tested the biodegradation of diesel with a bacterial population isolated from marine sediments under aerobic conditions, these authors found after 8 days of incubation, a death rate of 39 fuel % with a loss of 80% aliphatic components.

Moreover, it is known that the microbial needs minerals for its growth, particularly nitrogen, which the optimal proportions, generally accepted, are 10g of nitrogen and phosphorus 1g per 100g of carbon (Ballerini, 1999). These elements are included in the construction of cellular constituents in the multiplication of microorganisms (DNA synthesis, protein ... ect.). Prince *et al.* (2003) reported that in aerobic environments, the factors limiting the more likely are nitrogen and phosphorus.

Our test of biodegradability we found that xylem is very little in these biodegradable experimental conditions. A tire comparison, Arabian Light crude oil is degraded more than 70% (Oudot, 1984-2000). This is due to the nature of oil, which is composed of several types of hydrocarbons composed of different families assimilated by microorganisms and causing a significant increase in the rate of carbon mineralization (*Enterobacter cloacae* > *Pseudomonas* spp > *Escherichia coli*).

Our research work clearly shows that CMHs contaminated seawater can be a principle source for potent oil degrading bacteria. Genus *Enterobacter cloacae* was seawater dominant in all the samples analyzed. It was observed that bacterial consortium degraded Arabic high

crude oil efficiently when compared to the individual bacterial cultures tested.

CONCLUSION

From the shape of the curve of the variation rate of degradation over time for *Enterobacter cloacae* and *Pseudomonas* spp. and very remarkable, we note that in the early days threat of CO_2 is high in phases and 3, which shows a high degradation, but in phase 4, we observe that the biodegradable tolerance of *Enterobacter cloacae* higher then *Pseudomonas* spp., but for *Escherichia coli* is almost zero in all period of incubation. Belhaj et al., (2000) reported a correlation between the chemical composition of crude oil and its potential for biodegradation in the sense that oils with high contents of alkanes are more susceptible to microbial degradation phenomena.

Comparing CO_2 levels indicated the following order of biodegradability: *Enterobacter cloacae* > *Pseudomonas* spp. > *Escherichia coli*. Tow microorganisms capable of degrading petroleum components have been isolated. The results of this study revealed that *Enterobacter cloacae* and *Pseudomonas* spp., are the most versatile species of bacteria that could utilized petroleum products.

However, one important for petroleum biodegradation in natural environments is *Enterobacter cloacae* become predominant in an oil-contaminated marine environment. This shows that the strain of Enterobacter cloacae is able to better assimilate petroleum substances.

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