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Research article

Treatment of an oil polluted soil by injecting *Pseudomonas aeruginosa* and produced rhamnolipid.

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ABSTRACT

In order to remedy a possible pollution by oil since our country is a producer and consumer, we undertook a laboratory test decontamination of a contaminated soil. We tried to demonstrate the effectiveness of a combination of two biological techniques which are bioaugmentation by providing a bacterial suspension of *Pseudomonas aeruginosa* and biostmulation by incorporating to a contaminated soil a rhamnolipid biosurfactant produced by *P. aeruginosa*. The results showed that this combination improved the biological activity of the contaminated soil, estimated by the amount of CO_2 produced, and its microbial biomass. The germination of wheat seeds was also improved traducing diminution of the toxicity. The rate of hydrocarbons extracted after 28 days incubation decreased significatively in comparison with the untreated contaminated soil or the soil treated with either bioaugmentation or biostimulation.

Keywords: Hydrocarbon, biosurfactant, *Ps. aeruginosa*, bioaugmentation, biostimulation.

Introduction

The soil is a place of passage or stay of most major types of pollutants produced by human activity (Girard&*al.*, 2005). Several decontamination methods are adopted, such as soil flushing (Lecomte, 2002), the electric extraction (Delage&Schrefler, 2005), chemical methods (Colin, 2000) and phytoremediation (Girard&*al.*,2005). In recent years, the biodegradation has become the most used method in the restoration of contaminated sites by hydrocarbons (Kaczore&*al.*,2004). Diversified bacteria and fungi of soils are able to degrade organic pollutants as hydrocarbons and products of their degradation. However, a part of the hydrocarbons is bounded to soil organic matter and becomes less available to the microorganisms. Thus, the focus is also on the remediation assisted by surfactants of biological origin or biosurfactants (Gabet, 2004). These are synthesized by microbial cells(Perraud,2009).

This work is an attempt to the rehabilitation of a hydrocarbon contaminated soil by combining two biological techniques, which are bioaugmentation, by introducing into soil of bacterial suspension of *Pseudomonas aeruginosa*, and biostmulation by incorporating a rhamnolipid produced by this strain.

Materials and methods

Soil

The soil used in this study is a subsurface soil (0-25 cm) from an agricultural land located on the neighbourhood of the oil refinery at El Harrach in a suburb of Algiers. It is a sandy clay with pH 7.71.

Hydrocarbons

Analysis of hydrocarbons in the soil was carried out at the Center for Research and Development of Sonatrach. Their composition is presented in Table I and Fig. 1.

Table 1: Rate of hydrocarbons extracted from the soil

Type HC	Saturates	Aromatics	Résins	Asphaltenes
In soil (%)	45.125	28.347	24.396	2.132

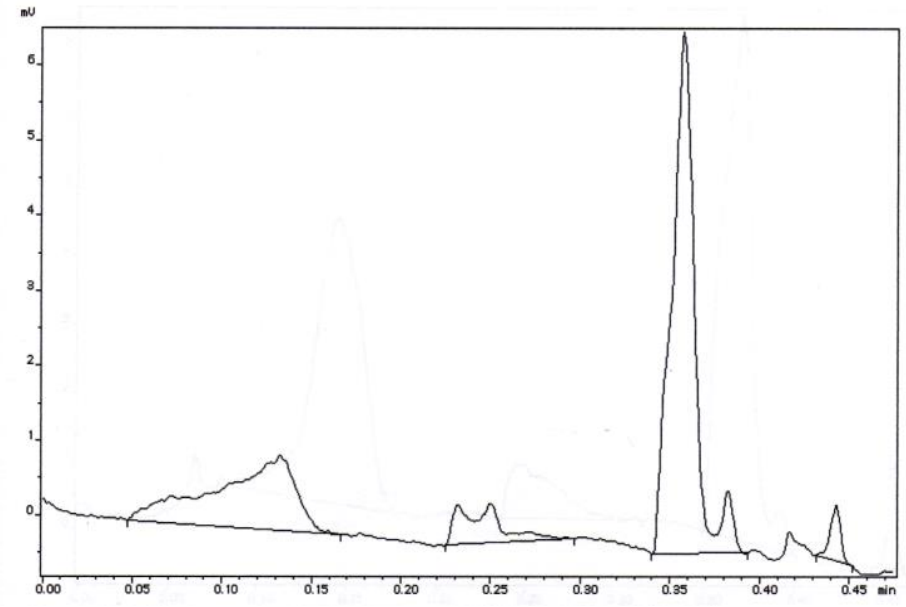

Figure 1: TLC-FID analysis for soil hydrocarbons.

Soil analysis byTLC-FID indicated the presence of four fractions dominated by saturated hydrocarbons.

Biological material

Bacterial strain

The bacterial strain is *Pseudomonas aeruginosa* which is the type specie (Singleton, 2008). It is able to grow on toxic organic compounds such as aliphatic and aromatic hydrocarbons (Kenneth, 2000), with an important production of rhamnolipids. The strain used is furnished by the laboratory of Microbiology of the University Mouloud Mammeri and the used biosurfactant is a rhamnolipid produced by *Ps. aeruginosa* in this laboratory.

Plant material

The germination test is effectuated using Wheat seeds of the variety Vitron. Wheat resists well to hydrocarbon contamination (Chaineau&*al.*,1997).

Biological analysis methods
Soil treatment

The soil was amended with 3.44 g of $(NH_4)_2SO_4$ and 0.53g of KH_2PO_4, and then divided intofour batches of 400g : the first, considered as the control, consists in contaminated soil, the second was enriched with 30ml of bacterial suspension of *Ps. aeruginosa* incubated for 48 h at 28°C in 0.9% NaCl solution. In the third one was incorporated the rhamnolipid produced by *Ps. aeruginosa* and the fourth is enriched with both 30 ml of the bacterial suspension and the rhamnolipid. Each treatment is replicated four times.

Respirometric test

A respirometric test was performed to assess the impact of different treatments on soil biological activity. Carbon mineralization potential was measured using the method proposed by Chaussod & Nicolardot (1982). After bringing soil moisture to 85% of field capacity, the different batches were incubated at 28°C in sealed glass vials each containing a test tube with 5 ml of 0.5N NaOH. The CO_2 is then trapped by soda as sodium bicarbonate (Na_2C0_3). The carbon is captured and measured after 7and 28 days by the spectrophotometric method at a wave length of 630 nm.

Microbial biomass

The total microbial biomass of soils was measured by the method of fumigation - extraction proposed by Jenkinson & Powlson (1976), adapted by Chaussod & Nicolardot (1982). The fumigation is carried out by exposure of soil samples to chloroform vapor for 16 hours at 20°C. The carbon is extracted in a 0.05N solution of K_2SO_4 (soil / solution = 1/5), with stirring for 40 minutes, then centrifugating for 15 minutes at 4500 rds / mn. This procedure is applied on fumigated and non fumigated batches. Carbon is determined by spectrophotometry after plotting the standard curve of glucose in the K_2SO_4 to 0.05N.

Determination of microbial biomass carbon

The difference of the amount of soluble extracted carbon between fumigated and non fumigated soil is calculated by the following formula:

$$Bc = [CF - CNF] / kec$$

Bc: Carbon microbial biomass;

CF: Carbon in the filtrate of K2 SO4 fumigated soil;

CNF: Carbon in the filtrate K_2 SO_4 non fumigated soil;

Kec: Coefficient of efficiency of extraction of microbial biomass carbon.

Germination test

This test is performed after 28 days of incubation to evaluate the effect of the different applied treatments to contaminated soil on seed germination of wheat in order to assess which of them induced an improvement of the soil biological quality.
In Petri dishes, 100 seeds of wheat are covered with soil of each treatment and are moistured. After 21 days, the germination rate is calculated by dividing the number of germed seeds on the total of seeds.

Statistical analysis

The experimental results were analyzed by ANOVA test completed with a comparison of means by the Newman-Keuls test which allowed establishing homogeneous groups.

Results

CO_2 production

In this work a strain of *Ps. aeruginosa* and its produced rhamnolipid are used to decontaminate a hydrocarbon polluted soil.
The results showed that both treatment with biosurfactant and the association of the tested strain and the biosurfactant was interesting for degradation of the hydrocarbon (Fig.2). After 7days of incubation, the higher amount of released CO_2 is obtained in the soil treated with the biosurfactant (Bs) of about 563.8 mg/100 g of soil, followed by soil treated with the mixture bacterial suspension-biosurfactant (Sus + Bs) with 521.48 mg/100 g of soil.

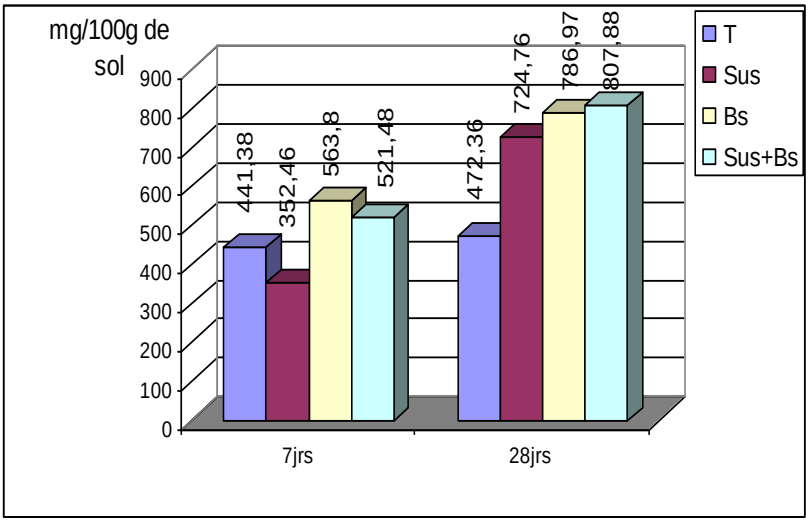

Figure 2: CO_2 production (mg CO_2/100 g soil) after 7days and 28days of incubation

(T: control, Sus: bacterial suspension, BS: biosurfactant, Sus+BS: bacterial suspension + biosurfactant)

After 28 days of incubation, the rate of CO_2 has increased reaching a maximum value of 807.88 mg/100 g in the soil treated by the mixture biosurfactant-bacterial suspension (Sus + Bs). In the soil treated by the biosurfactant (Bs), this rate was about 786.97 mg/100 g soil. For the soil treated with the bacterial suspension (Sus), we recorded a high amount of724.76mg/100g of soil after 28 days of incubation and only 352.46mg/100g of soil after 7days of incubation.
On the untreated contaminated soil, the amount of CO_2 obtained is low after 7 and 28 days of incubation (441.38 and 472.36mg/100g of soil respectively). The ANOVA results for the released CO_2 after 7 and 28 days of incubation (Table II) confirm that there is a significant difference in the amount of CO_2 released in the four batches of soil. The Newman-Keuls test (NK test) ranged the soil treated with the biosurfactant and the bacterial suspension-biosurfactant mixture in the group A. The soil treated with the bacterial suspension in the group B.

Microbial biomass

The results show that the microbial biomass recorded in the control was very low in the range of 1023.988 mg/100 g of soil. Higher amounts are recorded for the bacterial suspension (*Ps. aeruginosa*) and the mixture

Table II: Analysis of variance for the produced CO_2 in different batches of soil after 7and 28days of incubation (α=0.05).

	Factor	Means (NK test)	df	F value	Pr
After7 days of incubation	Bs	563,8 (A)	15	4,405932	0.02612
	Sus+Bs	521,48(A)			
	T	441,38(AB)			
	Sus	352,46(B)			
After 28days of incubation	**Sus+Bs**	807,88(A)	15	32,48151	0,00001
	Bs	786,97(A)			
	Sus	724,76(A)			
	T	472,36(B)			

treatment (biosurfactant-suspension) which are by 7868.958 and 9529.986mg/100g of soil respectively. The biosurfactant gave the highest biomass and satisfactory amounting to 15,351.95 mg/100 g of soil (Fig.3).

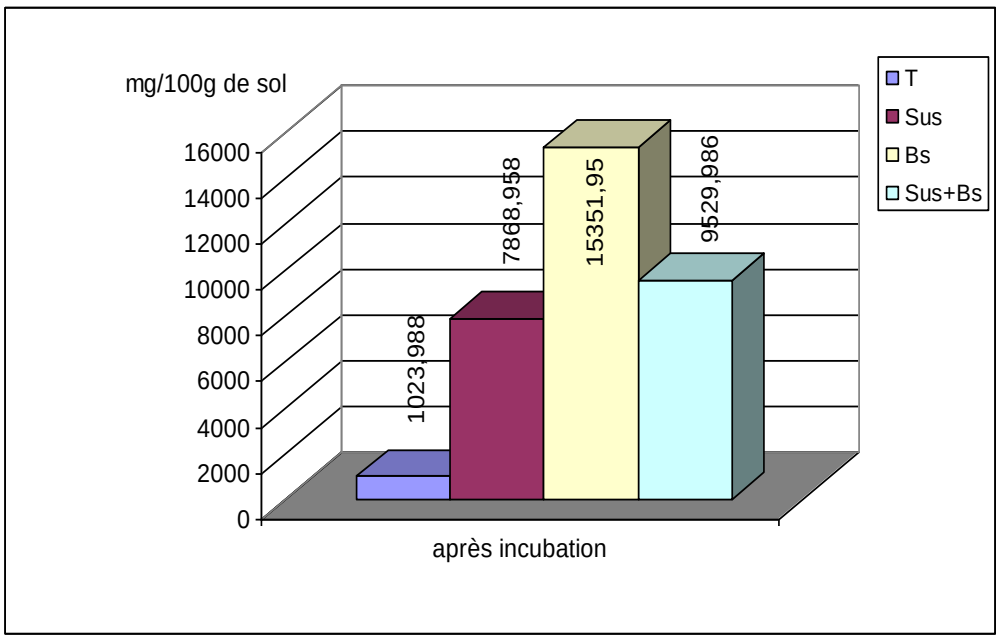

Figure 3: The microbial biomass after 28 days of incubation.

(T: control, Sus: bacterial suspension, BS: biosurfactant, Sus+BS: bacterial suspension + biosurfactant)

The ANOVA results confirmed that the difference in microbial biomass is very highly significant between the four batches of soil (P = 0.00001) (Table III). The Newman-Keuls test revealed three homogeneous groups, the

group A including the batch Bs, the group B including Sus + Bs and Sus batches, and the group C containing only the control.

Table III: Analysis of variance for microbial biomass in soils of different batches after incubation (α = 0.05).

	Factor	Means(NK test)	ddl	F	P
After incubation	BS	15351,95(A)	15	30,78007	0.00001
	Sus+Bs	9529,986 (B)			
	Sus	7868,958(B)			
	T	1023,988(C)			

Germination test

The rate of germination shown in Fig.4 is important in the soils treated with the bacterial strain *Ps. aeruginosa* and the soil treated with the biosurfactant with 80% and 77.50% respectively. In the soil treated with the combination of *P. aeruginosa* and biosurfactant, the germination rate was of 65%. However, the inhibition of seed germination is observed in the control soil with a germination rate of 52.50%.

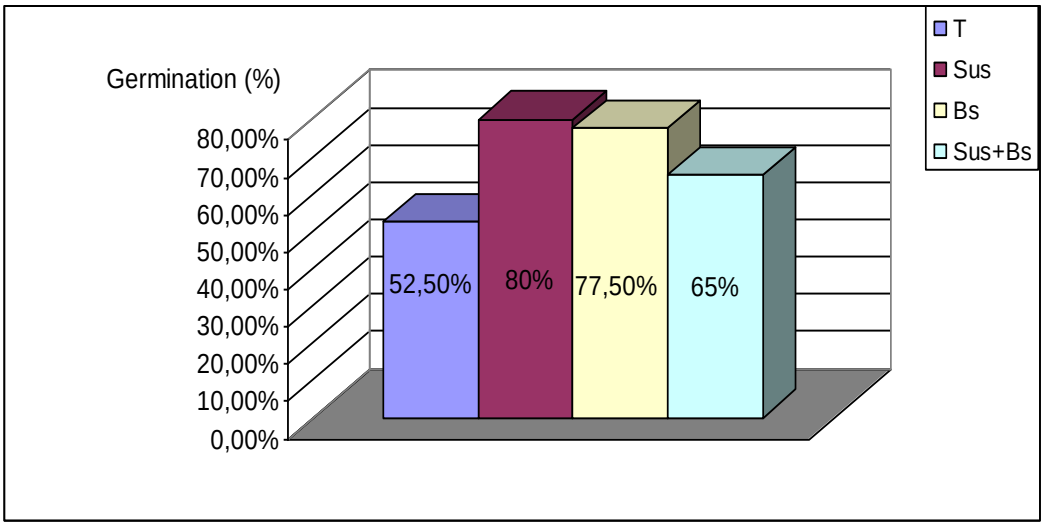

Figure 4: Rate of germination of wheat seeds in soils treated differently and the witness after the incubation.

(T: control, Sus: bacterial suspension, BS: biosurfactant, Sus+BS: bacterial suspension + biosurfactant)

The ANOVA results show that there is no significant difference for the rate of germination in the four batches of soil (P = 0.07787) (Table IV).

Table IV: analysis of variance for germination rate in different batches of soil after 28 days of incubation.

Variation	Sum. Sq.	ddl	Mean Sq.	F value	Pr	E.T.	C.V.
Total	4575	15	305				
Factorial	1925	3	641,6667	2,905661	0,07787		
Residual	2650	12	220,8333			14,8604619	21,62%

Discussion

CO_2 production

The results suggest that the presence of hydrocarbons in the soil causes inhibition of biological activity. This inhibition is particularly important when the concentration of hydrocarbons in the soil is high. This may be explained by inhibition of the metabolic activity of microorganisms.The toxic action of hydrocarbons causes a slowing of the soil microbial activity, resulting in physiological changes of microflora, among others, the denaturation of the enzyme system but also the diversity of microorganisms. Duchaufour (2001), notes in the same way, that when oil residues are unfavorable to the life of organisms both micro-organisms in the soil and plants growing on this soil.

In the untreated contaminated soil, microbial respiration was lowered after the first week of incubation. This is due to stagnation of the biological activity and thus breathing during the three last weeks after the acceleration recorded during the first week of incubation. The production of CO_2 in the control batch is higher during the first week compared to the cumulated amount produced after 28 days of incubation.This may be explained by the mineralization rate of microbial bodies (Chaussod et *al.*, 1986). The low amount of CO_2 released after 7 days of incubation from the soil treated with the bacterial strain can be explained by a less adaptation of this strain to the soil conditions during the first days of incubation. The biological activity improved significantly in soils treated with *Ps. aeruginosa* and biosurfactant and the combination of both compared to control. This improvement would result in degradation of hydrocarbons, including the simplest (saturates and aromatics) in the presence of the biosurfactant in relation with their properties. These amphiphilic substances may have emulsifying properties, foaming, wetting or dispersing. They can be stored in extreme conditions such as acidic pH and high temperatures (Meylheuc, 2001). According to D'Aes & *al.* (2010), biosurfactants can increase nutrients availability. The addition of the biosurfactant led to solubilisation and desorption of hydrocarbons from the soil matrix and the inoculation of the strain enhanced their biodegradation. According to Nasseri & *al.* (2010), the contribution of *Ps.aeruginosa* in a soil contaminated with phenanthrene led to the improvement of its degradation with a rate of 85.5% while the rate of 17.5% only was recorded in the soil non-inoculated with this strain. Gabet (2004) has achieved results demonstrating the ability of the biosurfactant (rhamnolipid) to remobilize trapped aromatic hydrocarbons in contaminated soils. Bordas & Lafrance (2001) have also shown that the rate of pyrene, aromatic hydrocarbon, remobilized by the rhamnolipid biosurfactant produced by *Ps. aeruginosa* reached a rate of 70% in their remediation test of soil contaminated with pyrene. According to Bard (2006), among the compounds of oil, the alkanes are the most biodegradable, whereas the more refractory are asphaltenes. The amount of remobilized hydrocarbons increases with the rate of contamination of soil, until saturation of biosurfactant micelles. Also, Chia-Wei &*al.* (2013) observed a positive correlation between total petroleum hydrocarbons degradation and the reduction of surface tension which indicates the presence of biosurfactants in the medium.

Microbial biomass

Microbial biomass is the active and the labile fraction of the soil organic matter since it regulates the processing and the storage of nutrients necessary for plant growth. It depends on the available carbon to meet the energy needs of micro-organisms (Chaussod & *al.*, 1986).
Hydrocarbons have a depressive effect on the microbial biomass. This may be due to their toxicity on microbial cells. Indeed, Jennings and Tanner (2000) noted that the number of heterotrophic aerobes is reduced less than a half in a grassland contaminated with oil. Sparrow & *al.* (1988) stressed that the microbial biomass reaches its lowest level in the soil contaminated by oil.

Germination test

The low germination rates recorded in the treated soil can be explained by the fact that some seeds of wheat rot. We believe that the low germination rate in the control is due to the toxic effect of hydrocarbons exerted on wheat seeds. Indeed, Chaineau & *al.* (1997) noted that oil can get into the seeds, reach and alter the metabolic and / or kill the embryo in direct contact. It is mainly the light fraction of oil that induces a significant enough reduction in germination (Chaineau & *al.*, 1997). In addition, germination is correlated with the hydrophobic properties of oil that prevent and / or reduce the exchange of water and gas (Amakiri & Onofeghara, 1984; Amadi & *al.*, 1992; Udo & Fayemi, 1975 in Chaineau & *al.*, 1997). Otherwise, the cyclic lipopeptide produced by a strain of *Pseudomonas* limited the pathogenicity of *Rhizoctonia solani* by its antibiotic action (D'Aes & *al.*, 2011).

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