Research on the Influence of Diesel Oil on the Bacterial Stems of *Bacillus Subtilis* and *Pseudomonas Aeruginosa*

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In this experiment we tested separately the evolution of turbidity for two pure bacterial suspensions, Bacillus subtilis ATCC 2589 and Pseudomonas aeruginosa ATCC 15442, in a concentration of 1 Mc Farland. In comparison with the evolution of pure suspensions we have analyzed the evolution of suspension turbidities in contact with diesel fuel in different concentrations: 100µL Diesel, 200µL Diesel and 300µL Diesel. The samples were incubated at 30-37 degrees Celsius for 72 h ; after 6 h, 24, 48 and 72 h thermostating time, bacterial suspension was photo metered at a wave with 530 nm length for measuring the suspension turbidity. In order to determine the viability of the microorganisms tested in the presence of the analyzed contaminators were made counts of bacterial colonies grown on nutrient agar culture medium (CFU / mL).

Keywords: Diesel oil, Bacillus subtilis, Pseudomonas aeruginosa, turbidity

Toxic substances from wastewater in most cases act as inhibitors of cellular enzymes or through other ways, such as all groups of inhibitors that react with protein not only attack enzymes but also various proteins in cell structure. These proteins can be found in the cytoplasmic membrane and their changes cause changes in permeability which will secondarily affect the metabolism systems.

The cellular metabolism can be altered by the inhibition of the transport system. The inhibitor can intervene through competition with substrates for transport system, inactivation of a membrane component, inhibiting an enzyme involved in energy supply for active transport, decreasing the synthesis efficiency of a protein or enzyme that interacts with the membrane substrate [1,2].

The contamination of soils and groundwater with petroleum compounds is among the most prevalent problems in environments worldwide. In situ biodegradation is one of the primary mechanisms by which petroleum and other hydrocarbons are eliminated from the environment. Hydrocarbon-degrading bacteria are widely distributed in marine, freshwater, soil habitats and their use in bioremediation of hydrocarbon-contaminated soils, which exploits their ability to degrade and/or detoxify organic contaminants, has been established as an efficient, economical, versatile and environmentally sound treatment.

Petroleum compounds with straight-chain *n*-alkanes of the saturated hydrocarbons are most susceptible to biodegradation, whereas branched alkanes are less vulnerable to microbial attack. The aromatic fraction is more difficult to biodegrade and the susceptibility of its components decreases as the number of aromatic or alicyclic rings in the molecule increases [3]. Polycyclic aromatic hydrocarbons occur extensively as pollutants in soil and water and are important environmental contaminants because of their recalcitrance. These compounds also constitute a potential risk to human health, as many of them are carcinogens .

Among several clean–up techniques available to remove petroleum hydrocarbons from the soil and groundwater, bioremediation processes are gaining ground due to their simplicity, higher efficiency and cost–effectiveness when compared to other technologies [4,5]. These processes rely on the natural ability of microorganisms to carry out the mineralization of organic chemicals, leading ultimately to the formation of CO_2, H_2O and biomass [6].

Strategies to accelerate the biological breakdown of hydrocarbons in soil include stimulation of the indigenous microorganisms by optimizing the nutrients and oxygen supply and the temperature and *p*H conditions (biostimulation), and through inoculation of an enriched mixed microbial consortium into soil (bioaugmentation). Besides to provide these optimum conditions, it is also important to know that the pollutant degradation in soil is influenced by mass transfer phenomena. Providing a way to reduce the sorption of the hydrophobic organic contaminants to the soil matrix can increase the rate and extent of biodegradation [7-11]. For this purpose, the addition of surfactants into the soil aims to enhance the emulsification of hydrocarbons and therefore they have the potential to solubilize hydrocarbons and increase their bioavailability and subsequent biodegradation. In biological treatments it is always necessary to perform laboratory feasibility tests to determine the microbial potential to degrade the pollutants and to evaluate strategies to optimize the degradation rates before the design of real scale in–situ or ex–situ (bioreactors, landfarming and others) treatments.

Bacteria appear to be relatively simple life forms. In fact, they are bodies with an incredible degree of adaptation, which involves complexity. Many bacteria multiply to very high speeds, and different species may use as food a huge variety of organic substances, including phenols, oil and other toxic contaminators [12-16].

On the other hand, cells can adapt, and can develop resistance to an inhibitor by different mechanisms. Although usually the adaptation of cellular metabolism to the inhibitor action is too slow to affect the speed of reaction, sometimes the adaptation is very fast and changes occur in the metabolic adjustments of cellular metabolism from enzymatic activity.

In this experiment it was attempted to find a combination of effective microorganisms in the degradation of the contaminators present in the wastewater coming from an oil refinery. It was studied

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the behaviour of different bacterial suspensions in the presence of Diesel fuel [17-21].

Experimental part

It was studied the inhibition effect of Diesel on two standard strains of bacteria: *Bacillus subtilis ATCC 2589 and Pseudomonas aeruginosa ATCC 15442.*

Materials and equipment used: sterile glass tubes (10 mL graduated tubes), 2-mL sterile syringes, sterile Petri-plates, incubator, colony counting device, spectro-photometer, densitometer Mc Farland, strains: *Bacillus subtilis, ATCC 2589, Pseudomonas aeruginosa* ATCC 15442, autoclave, hood with laminar air flow, Vortex homogenizer.

The culture medium used to promote the bacterial growth was prepared from: peptone 10 g, sodium chloride 5g, meat extract 10 g, water to 1000 mL. *The nutrient agar culture medium* for bacterial colony counting was made from: 10g bacto-peptone, 5 g Na Cl, 20 g agar, 10 g meat extract and water to 1000 mL, *p*H adjusted to 7.4. Medium cultures of nutrient broth and nutrient agar which were sterilized in autoclave at 121^{0}C for 15 min were prepared.

Hydrocarbon test preparation: 50 mL of Diesel was introduced in 50 mL glass bottle which was capped and autoclaved at 121^{0}C for 15 min for sterilization. Under the hood with laminar air flow**,** from each strain was taken 1 mL suspension was inoculated into 9 mL broth. It was incubated for 24 h at 30 - 37 degrees Celsius for activation and rejuvenation of micorbial culture. After the incubation, from each bacteria were made suspension cultures of 1.0 Mc Farland concentration, the dilutions were made in sterile saline. From each dilution made, it was taken 5 mL bacterial culture with 1 Mc Farland units concentration by adjusting the bacterial culture with DEN-1 densitometer over which it was added a set of samples and tested the evolution of the bacterium *Bacillus subtilis* : in the first tube 100µL Diesel oil, in the second tube 200 µL Diesel oil and in the third tube 300 µL Diesel oil. The hydrocarbon concentrations incubated in test tubes were: 20 mL / mL, 40 mL / mL or 60 mL / mL.

In one tube it was analyzed the evolution of the control sample of bacterium *Bacillus subtilis* incubated under the same conditions of temperature and time like the samples treated with contaminators (72h at 30-37^{0}C in the incubator). It was used pure bacterial strain of 1.0 Mc Farland units concentration.

The same procedure was used with the strain *Pseudomonas aeruginosa*. The incubation period after a thermostating time of 6, 24, 48 and 72 h, bacterial suspension which was photometered at 530 nm.After removing the tested samples from the incubator, before *fotometering*, the tubes were homogenized using *V*ortex homogenizer at 1200 rpm for 1 min.

In order to observe viability of the tested bacterial stem in polluted environments there have been made countings of bacterial colonies on nutrient agar culture medium (CFU / mL). From each tube, after fotometering were taken 0.5 mL suspension and decimal dilutions were made of 10^{-1} in saline (0.5 mL diluted to 5 mL). From the decimal dilutions were inoculated amounts of 1 mL in Petri dishes that contained nutrient agar and cooled to 45 ^{0}C. After cooling and incorporation, the plates were incubated at 30-37^{0}C with the lid down for 48 h for counting the surviving bacterial colonies. It was followed the bacterial viability in the presence of the contaminators with different concentrations. After incubation were there counted the bacterial colonies grown on nutrient medium.

Results and discussion

The studied bacteria have a different growth in incubation conditions in the absence of contaminators. *Bacillus subtilis* bacteria can metabolize the Diesel fuel which is used as a carbon source and which provides support for growth and development leading to bacterial multiplication. The studied concentrations are tolerated by the bacterium *Bacillus subtilis* studied which has a maximum bacterial population growth at 48 h and then decreases slightly compared with the pure suspension which has weaker development in the absence of the Diesel fuel.

The results obtained in this experiment are presented in graphic representation. In the first graph were represented the variation of the studied *Bacillus subtilis* bacterial strains, of the control samples that were treated in the same conditions of temperature and incubation. In the same dilutions it was tested the variation of their multiplication without having Diesel oil.

Concentrations of 60 µL/mL of Diesel fuel in the case of bacterium *Pseudomonas aeruginosa* are a growth factor which helps after 72 h bacterial multiplication but in comparison with the pure control bacterial suspension of bacterium *Pseudomonas aeruginosa* suffers an inhibition of cell growth.

Between the two studied strains, *Bacillus subtilis* has the best adaptation to Diesel fuel contaminator, bacteria *Pseudomonas aeruginosa* also supports concentrations of 60 µL/mL Diesel fuel but the pure suspension evolves better in the absence of the contaminator. From the two strains studied, *Bacillus subtilis* has the highest viability in the presence of oil. *Bacillus subtilis* support the oil used as a carbon source and can multiply in the presence of this cellular material. Viability of bacteria after 72 h of contact with oil is bigger in comparison with the viability of bacteria in environment without contaminator and without source of food. In case of accidental pollution with oil, the use of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains may help for the biodegradation of this type of contaminator.

Experimental results on the turbidity of the analyzed samples were compared by plotting (through graphic representations), figures 1 and 2. The graphs show the variation of turbidity for culture media that contained different concentrations of Diesel compared with control samples without contaminators.

Viability of bacteria after 48 h of incubation on nutrient media was compared by plotting the log CFU / mL in relation to oil contact time in the presence of various concentrations compared with control samples which were not contaminated (fig. 3 and 4).

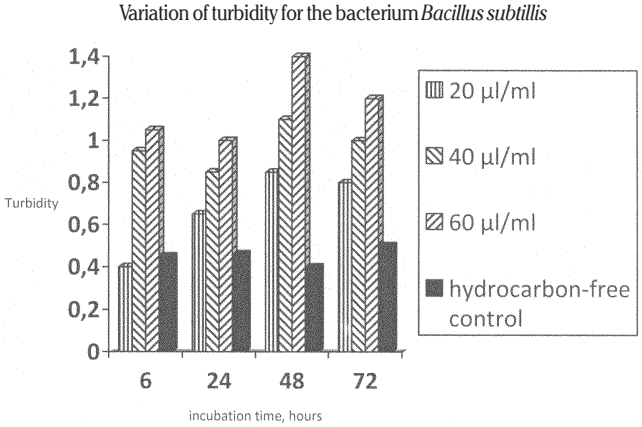

Fig. 1. Evolution of turbidity for the bacterium *Bacillus subtillis*

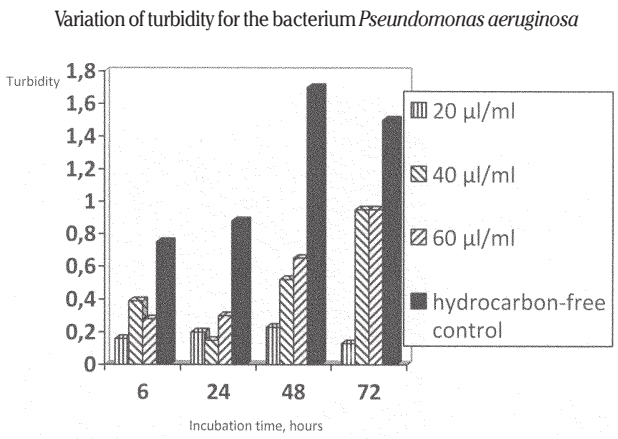

Fig. 2. Evolution of turbidity for bacterium *Pseudomonas aeruginosa*

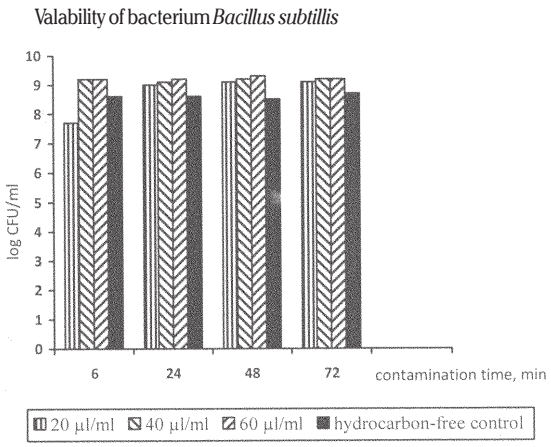

Fig 3. Variation of viability for bacterium *Bacillus subtilis*

Conclusions

Adaptation of some bacteria to different contaminators is due to phenomena related to physical, chemical and surface properties of bacterial cells coating which has the role to protect the cell and to absorb the contaminators.

Studies conclude that the bacteria *Bacillus subtilis ATCC 2589, Pseudomonas aeruginosa ATCC 15442*, can be used in processes of bioremediation of contaminated ecosystems.

References

1.ALQUATI C, PAPACCHINI M, RICCARDI, SPICAGLIA C, BESTETTI, Ann Microbiol 55, 237-242, 2005
2.MARGESIN R, SCHINNER F., Appl Environ Microbiol 63, 2660-2664, 1997
3.BARTHAKUR H.,. In Ecological Issues and Environmental Impact Assessment, ed. P. N. Cheremisrnoff, Houston, TX: Gulf Publishing Company 371-389, 1997

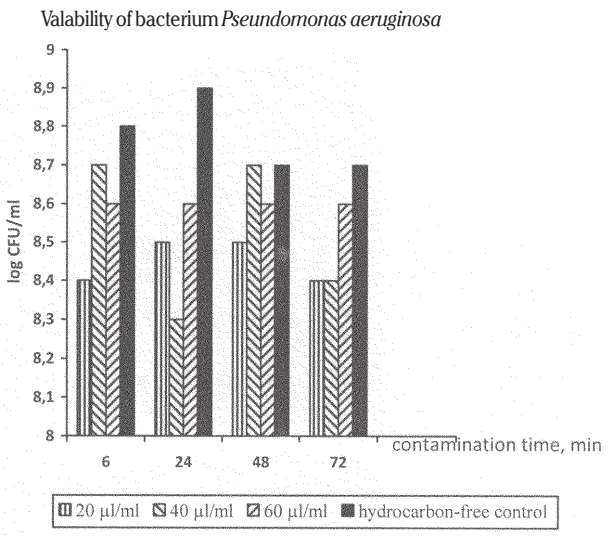

Fig 4. Variation of viability for bacterium *Pseudomonas aeruginosa*

4.LF TOLEDO, C. CALVO, B. RODELAS, Systematic and Applied Microbiology Volume 29, Issue 3, Pages 244-252, 2006
5.ALEXANDER, M., Academic Press, San Diego1994
6.DUARTE DA CUNHA, C.; LEITE, Braz. J. Microbiol., 31, 45–49, 2000
7.RIZZO, A.C.L.; CUNHA, C.D.; RAIMUNDO, R.S.; MAGALHÃES, M.H.; LEMOS, J.L.S.; MILLIOLI, V.S.; LEITE, S.G.F.; SANTOS, R.L.C.; SORIANO,
10th Annual International Petroleum Environmental Conference, Houston, USA, 2003
8.D. BUIUC, M. NEGUT, Medical Printing House, 1999
9.BEYCHOK, MR., Chemical Engineering Progress Symposium Series 67 (107): 322-339. Available at the CSA Illumina website, 1971
10.KHOPKAR, MS., Chemical Engineering Progress Symposium Series 67 (107): 322-339, 2004.
11.LARRY D. Ibis Publishing, 1993
12.PITT, T. L Pag. 1109-1113, 1998
13.GALLEGO, J. L. R., J. LOREDO, J. F. LLAMAS, F. VA´ZQUEZ, Bioremediation, 12, 325, 2001
14.JAIN, D. K., H. LEE, AND J. T. TREVORS, J. Ind. Microbiol., 10, 87, 1992
15.MORAN, A. C., N. OLIVERA, M. COMMENDATORE, J. L. ESTEVES, F. SINERIZ, Biodegradation, 11, 65, 2000
16.LIANG JIN0, XIAO-JUAN WANG, ZONG-LIAN GU, De-Zhi ZHOU, Si-Qin XIE, Appl. Microbiol., p. 540-544, 2006
17.ROESSLER, W.G., C.R. BREWER.. Appl. Microbiol. 15:1114-1121
18.MALLETTE, M.F.. XV., p. 521-566
19.*** EUROPEAN PHARMACOPOEIA 5.0. Cap. 2.6.12. Biological tests. Microbial examination of nonsterile products (Total viable aerobic count), Plate count methods, p. 154.
20.*** USP PHARMACOPOEIA 29 Validation of microbial recovery from pharmacopeial articles, cap. 1227 Estimating the number of colony forming units
21.*** EUROPEAN PHARMACOPOEIA 5.0.Cap. Alternative methods for controlof microbiological quality, 2.7.2. Turbidimetric method p. 4132

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