Testing the Behaviour of Pure Bacterial Suspension (Bacillus Subtilis, Pseudomonas Aeruginosa and Micrococcus Luteus) in Case of Hydrocarbons Contaminators

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The tubes in which the tests were made contained a 5 mL bacterial suspension 1 Mc Farland diluted in sterile saline. Over the content of each tube (except control tubes) were placed 200 µL of three contaminators (a tube corresponding to a contaminator). The concentration of the contaminated tubes was 40 µL/mL. Incubation between fotometerings was made at 30°C. After each fotometering, decimal dilutions were made in sterile saline. It was followed the viability of bacteria in environments with hydrocarbons, compared with the viability of bacteria without contaminators. From these dilutions, inoculations were made in Petri dishes containing agar solid medium. The surviving bacterial colonies were counted after 48 h of incubation. During incubation, the tested medium did not contain nutrients to promote the development of cell mass (bacterial suspensions were diluted in sterile saline). Thus, hydrocarbons were used as the sole carbon source to observe the viability of various bacteria in similar environments compared with their viability in environments without contaminators and nutrients (control samples).

Keywords: benzene, hexane, methylene chloride, Bacillus, Pseudomonas, Micrococcus

Toxic substances from wastewater in most cases act as inhibitors of cellular enzymes or through other means, for example all inhibitors that react with groups of proteins attack not only enzymes but also different structure proteins from the cell [1].

These proteins can be found in the cytoplasm membrane and their changes cause changes in the permeability which will secondary affect the metabolic systems. The cellular metabolism could be altered also by the inhibition of the transport system. The inhibitor can interfere through the competition with the substrate for the transport system, inactivation of a component of the membrane, inhibiting an enzyme involved in energy supply for the active transport, decreasing the synthesis efficiency of a protein or enzyme that interacts with the membrane substrate [2].

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. On the other hand, cells can adapt, and can develop resistance to an inhibitor by different mechanisms. Although, usually, the adaptation of the cellular metabolism to the inhibitor action is too slow to affect the reaction speeds, sometimes the adaptation is very fast and changes occur in the metabolism and adjustments of cellular metabolism against the enzymatic activity [3-5].

Petroleum compounds consist of four fractions: saturated hydrocarbons, aromatic hydrocarbons, nitrogen-sulphur-oxygen containing compounds and asphaltenes. Normally, of the saturated hydrocarbons, the straight-chain n-alkanes 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. 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 [5]. These processes rely on the natural ability of microorganisms to carry out the mineralization of organic chemicals, leading ultimately to the formation of CO₂, H₂O 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 pH conditions (biostimulation), and through inoculation of an enriched mixed microbial consortium into soil (bioaugmentation). In addition 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 [7-10]. 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 [11-14].

In the present experiment it was tried to find a combination of effective microorganisms in the degradation of the contaminators from the wastewater
coming from an oil refinery. We studied three compounds: benzene, hexane and methylene chloride. The exact composition of hexane fraction depends largely on the oil source (crude or reformed) and refining constraints. Benzene, known cancirogen, is an important industrial solvent and precursor in the production of medicines, plastics, synthetic rubber, and colorants. Methylene chloride is used in various industrial processes, manufacture of paints industry, pharmaceutical industry etc. It is an irritating solvent with repercussions on health, being aggressive pollutant[15-19].

Experimental part

Equipment used for experiments was: incubator MLV, analytical balances Ohaus, densitometer Mc Farland, standard suspension kits for the concentrations of germs, Areca Velp electric shaker, electric oven, horizontal laminar flow hood, autoclave with wet air, Magnifier spectrophotometer (colony counter), sterile swabs, sterile syringes, tubes stoppered, graduated flasks 100ml, 500mL, quoted Pipettes, Pasteur pipettes, physiological ser, Petri plates with 10 cm diameter; and biological material used was strains of: Bacillus subtilis, ATCC 2589, Pseudomonas aeruginosa ATCC 15442 and Micrococcus luteus ATCC 9341.

Used culture medium and chemicals: the culture medium used to promote the growth of bacterial germs was prepared from peptone 10 g, sodium chloride 5g, meat extract 10 g and water up to 1000 mL. Nutrient agar culture medium for counting the bacterial colony has been prepared from 10 g bacto-peptone, 5 g Na Cl, 20 g agar, 10 g meat extract and water to 1000 L, pH adjusted to 7,4. The medium is autoclaved at 121°C for 15 min for sterilization.

Analytes: hexane, benzene and methylene chloride (30 ml each) were placed in 50 mL glass containers and then capped and autoclaved at 121°C for 15 min for sterilization.

From each strain was taken a 1 ml suspension and was inoculated into 9 ml broth (nutritive medium). Was incubated for 24 h at 30-37°C for activation and rejuvenation of the microbene culture. After the incubation, from each bacteria were made suspensions of bacterial cultures of 1.0 unit Mc Farland concentration, and the dilutions were made in sterile saline.

Four tubes, each containing 5 mL of a dilution for each strain of 1.0 unit Mc Farland have been used. Over the content of each tube (except control tubes) were placed 200 μL of three contaminators (a tube corresponding to a contaminator). The concentration of the contaminated tubes was 40 μL/ mL. Incubation between fotometerings was made at 30°C for a period of 18 h. During this period, the samples were fotometered three times at 530 nm: after 30 min, 120 min and 18 hours. The microbial growth leads to detectable changes in environmental opacity. This could be quantified by measuring optical density with a spectrophotometer at a wavelength of 530 nm. Turbidity does not indicate the viability of microorganisms, the method does not distinguish between viable and nonviable microorganisms.

To observe the viability of polluted environments, there have been made counts of bacterial colonies developed on nutrient agar culture medium (CFU/mL). After each fotometering, separately were made decimal dilutions in sterile saline. Tubes were marked and were prepared Petri dishes containing nutrient agar medium at 45°C. The diluted samples were inoculated (1 mL of decimal dilutions of 10⁻¹) in nutrient agar medium poured into Petri dishes. The samples were scrambled for incorporation and after cooling were incubated in thermostat at 30-37 °C for 48 h. The bacterial colonies grown on nutrient medium were counted after incubation.

Results and discussions

Experimental results on the turbidity of analysed samples are shown in graphic representation. The results were compared by plotting (figs. 1,3,5). The graphs show the turbidity variation of culture medium containing contaminators in comparison 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 the contamination time. The results showed that the viability of bacteria was significantly reduced in the presence of contaminators compared to control samples without contaminators.

Fig. 1 Variation of turbidity for medium with Bacillus subtilis

Fig. 2 Variation of viability for bacterium Bacillus subtilis
to contact time in the presence of contaminators in comparison with control samples without contaminators. It was followed the viability of bacteria in environments with hydrocarbons, in comparison with the viability of bacteria without contaminators (figs. 2, 4, 6).

Bacterium *Bacillus subtilis* has a significant increase in contact with benzene in comparison with the control sample in the first 30 min. In the next period, up to 120 min, the bacterial mass suffers inhibition, and the viability decreases again after 18 h, the cell density is lower than in the first half hour. In terms of hexane in the first 30 min bacterium *Bacillus subtilis* has a slight increase of cell mass in comparison to the control samples (fig. 1).

Bacterium *Bacillus subtilis* in contact with methylene chloride in the first 30 min of incubation, multiply the cell density and after 120 min suffers inhibition and decay, decreasing its biosynthesis and biodegradation efficiency and leading to lower cell density compared with that of a control sample (fig. 2).

Microcococcus strain, in the first 30 min of contact with benzene undergoes a phenomenon of inhibition of the bacterial growth in comparison with the bacterial growth from the control sample (witness sample) (fig. 3). After 120 min of incubation the cell mass adapts to the contaminator and generates the bacterial multiplication in contact with the benzene in comparison with the control sample.

For a bacterium *Micrococcus luteus* a hexane type contaminator affects the cell development and is not tolerated leading to enzyme inhibition after 18 h of contact. Methylene chloride also causes inhibition, but later bacterium begins to regenerate after a period of adaptation to the contaminator and after 120 min lowers the cell density below the density of the control sample (fig. 4).

*Pseudomonas* strain had a decrease in bacterial growth in contact with benzene in comparison with the control sample in the first 30 minutes; then, the strain has adapted to environments contaminated by benzene and sparked bacterial development after 120 min, in comparison with the control sample which has a decrease of bacterial development due the shrinking of the nutrient substrate in the test vial (fig. 5).

In the presence of methylene chloride it does not show large variations of inhibition for the cell density multiplication in comparison to the control sample incubated without contaminator. After 18 h the ampoule with benzene has a spectacular adaptation and an increased resistance and uses as nutrient substrate the tested contaminator which leads to an exponential increase in bacterial mass. The bacterium *Pseudomonas aeruginosa* suffers the cell multiplication inhibition but up to 120 min contact adapts and synthesizes the cellular material (fig. 6).
Conclusions

Experiments have shown that bacteria *Bacillus subtilis* ATCC 2589, *Pseudomonas aeruginosa* ATCC 15442 and *Microccocus luteus* ATCC 9341 are able to utilize benzene, hexane and methylene chloride to increase biomass, cell viability in the presence of these contaminants is different for the three tested bacterial species which established competing relationships for the substrate. Tests showed that the most resistant bacterial strain tested in the presence of benzene and hexane is the bacteria *Bacillus subtilis* and methylene chloride is well tolerated by the bacterium *Pseudomonas aeruginosa*.

Studies conclude that the bacteria *Bacillus subtilis* ATCC 2589, *Pseudomonas aeruginosa* ATCC 15442, *Microccocus luteus* ATCC 9341 can be used in processes for bioremediation of contaminated ecosystems.

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