USING A BACTERIAL CONSORTIUM IMMOBILIZED IN CHITOSAN BEADS AS A TOOL FOR BIOREMEDIATION

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ABSTRACT – *Bioremediation consists of using living organisms to mitigate the effect of environmental pollutants. This work evaluated the capacity of a bacterial consortium immobilized in chitosan beads to degrade n-hexadecane. The consortium was tested in sterilized and unsterilized sediments, both in free form and immobilized in chitosan beads. The microcosms were contaminated with 10 mg of n-hexadecane/g of sediments and inoculated with 10^6 CFU/g of bacterial cells. The biodegradation was evaluated by monitoring the dehydrogenase activity (DHA), estimated by measurements of the product formazan. The immobilized consortium showed significant dehydrogenase activity compared with controls, confirming the capacity to metabolize the pollutant. This product represents a new option of bioremediation technology, and can be applied in mangroves contaminated with n-hexadecane, commonly found in oil spill areas, with the advantage of being an ecologically safe technology.*

1. INTRODUCTION

Mangrove ecosystems are known for acting as pollutants reservoirs due to the high retention capacity of their sediments and thus are considered to be very sensitive to oil spills (Duke et al. 2000). Due to urbanization and the growth of activities related to the oil industry, the emission of pollutants has also increased, as well as the number of accidents involving oil spills in coastal and estuarine areas (Gentili et al., 2006).

Environmental contaminants derived from oil include hydrocarbons such as n-hexadecane, which is considered as a long persistence contaminant due to its low solubility in water, making its degradation far more difficult (Ciric et al., 2010). The disturbance generated in the environments affected by petroleum hydrocarbons is harmful to both local biodiversity and to human health, so the remediation of impacted environments is extremely necessary.

Bioremediation consists of a set of processes in which living organisms, usually plants or microorganisms, are used in order to remove or reduce environmental pollutants. This technique is

highly recommended as a viable alternative for treating contaminated environments, such as surface and groundwater, sediments, industrial and domestic effluents (Alexander, 1999).

The immobilization of microorganisms as a bioremediation strategy presents advantages compared with the use of cells in suspension, such as facilitating the recovery, collection and isolation of the products; higher growth rates and higher biomass when used in reactors, higher catalytic stability, increased tolerance to high concentrations of toxic compounds, reusability of microorganisms, lower susceptibility to contamination, as well as greater stability and lifetime of the immobilized cells (Matsumura et al., 1997). Still, the degradation of immobilization matrix and its use as a carbon source acts as a biostimulator to the growth and proliferation of microorganisms, which may accelerate the bioremediation process (Hsieh et al., 2008).

Among the materials used in the immobilization process, chitosan, a cationic polysaccharide produced by the deacetylation of chitin, stands out due to several factors. Chitin is the main component of the shells of crustaceans, exoskeleton of insects and fungi cell walls, making it an abundant natural resource and it also has a low cost, taking into account that can be obtained as a byproduct of crustacean processing (Hsieh et al., 2008).

In this context, this study aimed to test the hypothesis that mangrove sediments contaminated with n-hexadecane can be recovered by applying a consortium of bacteria immobilized on chitosan beads in a fast, efficient and environmentally safe manner.

2. METHODS

2.1. Bacterial strains

In this study we used 7 bacterial strains belonging to the Microbial Ecology and Biotechnology Laboratory (LEMBiotech) from the Federal University of Ceará (UFC) microbial collection, which were isolated by enrichment technique in mineral medium containing n-hexadecane as sole carbon source from sediment samples in an oil chronically contaminated area within a mangrove in Baia de Todos os Santos, Brazil (Angelim et al., 2013).

For reactivation, the cultures stocks were inoculated into agar plates containing Tryptone Glucose Yeast Extract (ATGE), plus NaCl 2% (w/v) (saline ATGE). The isolated colonies were used for preparation of pure cultures in saline TGE broth and incubated at 150 rpm, 30 °C for 24 h. From these cultures the inocula were obtained and were adjusted to 0.1 OD at $\lambda = 600$ nm.

2.2 Preparation of the microcosms

The microcosms were assembled in 50 ml Falcon tubes containing 10g of sediment. The tubes pellets were sterilized by autoclaving at 121 °C for 1 hour and after this time colony forming units (CFU) were counted by dilution technique in saline ATGE plates. The plates were incubated at 30 °C and observed daily up to 7 days.

Sterile sediments were added to 2500 uL of sterile distilled water, 50 uL of culture and 100 mg of n-hexadecane. The seven bacterial strains were tested alone or in consortium. For the consortium, 50 uL of each culture selected to compose the consortium were mixed vigorously and from this mixed cultures 50 uL were taken to be inoculated to the microcosm. At 0, 3, 6, 9, 12, 18, and 24 days after inoculation, triplicates of each microcosm were sacrificed to monitor the pH, total bacterial counts and measurement of dehydrogenase activity. Two independent experiments were executed.

2.3 Dehydrogenase Activity

In order to measure the metabolic status and the respiration rate of the microorganisms in the microcosms, dehydrogenase activity (DHA) was employed, using the method of the tetrazolium salt (Grabe, 1970).

At the end of each period, 5 ml of a 2% (w/v) Triphenyl tetrazolium chloride (TTC) prepared in 100 mM Tris-HCl buffer, pH 7.0, were added to the microcosms, which were incubated for 24h protected from light. The product, formazan, was then extracted by adding 20 ml of acetone under vortexing for 5 seconds, followed by centrifugation at 5000 g for 10 min to separate supernatant and sediment. The supernatant containing formazan was collected and measured in a spectrophotometer at 485 nm. The concentration of formazan was determined from a standard curve previously prepared from known concentrations of commercial formazan and the activity expressed in mass of triphenylformazan (TPF) formed per microgram of sediment (TPF.g-1 ug) (Figure 1).

2.4 Immobilization of microorganisms in chitosan beads

The selected bacterial strains were immobilized in chitosan beads following the method described by Angelim et al., 2013, which consists in solubilizing 3% (w/v) chitosan (brand Galena, 86.5% deacetylation) in 100 ml of an acetic acid solution 1% (v/v) under slow agitation for at least 3 hours and left to stand for 24 hours. Concomitantly, the inoculum was prepared from the resulting biomass from the 100 ml of bacterial culture centrifuged at 10,000 g for 5 min. The biomass was resuspended in 1 ml of 0.9% NaCl and the suspension was mixed with chitosan gel and the mixture was slowly agitated for 3h. The chitosan gel containing the microorganisms was dripped using syringes in 200 ml of a 1% tripolyphosphate (TPP) (w/v) solution pH 9.0 for the formation of beads and entrapment of cells. The beads remained in contact with TPP solution for 3h allowing the proper formation of cross-links in the chitosan chains, ensuring the integrity of the beads. Subsequently, the beads were washed in a 0.15M K_2HPO_4 solution pH 8.0 three times. In order to monitor the microbial biomass, 3g of pellets were weighed, grinded, diluted in 30 ml of sterile saline and plated on saline ATGE for total viable cell count expressed as Colony Forming Units (CFU) per gram.

The degradation of chitosan beads was visually monitored, comparing the two employed conditions: sterilized and unsterilized sediment. The microcosms were sacrificed and poured into plates and washed with distilled water in order to detect the beads and/or their residues.

2.5 Statistical Analysis

Quantitative tests were analyzed in at least two independent experiments in triplicate and data were subjected to analysis of variance by ANOVA with Tukey post-test using a confidence interval of 95% ($p < 0.05$). Statistical analysis and graphs were made using the GraphPad Prism 5.0 software.

3. RESULTS AND DISCUSSION

3.1 Testing in microcosms

Dehydrogenases are among the most commonly found enzymes in soil. The dehydrogenase activity is a measure of the intensity of the microbial metabolism in the soil, thus the microbial activity in the soil. Studies have shown a positive correlation between an increase in DHA and bioremediation of soils contaminated with petroleum hydrocarbons (Benedict et al. 2003)

Seven strains were tested and only HEXBA01, HEXBA04, HEXBA05 and HEXBA06 presented DHA, especially HEXBA05 that produced more than 10 g of TPF/g on the sixth day, the activity dropped slightly on the 18th day and then increased on the 24th day. Although HEXBA01 and HEXBA04 have also shown DHA, this activity was negligible compared to HEXBA05. HEXBA06 also had high DHA, although with it peaked only at day 24 (Figure 4).

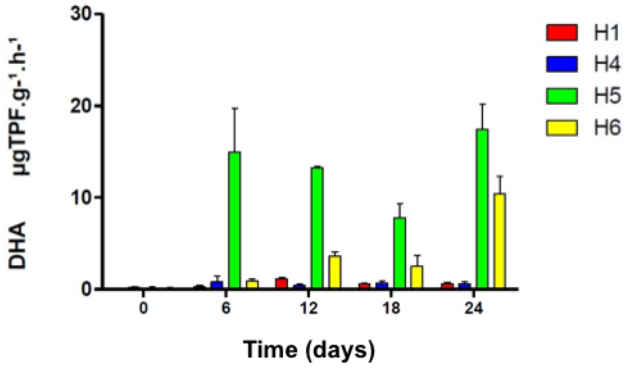

Figure 1 - Dehydrogenase activity (DHA) monitoring during the biodegradation of n-hexadecane in mangrove sediments microcosms inoculated with the bacterial strains.

In general, the DHA in all strains increased from day 6 to 12, then decreased and a further increase in the 24th day was observed, this pattern was observed in all the repetitions. This suggests the occurrence of changes in the metabolic state of the strains between these periods, due to the many possible starting routes of the n-hexadecane as well as the use of by-products, such as fatty acids, formed in the first stage (Callaghan et al ., 2009) The most sudden drops in activity and cell growth observed (Figure 2) should be related to the degradation of intermediate products generated from the degradation of n-hexadecane, leading to a community adaptation.

As shown in Figure 2, four strains of bacteria remained viable over 30 days of culture in the

microcosms. Overall, there was no temporal correlation between the increase in the number of viable cells and increased DHA. This result is corroborated by the fact that HEXBA06 had a significant increase in the number of CFU/g on the 6th day while an increase in the DHA only happened on the 24th day. Likewise, a reduction in HEXBA05 counts in the 24th day and a peak DHA occurred. Benedict et al (2003) found no relationship between the increase in cell counts with metabolic activity.

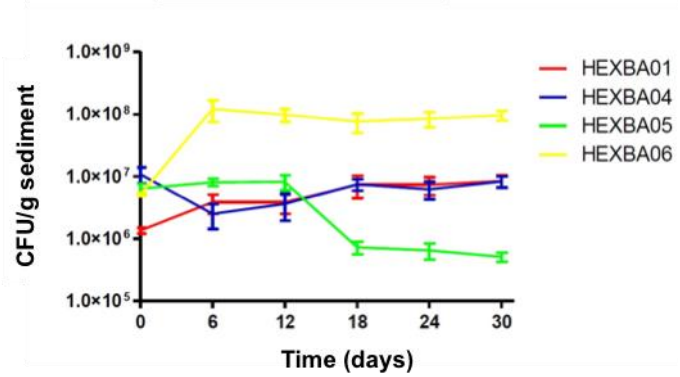

Figure 2 - Growth curves of HEXBA01 strains HEXBA04, HEXBA05 microcosm and sterile HEXBA06 in mangrove sediment contaminated with 10 mg / g of n-hexadecane.

The tested strains had higher DHA values, HEXBA05 (DHA max. = 20.14 µgTPF) and HEXBA06 (DHA max. = 11.46 µgTPF). Therefore these two strains were combined in a consortium called CHB56, which was tested and the results are shown in figure 6.

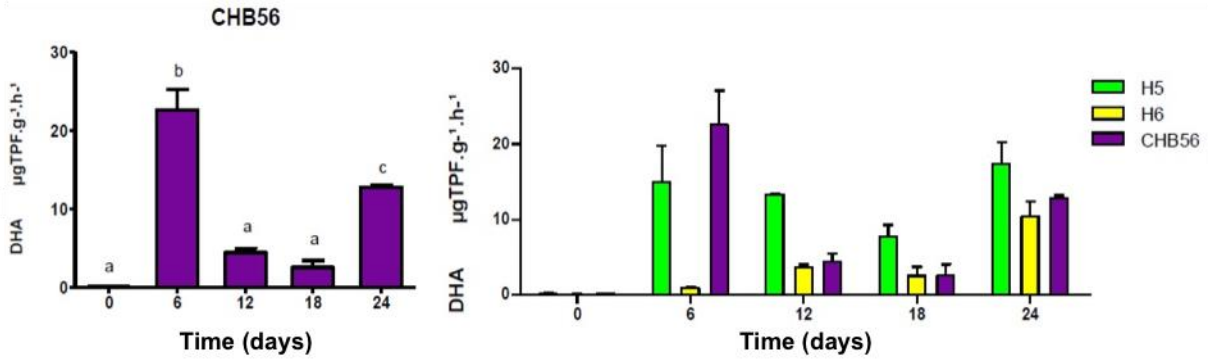

Figure 3 - DHA in CHB56 consortium and comparison with the results of individual strains.

The same pattern of DHA by the consortium was observed, except that there was an increase of enzyme activity on the sixth day. This improved performance can be explained by the fact that during the degradation of n-hexadecane intermediate byproducts are produced and oil degradation by microbial co-metabolism becomes essential, since compounds considered toxic to a particular micro-organism can serve carbon source to another (WETLER-TONINI; Rezende; GRAVITOL, 2011). Based on these results, the CHB56 consortium was selected for the following tests.

3.2 Testing in microcosms with CHB56 consortium

The next step, after the consolidation of an efficient consortium, we tested two additional conditions in microcosms: i) the presence of indigenous sediment organisms (sterile or natural sediment), and ii) the inoculation of microorganisms: in liquid medium or imprisoned in chitosan beads (free or immobilized consortium). The combination of these variables generated the 4 conditions used in the test: Free Consortium, Sterilized Sediment (CLSE); Immobilized Consortium, Sterilized Sediment (CISE); Free consortium, Natural Sediment (CLSN); Immobilized Consortium, Natural Sediment (CISN).

In these tests, after inoculation at time zero, DHA was measured earlier, within 3 days, instead of the 6 days of previous tests (Figure 4). It is noteworthy that in both types of microcosms activity was detected on the third day, i.e., the metabolic activity in fact occurs earlier than it had been measured in the first tests (Figure 3). Analyzing the two microcosms prepared with sterilized sediments, we clearly observed that DHA detected in microcosm inoculated with the immobilized consortium (CISE) exhibited twice the DHA than in the microcosms inoculated with the free consortium This difference can be explained by the protection provided by the chitosan matrix to the microorganisms so that it is not exposed to the direct toxicity of the contaminant. Also, chitosan matrix provides a favorable environment for the growth of the entrapped bacteria, ensuring gas exchange and nutrient input through their pores. In addition to offering advantages to the immobilized consortium, chitosan also has prebiotic activity and favors the growth of indigenous microbial communities (Angelim et al., 2013).

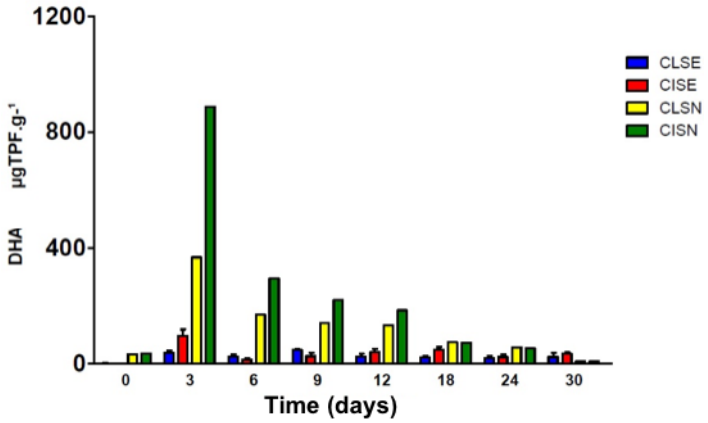

Figure 4 - Dehydrogenase activity (DHA) monitoring during the biodegradation of n-hexadecane in mangrove sediments microcosms inoculated with CHB56 consortium under different conditions.

In microcosms prepared with non-sterile sediment, ie, that simulate a natural scenario, since it has the influence of the native flora, the result with the immobilized consortium differed even more from the free consortium with DHA values twice as high. These microcosms DHA was also significantly higher than in the microcosm that used sterile sediment, demonstrating unequivocally the

participation of native flora in the biodegradation of n-hexadecane.

As shown by Angelim et al. (2013) HEXBA05 and HEXBA06 do not produce chitosanases, which implies that the condition in which the sediment is sterilized, increasing DHA should only be by the consortium activity. In the non-sterile condition, in which indigenous microorganisms capable of degrading chitosan may occur, the large difference in the peak of DHA may be attributed to the metabolization of the N-acetyl-glucosamine chains of the chitosan. Chitosanases are widely distributed in nature, being produced by bacteria and fungi, assuring the biodegradation of chitosan under natural conditions.

Therefore, even if the strains of immobilized microorganisms in chitosan beads do not produce chitosanases, inevitably the trapped biomass will be released after degradation of the matrix by the environmental microbiota. This explains the progressive wear observed in the chitosan beads in the microcosms prepared with non-sterilized sediment and inoculated with CHB56 consortium, with visible reduction of size and rupture up to the ninth day. In the 12th day of the beads had been completely degraded. This observation shows the potential of the indigenous microbiota to degrade the chitosan beads.

In an attempt to assess all possible interfering factors in determining the DHA in the consortium, we analyzed n-hexadecan degradation under the following conditions: i) microcosm with unsterile sediment and chitosan beads without bacteria - to evaluate the metabolism of chitosan by the indigenous microbiota (C1); ii) microcosms with unsterile sediment enriched only with n-hexadecane (10 mg/g) - to evaluate the ability of the indigenous microflora to metabolize n-hexadecane (C2); iii) Microcosms with unsterile sediment without addition of any carbon source (SED) - to evaluate the basal sediment DHA

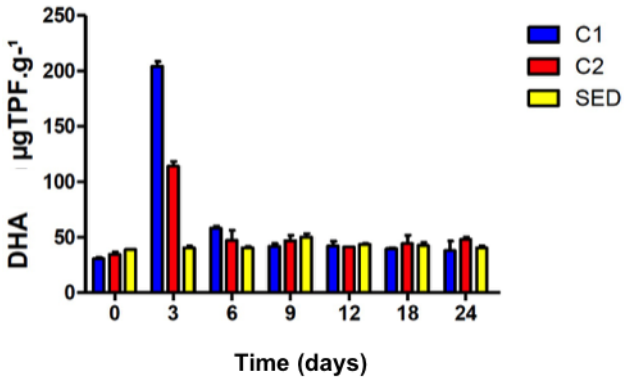

Figure 5 - Results of DHA conditions control of the microcosms.

The results clearly showed that the basal activity of the sediment (SED) is very low and this result was essential to support all the results obtained so far with the consortia. We then observed that an enzymatic activity with the addition of chitosan, proving that the indigenous microbiota are able to metabolize this polymer and this is quite notable, since it is important that the beads are biodegraded in the natural environment. Activity in the C2 control proved that the native flora has a low capacity

to metabolize n-hexadecane, proving the value of bioaugmentation with the selected strains.

4. CONCLUSION

The results of this study showed that CHB56 consortium immobilized on chitosan beads was more effective in degrading n-hexadecane in mangrove sediments contaminated with the pollutant than the free consortium, thus, it may become a new bioaugmentation technology option with the advantages of the easy handling, storage, transport and application.

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