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Comparison of different slow-release nutrient composites produced to stimulate microorganisms

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ABSTRACT

Concern for environmental quality has increased in society because industrial and technological development has released high levels of contaminants into the environment, such as hydrocarbons. A technique widely used for bioremediation is biostimulation, which may be enhanced by microencapsulation. This research formulated slow-release nitrogen and phosphorus compounds using different polymer (Alginate/Capsul®, carboxymethyl cellulose) matrices and compared them with the agricultural product Osmocote® and mineral medium Bushnell-Haas as hydrocarbonoclastics biostimulation agents in the environment for pollutant bioremediation. N (nitrogen) and P (phosphorus) were immobilized using lyophilization and ionic gelation techniques. Experiments were conducted using encapsulated material and evaluated for biomass production, glucose consumption as organic carbon source and N and P supply. The immobilized carboxymethyl cellulose compound showed the best results of glycosidic degradation (66.7%) and microbial biostimulation (350 mg L-1 protein) compared to systems containing free nutrients (11.3% and 150 mg L-1 degradation glycosidic and microbial biostimulation, respectively). Thus, this compound is a potential slow release product for bioremediation processes.

Keywords: biotechnology, environmental, pollution.

Comparação entre diferentes compósitos de liberação lenta de nutrientes para estimulação de microrganismos

RESUMO

A preocupação com a qualidade ambiental aumentou na sociedade porque o desenvolvimento industrial e tecnológico liberou altos níveis de contaminantes no meio ambiente, como os hidrocarbonetos. Uma técnica amplamente utilizada para a biorremediação é a bioestimulação, que pode ser reforçada por microencapsulação. O objetivo desta pesquisa foi formular compostos de nitrogênio e fósforo de liberação lenta usando diferentes matrizes de polímeros (Alginato/Capsul®, Carboximetilcelulose) e compará-los com o produto agrícola Osmocote® e meio mineral Bushnell-Haas como agentes de bioestimulação hidrocarbonoclasticos no meio ambiente para biorremediação poluente. N (nitrogênio) e

P(fósforo) foram imobilizados usando técnicas de liofilização e gelificação iónica. As experiências foram conduzidas usando material encapsulado e avaliadas para produção de biomassa, consumo de glicose como fonte de carbono orgânico e fornecimento de N e P. O compósito imobilizado composto por carboximetil celulose apresentou os melhores resultados de degradação glicosídica (66,7%) e bioestimulação microbiana (350 mg L-1 de proteína) em comparação com os sistemas contendo nutrientes livres (11,3% e 150 mg L-1 de degradação glicosídica e bioestimulação microbiana respectivamente). Assim, este composto é um potencial produto de liberação lenta para processos de biorremediação.

Palavras-chave: ambiental, biotecnologia, poluição.

1. INTRODUCTION

Concern for environmental quality has increased in society. The increase in industrial and technological development has led to highly contaminated soils, rivers, lakes, oceans, groundwater and sediments (Röling and Versevel, 2002). Because many contaminants have mainly originated from oil industries, high levels of carbon (originating from petroleum hydrocarbons) have been released to the environment.

According to the US Environmental Protection Agency (USEPA), one of the most widely reported processes in the literature for treating areas contaminated with high levels of available carbon is the bioremediation process. Bioremediation is a treatment process that uses naturally occurring microorganisms to degrade hazardous substances and transform them into less- or non-toxic substances (USEPA, 2004).

Bioremediation technologies can be classified as *ex situ* or *in situ*. *Ex-situ* technologies are treatments that remove contaminants to a location outside the contamination place, and *in situ* technologies involve treating the contaminants where they occur. *In situ* bioremediation techniques are advantageous because they can eliminate transportation costs with less intervention (Iwamoto and Nasu, 2001; Tyagi et al., 2011).

Different techniques can be used for *in situ* bioremediation, including natural attenuation, bioaugmentation and biostimulation. Natural attenuation is a reduction of toxicity, mobility or volume of the contaminant without human intervention and can occur by physical, chemical and biological processes. Bioaugmentation, however, involves stimulating the native populations that are reintroduced to the contamination site or the addition of wild strains or non-native mixed cultures to the contaminated site, which can degrade the pollutant. Finally, biostimulation is a technique aimed at stimulating native microbiota in a given environment due to an adequate supply of nutrients and favorable environmental conditions. However, the main idea is to influence development of indigenous microorganisms at a contaminated site to reduce the contaminant through its use as a carbon source in microbial metabolism, which reduces pollutants over time (Azubuike et al., 2016; Iwamoto and Nasu, 2001; Tyagi et al., 2011). Currently, many studies have used biostimulation, because it causes less damage to the environment. However, the stimulus is not always immediate and may require some time before results appear. The quantity and type of added nutrients depend on the carbon, nitrogen and phosphorus ratio (C: N: P) in the environment. This ratio should always be balanced to allow the microorganisms to grow adequately (Azubuike et al., 2016).

To provide such nutrients, cause less impact on the system and generate good nutritional relationships, many scientific investigations have used the microencapsulation technique for active materials in formulating slow-release fertilizers. Using this technique, the contents gradually release and meet the nutritional requirement of the involved organisms (Bansode et al., 2010; Reis et al., 2013; Favaro-Trindade et al., 2008).

Microencapsulation is a technology for packaging liquids, solids and gases in small, sealed

capsules, which isolates and protects them from adverse environmental conditions, such as light, oxygen, moisture and interaction with other compounds. Capsules can release their contents at controlled rates under specific conditions. These packages are spherical with a nanometer size; however, they are strongly influenced by the originating material structure (Bastos et al., 2009; Suave et al., 2006).

One of the key steps in coating is selecting appropriate wall materials. Coating materials are film-forming materials selected from a wide variety of natural and synthetic polymers, depending on the coated material and the desired microcapsule characteristics (Bastos et al., 2009; Dubey et al., 2009). Ideally, the wall material must be an emulsifier, promote adequate content release when reconstituted into the product, have good film-forming ability, have a low viscosity with high levels of solids, and have high hygroscopicity. The following are among the most commonly used wall materials: carbohydrates (starch, maltodextrins, sucrose and cyclodextrins), cellulose (carboxymethyl cellulose and derivatives), gum (Arabic and agar), lipids (waxes, paraffin and fatty acids), and proteins (gluten, casein, gelatin and others) (Dubey et al., 2009; Suave et al., 2006).

Recently, studies have evaluated the use of slow-release fertilizer, as a form of bio stimulus, to provide the concentrations of nutrients necessary for the bioremediation process. However, the majority of products that have been applied are usually used for different agricultural cultivars and provide nutrients (Darmayati et al., 2017; Becker et al., 2016). There are few studies on this line of bioremediation and the use of polymers such as encapsulation matrix has been reported for application mainly in the area of food and drugs.

Given the above considerations, the aim of this research was to formulate nitrogen and phosphorus slow-release compounds using different polymers (alginate / Capsul®, carboxymethyl cellulose) matrices and compare them to the agricultural product Osmocote® and mineral medium Bushnell-Haas as biostimulation agents of hydrocarbonoclastics in the environment for pollutant bioremediation.

2. MATERIAL AND METHODS

2.1. Preparing slow-release encapsulate

In order to obtain the first encapsulation, monopotassium phosphate, dipotassium phosphate and ammonium nitrate (all at 1 g L-1 and acquired from the Proquímios industry, Brazil) were weighed, dissolved in distilled water and mixed in a 3% sodium alginate solution (w v-1) (from the Proquímios industry) and 4% Capsul® (w v-1) (National Starch and Chemical Corporation USA). The final solution was transferred to a separator funnel and dripped into a 0.3 M calcium chloride solution (Vetec company, Brazil), which is the ionic gelation technique shown in Figure 1, where spherical capsules were formed at the end of the production and dried in an oven at 60°C for 1 h.

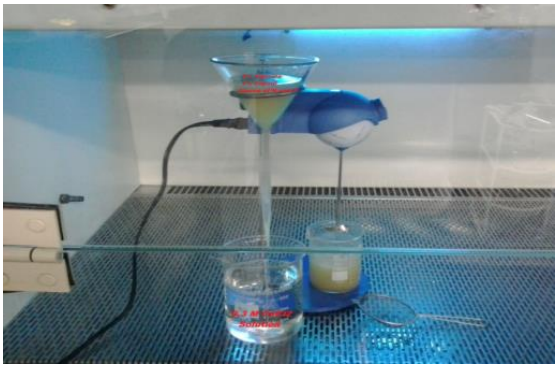

Figure 1. Alginate and Capsul® capsule production.

To produce the second encapsulated carboxymethyl cellulose (CMC), monopotassium phosphate, dipotassium phosphate and ammonium nitrate (all at a concentration of 1 g L-1 and from the Proquímios industry) were weighed, dissolved in distilled water and mixed in 1% carboxymethyl cellulose solution (Figure 2), which formed a solution that was freeze-dried in a countertop Enterprise I Terroni lyophilizer.

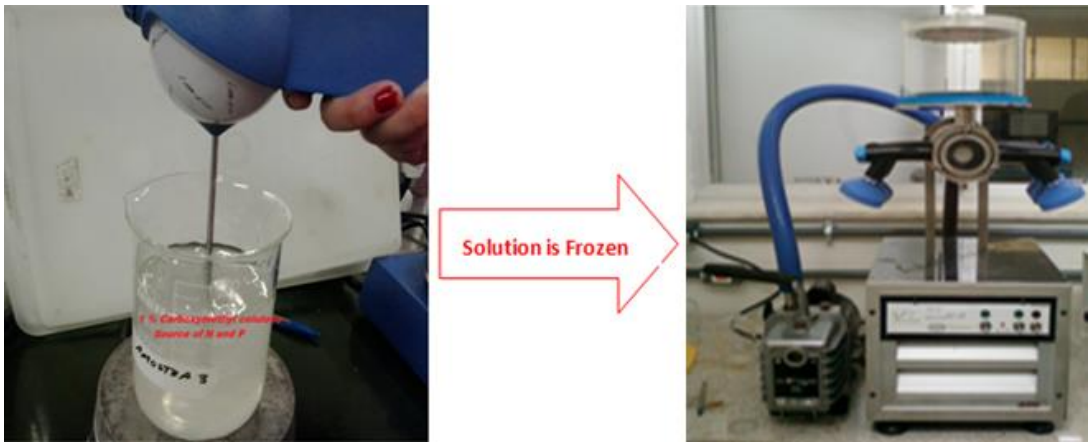

Figure 2. Schematic for producing immobilized carboxymethyl cellulose.

2.2. Morphology analysis of encapsulates

A morphological analysis of capsules was performed at the Ceramic Materials Laboratory of the Military Institute of Engineering in Rio de Janeiro. For this analysis, a JEOL® scanning electron microscope (SEM) Model JSM 5800W was used. The capsule sample was subjected to drying in an oven at 80°C for 24 h. Then, the samples were placed on metallic cylindrical holders (stubs) measuring 10 mm in diameter and secured with double-sided adhesive tape. The sample in the stubs was then coated with gold and placed in the SEM. The acceleration voltage used was 20 kV, with the secondary electron image as detector.

2.3. Biostimulation experiments

All experiments were inoculated with *Pseudomonas* sp. obtained from a marine environment with a history of hydrocarbon contamination and purchased by the laboratory of Industrial Microbiology of UFRJ (Lima et al., 2007). After growing in a nutrient broth, the strain was maintained in a refrigerator and transferred to fresh nutrient broth every month. For each experiment, the pre-inoculum came from a stock grown in a new sterile nutrient broth, which was maintained at 30°C for 24 h. The inoculum initially used was 5 ml for each 100 ml of experimental medium in the biostimulation system.

To understand the biostimulation process, experiments were performed using liquid systems composed of Bushnell-Haas (BH) (Hamdan and Fulmer., 2011; Reis et al., 2013) mineral medium and 2% glucose (w v-1). The mineral medium (BH) was then modified based on Reis et al. (2013) in which the nitrogen and phosphorus sources were replaced by immobilized matrices carboxymethyl cellulose. The biostimulation was performed in 500 ml glass jars with 200 ml of mineral medium of modified composition in which N and P were replaced by the encapsulated versions at the same concentrations as the original medium. The experiments with the commercial product Osmocote® were also conducted for comparison with the produced matrices, besides experiments with Bushnell- Haas (Reis et al., 2013) medium which was used as a positive control containing free nutrients.

All experiments were carried out in triplicate for 96 h, the samples were collected at intervals of 0, 24, 48 and 96 h; and the phosphorus, ammonia nitrogen and glucose concentrations were analyzed in all the proposed systems.

2.4. Analytical determinations

The ammonia nitrogen, total phosphorus and glucose were analyzed using a colorimetric kit specific for each analysis (Doles®, Brazil), and the protein concentration was determined using the Lowry method with BSA as the standard (Lowry et al., 1951).

3. RESULTS

3.1. Production and morphological characterization of slow-release nutrient encapsulate

The compound composed of 3% (w v-1) sodium alginate and 4% (w v-1) Capsul®, was diluted in distilled water and mixed with nutrients to form a solution source that was dripped in calcium chloride, which produced small whitish spheres that after drying had an average diameter of 0.2 mm (Figure 3).

Figure 3. Illustration of the encapsulation of alginate / Capsul® and nutrients (N and P) obtained before (a) and after drying in an oven at 60°C (b).

The compound composed of 1% (w v-1) carboxymethyl cellulose was diluted in distilled water and mixed with nutrients, forming a solution that was freeze-dried, which produced a compact product subsequently subjected to manual grinding for better handling (Figure 4).

Figure 4. Illustration of the encapsulation of carboxymethyl cellulose and nutrients (N and P) obtained before (a) and after freeze-drying process (b).

The scanning electron microscopy provides information on the morphological characteristics of the encapsulates, such as the presence of cracks and pores, allowing rapid and direct analysis of the efficiency of the encapsulation process (Figures 5 and 6).

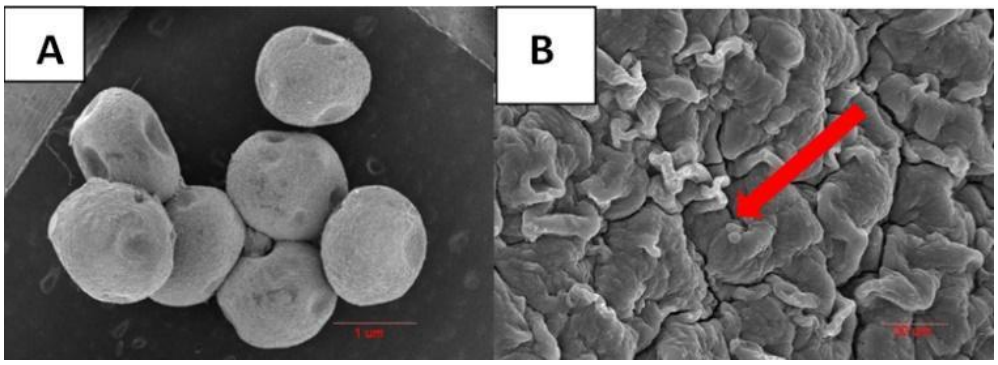

Figure 5. Encapsulate scanning electron microscopy of the polymers alginate/Capsul® with nutrients: a) An increase of 20 x was observed, the scale bar is1µm; and b) An increase of 1000 x was observed, the scale bar is 20µm.

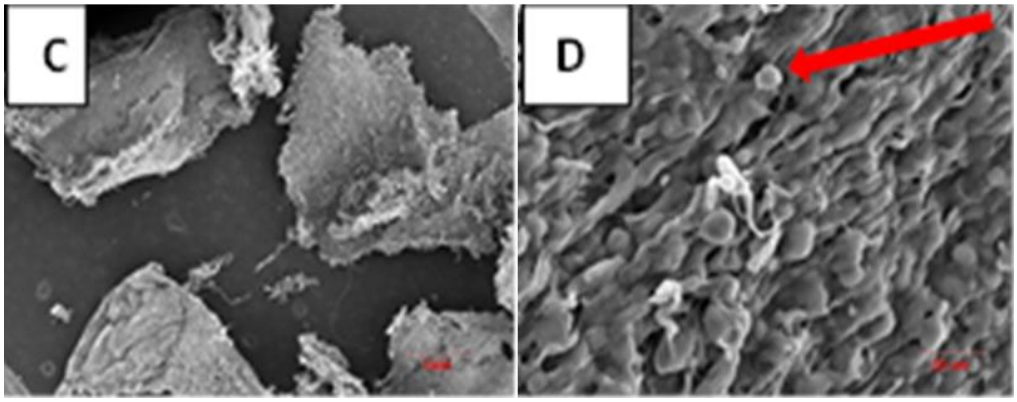

Figure 6. Encapsulate scanning electron microscopy of the polymer CMC with nutrients: c) An increase of 19 x was observed, the scale bar is 1µm; and, d) An increase of 1000 x was observed, the scale bar is 20µm.

Through the SEM images, it can be observed that both of the compounds formed Alginate / Capsul® as the carboxymethylcellulose presented in its polymeric walls granules scattered on the surface, as shown in Figures 5 and 6 (red arrows). According to Madene et al. (2006) and Matté and Rosa (2013), when this type of interaction between the encapsulated material (nutrients) and encapsulation material (polymers) occurs, the formed product is defined as microsphere.

In the images (Figures 5b and 6d), it can be seen that, in both cases during the encapsulation process, there are cracks on the produced materials' walls.

3.2. Assessing the biostimulation process

In the biostimulation process (Figure 7), the microorganism was evaluated as a function of time and increased the profile of the nutrient concentrations, the carbon-source consumption and a protein concentrations (microbial growth).

The results of Figure 7a show that the initial N concentration in the matrix containing systems was lower, indicating a possible retention of that nutrient when compared to the BH medium. An increase of N in the first 24 hours still appeared, with higher a value obtained in alginate/Capsul® system.

The free-nutrient system, Bushnell Haas (BH), in the same 24-hour period showed a visible consumption of nutrient by the decrease of its concentration. It is also observed that the BH and immobilized alginate/Capsul® systems exhibited nutrient stabilization after 48h of the process. The Osmocote and Immobilized CMC systems showed a drop profile in nutritional concentrations during the same period.

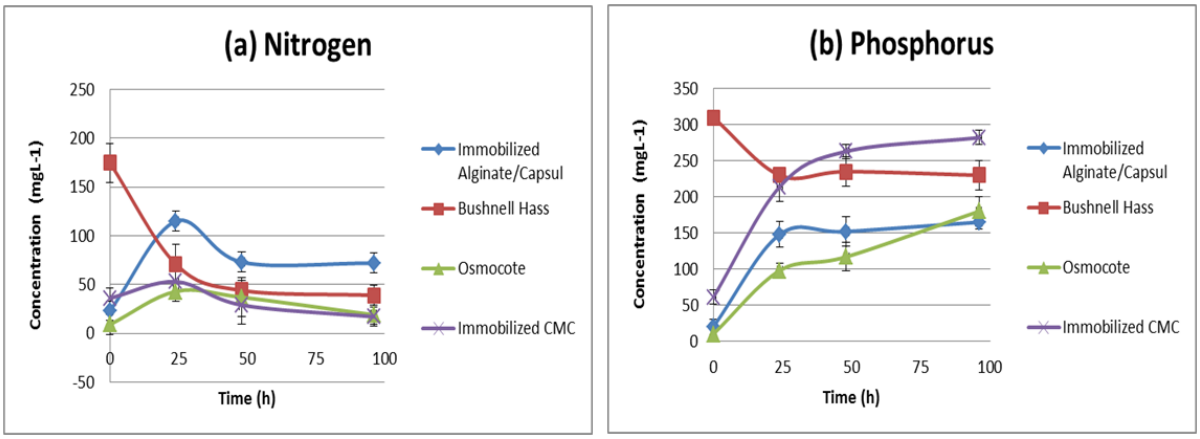

Figure 7. Variation of nutrient concentration over time in different systems: (a) nitrogen and (b) phosphorus.

The results of the nutrient phosphorus release presented in Figure 7b show that the polymer immobilization systems controlled the nutrients' release, but it is possible to observe that these systems provided a considerable phosphorus concentration in the medium already in the first 24 hours. It is also observed that throughout the experiment the mentioned systems were able to gradually increase the phosphorus concentration. The BH system showed a drop of the concentrations in the 24h of experiment and after that period the concentration profile remained stable until the end.

Microbial growth and carbon source consumption during the experiment in the different studied systems can be analyzed in Figures 8 (a) and (b).

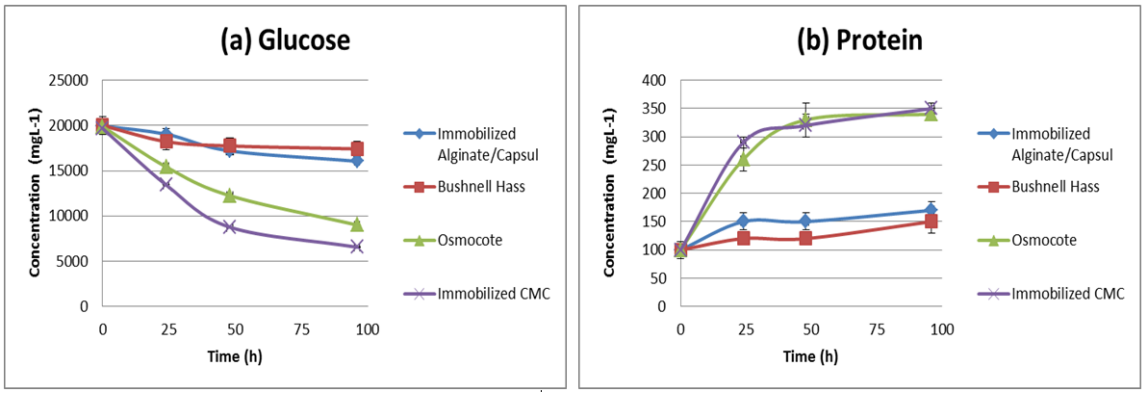

Figure 8. Variation of the carbon source consumption (a) and the estimated microorganism growth in different systems.

In Figure 8a it is possible to observe all the systems studied in the work presented a profile of the consumption of the carbon source throughout the experiment, with a concentration decrease in the medium. It is also verified that the system which provided the lowest concentration of carbon consumed was the experiment in the BH medium and the one that showed the highest decrease (higher consumption) in glucose concentration was the CMC immobilized system followed by the Osmocote system.

The results found in Figure 8b show that both alginate/Capsul® immobilized nutrient and the free nutrient (BH) systems provided a gradual profile in microbial growth. It is also possible to observe that both the CMC immobilized and the Osmocote system showed the highest values of protein concentration, unlike the other systems, whereas the system that exhibited a lower rate of protein concentration was the Bushnell Hass system.

The slow-release product developed in this study showed that, despite the experiment being conducted for 96 hours, there was no total release of nutrients (whose initial immobilized

concentration was equivalent to the Bushnell-Haas medium) and still has a residual concentration in the system (as seen in Figure 8) that would extend the time of biological activity. For an application in environmental systems, hence, not only the concentration of nutrients to be encapsulated but also the polymers could still be optimized, allowing a more adequate control of the release time thus prolonging the time of the process.

4. DISCUSSION

The polymer materials used for the production of slow release systems are good film-forming agents, so they were suitable to promote nutrient protection (Borrmann et al., 2011; Chan et al., 2009; Franchetti and Marconato, 2006), as shown in Figures 5 and 6.

Reis et al (2013) produced slow-release capsules for oil bioremediation using polymers based on alginate / NPK Capsul® to encapsulate and apply liquid systems with oil from a mineral medium. The authors observed degradation of 43.6% of total petroleum hydrocarbon.

In another study of oil spill bioremediation in ocean waters, Warr et al. (2013) produced a fertilizer nutrient clay base encapsulate using carboxymethylcellulose and simulated the application of this and obtained a reduction 98% of the concentration of total alkanes present in the oil in 1 month.

The data presented in Figures 7 (a, b) and 8 (a, b) show the efficiency of the polymer systems compared to the systems with free nutrients for stimulation of the environmental microorganisms.

The analyzes of the immobilized systems, alginate/Capsul®, Osmocote® and CMC, show that there was a gradual release in the first 24 h, seen in Figure 7 (a, b). This initial release provided a better rate of protein growth as observed in Figure 8b. This was possibly fundamental to provide a better C: N: P ratio, thus allowing the involved microorganisms maintain this more adequate relationship until the end of the experiment. This can be corroborated by the analyses of Figures 7 (a, b) and 8 (b) of the Bushnell-Hass system, since this system provided all the nutrients in the first hours but did not exhibit the best protein growth rate (Figure 8b). Wolicka et al. (2009), applying aerobic microorganisms in bioremediation *in situ* of soil contaminated by petroleum products, also observed that proper C: N: P ratio was necessary to obtain good microorganism growth. According to Das and Chandran (2011), the surplus of free nutrients can inhibit the activity of the oil-degrading microorganisms and so, instead of remedying, will cause a negative impact on the environment.

In Figure 7b, low nutrient consumption by microorganisms was well seen in all systems with immobilized nutrients. Also the Bushnell-Hass system showed a drop in phosphorus concentrations in the first 24 hours, and after that period until the end of the experiment that concentration remained constant, indicating that the medium showed an excess of this nutritional source.

In Figure 8b it was possible to observe that the best growth rates were in the immobilized CMC and Osmocote systems because they provided good stimulus to the involved microorganisms. This was likely due to the polymers' dissolution, since they dissolve in contact with the water and thus release their nutrients (Käistner, 1997; Mendonça et al., 2008).

In the literature some authors (Coulon et al., 2007; Yu et al., 2005) reported in their research the continuing need for nitrogen and phosphorus correction to stimulate degrading the microorganisms' hydrocarbons. Chaîneau et al. (2005) found in their studies a stimulatory effect of the oil-degrading microbial community for 150 days, as they increased the concentration of added nutrients. These studies support the need for the use of the encapsulated material as a source of slow release to the environment to be treated, allowing a reduction in the cost of bioremediation and increased effectiveness.

The encapsulates produced from freeze-dried polymer matrixes of Carboxymethylcellulose and Osmocote provided good bacterial estimation when compared to other systems. The use of immobilized CMC presents a great potential in the release of nutrients with a better cost / benefit ratio, as it provided a good use of the nutrients and produced lower residual quantities in the system.

5. CONCLUSION

Polymer matrices containing nitrogen and phosphorus proved to be effective in releasing nutrients to promote microbial growth, and became a good solution for the treatment of environmental areas with lower eutrophication risk.

Compounds immobilized with 1% (w v-1) carboxymethylcellulose formed good structures for nutrient release, producing biostimulation comparable to the commercial Osmocote® product.

6. ACKNOWLEDGEMENTS

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