Potencial of degradation of naphthalene, phenanthrene and pyrene from sediments collected area near the Panama Canal

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ABSTRACT

Considering the increase in accidental oil spills occurring around the Panama Canal, and given the fact that few investigations have been conducted to the application of biodegradation in coastal marine environments, we developed the present study with the aim of isolating and identifying bacterial strains capable of degrading hydrocarbon fractions. The isolation was conducted with a minimal medium of mineral salts of 2.3% NaCl. The inoculation was performed with 5 g of sediments in 250 ml Erlenmeyer flasks containing 0.3, 0.4, 0.5, 0.6 and 0.7 g/l naphthalene, pyrene and phenanthrene (the only carbon source). Successive transfers were made periodically to the new medium, and each of the cultures were incubated at 37°C, stirring them constantly at 150 rpm for 30 days in order to observe the bacterial turbidity growth. All isolates were examined by Gram's staining reaction to differentiate between from Gram positive and Gram negative bacteria. More than 8 strains that were capable of using naphthalene as the only carbon and energy source were isolated from marine sediment samples. An automated test system API 20 was used for determination and identification of them. This study showed that one species of bacteria, Rhodococcus equi that is able on naphthalene, pyrene and phenanthrene as a carbon and energy source. Our experiments demonstrated that microorganisms have the capacity of degrading hydrocarbons.
Keywords: Biodegradation, isolating, degrading, energy source, microorganism.

RESUMEN

Considerando el aumento de los vertidos accidentales de petróleo que ocurren alrededor del Canal de Panamá y dado el hecho de que pocas investigaciones se han llevado a cabo para la aplicación de la biodegradación en los ambientes marinos costeros, se ha desarrollado el presente estudio con el objetivo de aislar e identificar cepas de bacterias capaces de degradar fracciones de hidrocarburos. El aislamiento se llevó a cabo con un medio mínimo de sales minerales de 2,3 % de NaCl. La inoculación se realizó inoculado con 5 g de sedimento en matraces Erlenmeyer de 250 ml con 0.3, 0.4, 0.5, 0.6 and 0.7 g/l de naftaleno, fenantreno y pireno (la única fuente de carbono), las transferencias sucesivas se realizaron periódicamente, y cada uno de los cultivos se incubaron a 37 ° C con agitación constante a 150 rpm durante 30 días hasta observar la turbidez, característica de crecimiento bacteriano . Todos los aislamientos fueron sometidos por tinción de Gram para diferenciar las bacterias Gram positivas y Gram negativas. Más de 8 cepas, que fueron capaces de utilizar naftaleno, fenantreno y pireno, como la única fuente de carbono y energía se aislaron de muestras de sedimentos marinos. Un API 20 sistema automatizado de prueba se utilizó para la determinación e identificación de las bacterias. Este estudio demostro que una especies de bacterias, Rhodococcus equi que tiene la capacidad de tolerar naftaleno, fenantreno y pireno, como fuente de carbono y energía. Nuestro experimento demostro que los microorganismos tienen la capacidad de degradar hidrocarburos.
Palabras Claves: Biodegradación, aislamiento, degradación, fuente de energía, microorganismos.

1. INTRODUCTION

One of the most important sources of environmental pollution worldwide is the discharge of petroleum hydrocarbons into aquatic and terrestrial ecosystems (Rahman et al. 2003). United States Environment Protection Agency has listed 16 PAHs (Polycyclic Aromatic Hydrocarbons) as priority pollutants because of their toxicity, mutagenicity and carcinogenicity (Keith and Telliard 1979). Extensive research has been conducted on the transportation and transformation of PAHs in natural environments, including water (Zhang et al. 2009), soil (Thiele-Bruhn and Brümmer 2005) and sediment (Marcon et al. 2007). Some of hydrocarbon compounds pollutants are polycyclic aromatic hydrocarbons (PAHs). Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds with two or more fused aromatic rings in linear, angular, or cluster arrangements. PAHs are released into the environment from the incomplete combustion of fossil fuels and organic matter, the accidental spilling of processed hydrocarbons and oils, run off from asphalt pavements, coal liquefaction and gasification and natural geological processes (Zhang et al., 2004).

Biodegradation is an efficient-environment-friendly and cost- effective technology for both ex-situ and in-situ remediation of contaminated environments by PAHs. The main advantages of bioremediation are its cost and environmental friendliness over conventional treatments, such as dredging, capping and electrochemical remediation (Robles-Gonzalez et al. 2008; Perelo 2010). This phenomenon has been shown to occur both in terrestrial and aquatic ecosystems (Leahy and Colwell, 1990; Macnaughton et al., 1999; Roling et al., 2004; Margesin et al., 2007). So far, a few studies have been reported on aerobic (Zhang et al. 2009), anoxic (Bilal et al. 1990; Ramsay et al. 2003) or anaerobic (Coates et al.1996; Kim et al. 2008; Li et al. 2010; Sayara et al. 2010) biodegradation of PAHs. Several aerobic pure cultures degrading naphthalene, phenanthrene, or pyrene as sole carbon source (Desai et al. 2008; Zhang et al. 2009a; Lu et al. 2010) have been isolated. Most of them belong to Pseudomonas (Ahn et al. 1998), Mycobacterium (Pagnout et al. 2007), Rhodococcus (Samantaetal. 2002), Sphingomonas (Desai et al. 2008), Cycloclasticus (Kasai et al. 2002), plus Aeromonas, Corynebacterium and Micrococcus (Samanta et al. 2002), Alcaligenes (Weissenfels et al. 1990), Neptunomonas and Stenotrophomonas (Hoetal. 2000), Marinobacter, Staphylococcus, Micrococcus, Sp. (Gauthier et al., 1992; Gilewicz et al., 1997).

Aromatic with two, three or more than four aromatic rings (naphthalene, phenantherene and pyrene) are also efficiently biodegraded. However, those with four or more aromatic rings (eg., pyrene) are quite resistant to biodegradation (Sutiknowati, 2007). In addition, the physical processes are often limited to aquatic environments. Therefore, the microorganisms should provide all the necessary enzymes needed to degrade PAHs (Al-Thani et al., 2009)

Extensive studies have been conducted on the biodegradation of isolated bacteria from natural environments, leading to isolation of some bacteria which have the ability of using PAHs compounds as the only carbon and energy source (Shafiee et al., 2006; Shafie et al., 2003; Kafilzadeh et al., 2011). Isolating those bacteria capable of degrading organic pollutants such as naphthalene, pyrene and phenanthrene (fig. 1) in soil and water ecosystems can be the perfect solution for improving the microbial population in hydrocarbons-contaminated areas.

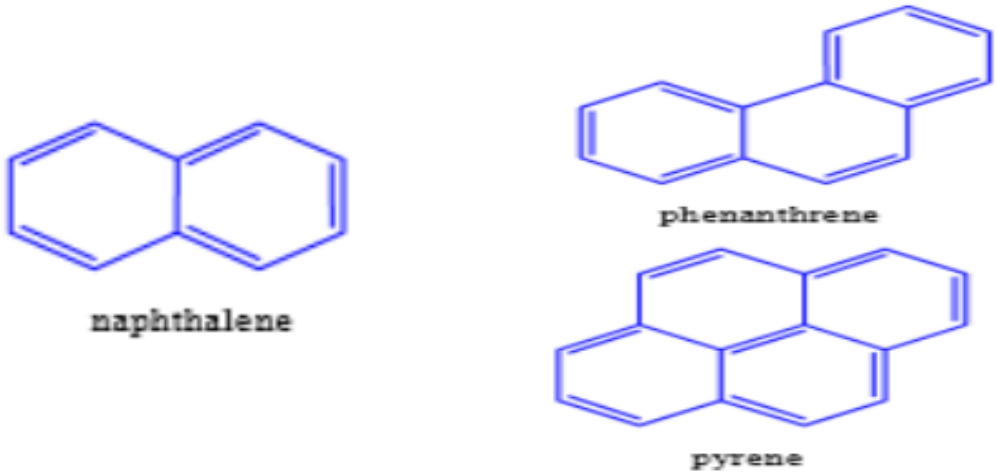

Fig. 1: Molecular structure of the PAHs of interest in this study

2. MATERIALS AND METHODS

2.1. CHEMICAL AND MATERIALS

Naphthalene, pyrene and phenanthrene purchased from Sigma–Aldrich, was used as the sole carbon source for enrichment of degrading bacteria. The bacterial consortium was isolated from a mixture of five different sampling sites in the area near the Panama Canal (Manzanillo Bay).

2.2. ENRICHMENT OF CULTURE

The samples were obtained by dredging at depth of 12 meters. As stated before, isolation was conducted with minimal medium of mineral salts of 2.3% NaCl. The minimal basal salts (MBS) medium used for enrichment and further experiments contained per liter: 1.0 g of (NH4)2SO4, 5.0 g KH2PO4, 0.1 g MgSO4.7H2O, 5 mg of Fe(NH4)2(SO4)2 and 1.0 ml of trace elements solution.

The trace element solution contained per liter: 23 mg MnCl2.2H2O, 30 mg MnCl4.H2O, 31 mg H3BO3, 36 mg CoCl2.6H2O , 10 mg CuCl2.2H2O, 20 mg NiCl2.6H2O, 50 mg ZnCl2, 30 mg Na2MoO4.2H2O. The basic salt environment and tracing solution for separate elements were autoclaved (20 minutes, 121 °C). After sterilization, the inoculation was performed with 5 g of sediment in 250 ml Erlenmeyer flasks with 0.3, 0.4, 0.5, 0.6 and 0.7 g/l of naphthalene, pyrene and phenanthrene (the only carbon source).

Successive transfers were made periodically to the new medium, and each of the cultures were incubated at 37 °C, stirring them constantly at 150 rpm for 30 days in order to observe the characteristic turbidity of bacterial growth. Once this period finished, 1 ml of each sample was taken in order to be inoculated on the plates containing the same medium solidified with agar. (fig. 2.).

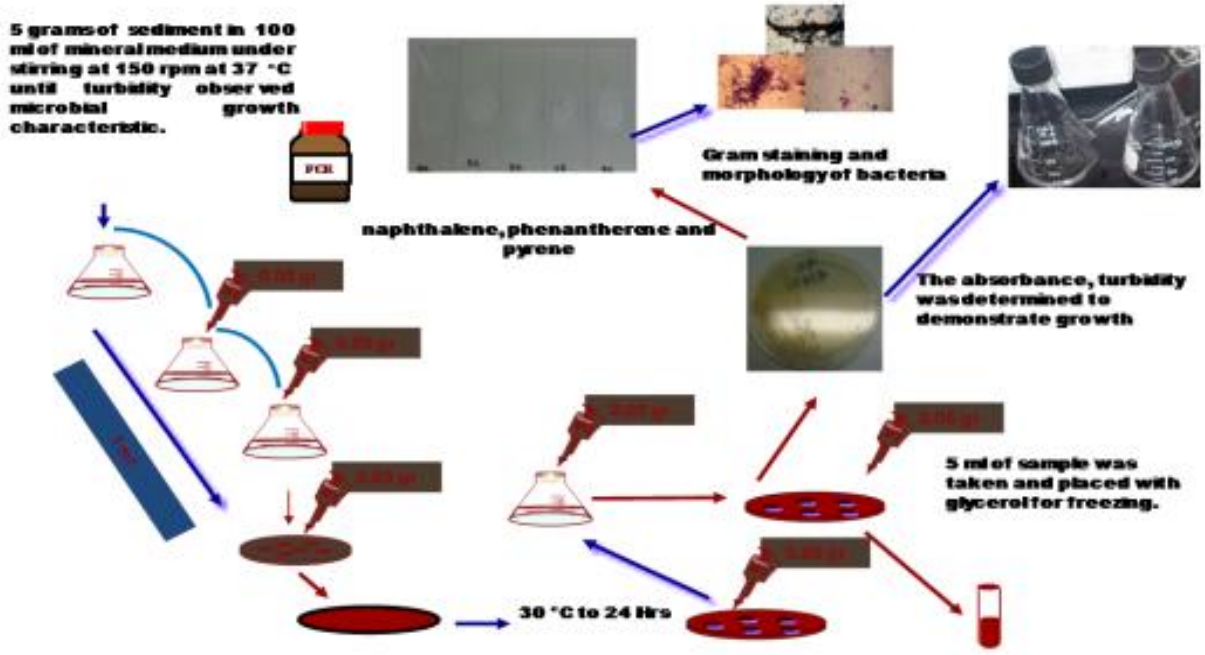

Figure 2. Methodology used for the isolation of bacterial strains capable of degrading Polycyclic Aromatic Hydrocarbons

2.3. DETERMINATION OF THE TIME COURSE OF GROWTH OF THE ISOLATES

The growth was determined by the colorimetric method which was measured by optical densities of the filtrates containing suspended cells were measured by spectrophotometer (Cecil, 1010, England) at wavelength of 525 nm. The Gram stained slides for the isolated microorganisms were observed under microscope (Olympus B071, Japan). Transmission Electron Microscopy (TEM) (Philips CM12, Netherlands) was preformed for the isolated organisms. Successive transfers were made periodically to the new medium. For this purpose, different concentrations of 0.3, 0.4, 0.5, 0.6 and 0.7 g/l of hydrocarbon were produced and added to the 100 ml mineral base medium of erlenmeyer flasks whose heads were covered with cotton. Pyrene and phenanthrene were also used in these concentrations in separate flasks. Each of the flasks was filled with half McFarland of the bacteria. Then, they were incubated at 300 C in the dark room. Within 12 hours, 2.0 ml sample was collected from each flask and assayed for OD at 525 nm in a UV spectrophotometer (Nnamchi et al., 2006).

To prepare solid culture medium, 20 g of agar per liter were added to the above medium and the mix was poured into petri dishes. Using sprayplate method, each of the compounds was sprayed onto individual plates. Agar plates were incubated at 100 C for 1-2 days. After the incubation, colonies that showed a precipitation were selected for identification and further characterization. The isolates were identified on the basis of morphological, trophic and biochemical traits according to the Bergey,s Manual of Systematic Bacteriology (Coral and Karagoz, 2005).

The microorganisms were identified according to general principles of microbial classification, using selective media and microscopic examination of its morphological characters. An automated test system API 20 (BioMerieux) was used for determination and identification of the isolates

2.4. STATISTICAL ANALYSIS

The statistical analysis of the results was conducted by ANOVA test and SPSS, at a level of significance of $p < 0.05$.

3. RESULTS AND DISCUSSION

The original naphthalene enriched mixed culture was obtained by adding 25 g of fresh sediment of the sample immediately after field collection. The mix was done with 150 ml sterilized medium minimum (MM) containing 50 mg/l of naphthalene in Erlenmeyer flasks, and shaken in an orbital shaker at 150 rpm at 30oC in the dark. Two weeks later, 10 ml aliquots were transferred to 100 ml of fresh MM containing 50 mg/l of naphthalene, and the flasks were shaken for another two weeks. Bacterial colonies were selected by using as a criterion its potential to degrade the naphthalene. All isolates were examined by Gram's staining reaction to differentiate between Gram positive and Gram negative bacteria. All isolates were examined by Gram´s straining reaction (table 1).

A total of 8 strains, that were capable of using naphthalene as the only carbon and energy source for growing, were isolated from marine sediment samples. The bacteria were identified as Rhodococcus equi and Corynebacterium propinquum. (fig. 3).

Table 1: Characteristics of the strains found in the area near the Panama Canal

GRAM	MORPHOLOGY	COLONIES
(+)	Pleomorphic bacillus	Bluish entire edge
(+)	Sporulated bacillus	Yellow, elevated, wavy edge

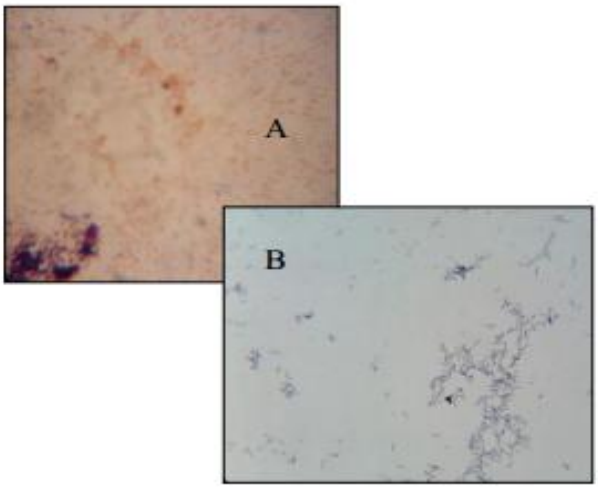

Figure 3. Hydrocarbon-degrading strains identified by API 20 NE as a) Rhodococcus equi, b) Corynebacterium propinquum.

3.1. KINETIC MODEL

To calculate the kinetic model of the growth of Rhodococcus equi strain that degrades Naphthalene biodegradation in different duplicate, with their biotic and abiotic controls were performed. (fig. 4).

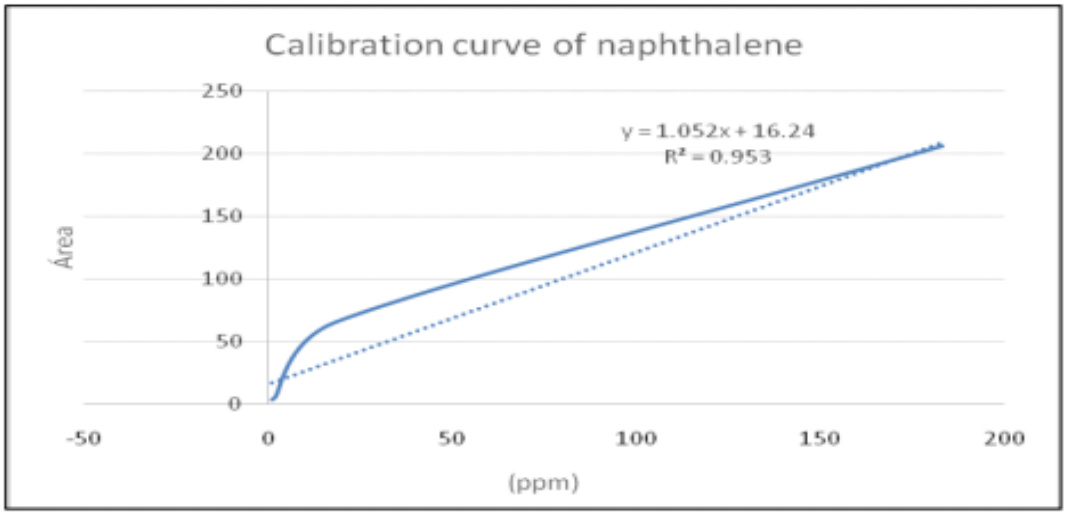

Figure 4. Calibration Curve of Naphthalene

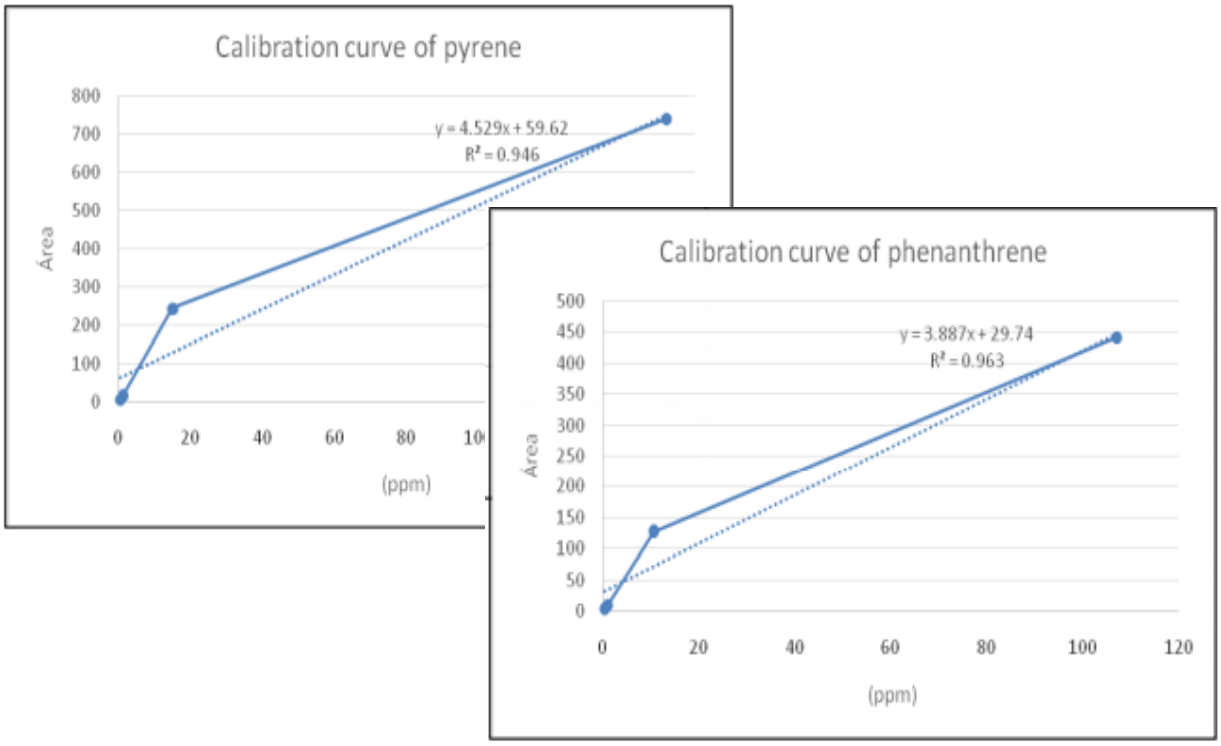

Figure 5. Calibration Curve of phenanthrene and pyrene

As explained in the bio-test activities, the isolated bacteria were incubated in Erlenmeyer flasks (250 ml) at 26 °C with constant stirring (150 rpm) according to Foght et al. (1999). The minimum medium was added to each of the six flasks with different concentration of naphthalene (125, 250, 500, 750, 1000 and 2000 mg / L naphthalene). Each test was performed twice in order to obtain the average. The only source of energy is coal and Naphthalene. Bacteria growth was measured in levels of absorbance (A) by UV spectrophotometry (fig. 5).

The results of measurements of bacterial growth where V represents the rate of bacterial growth, which is calculated based on the growth differential between the time differences, as established Mihelcic (2001). The first growth rate for each concentration of naphthalene, which is taken as representative for the calculation of the final kinetic curve, represents the trend line of the kinetic model. This can be adapted to a kinetic model of Monod (when no bacterial inhibition) or a kinetic model of Andrews (when bacterial inhibition).

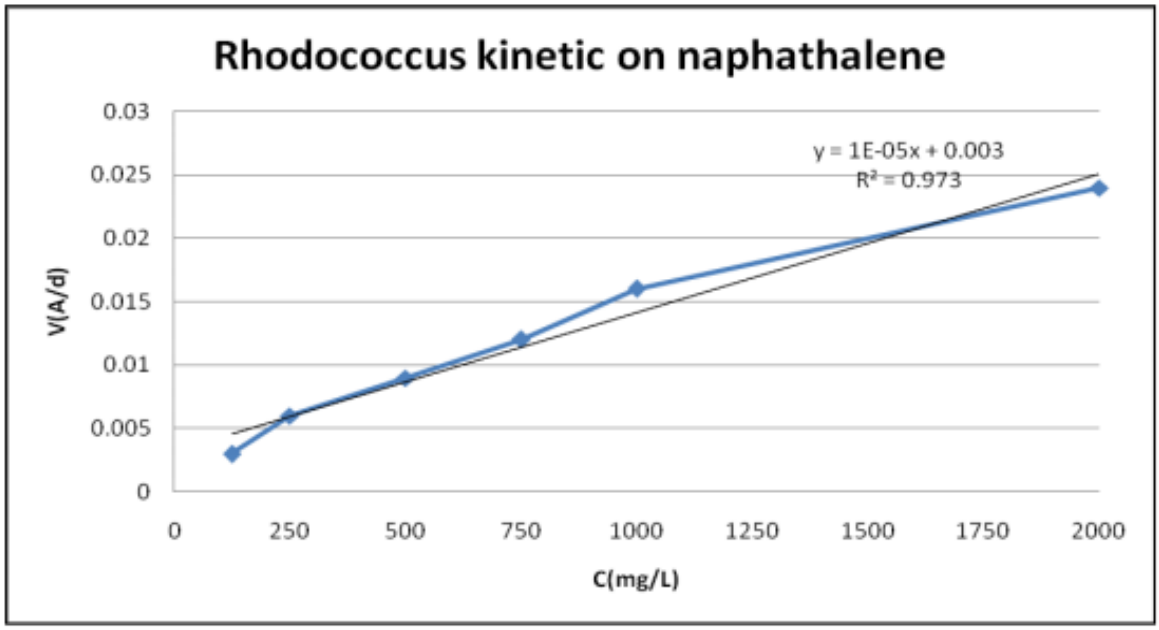

Figure 6. Rhodococcus Kinetic on Naphathalane

The Growth behavior of the Rhodococus equi strain is observed when it consumed the Naphthalene as the sole source of carbon. An unusual behavior was observed for the strain was consumes a recalcitrant compound, because in such cases, generally, inhibition occurs (Dominguez et al, 2002). The can be said because, according studied range of concentrations use in this study (125 and 2000 mg/l), the inhibition point was not observed. Therefore it is recommended to conduct other kinetic tests with phenanthrene, pyrene and naphthalene using higher concentrations. The logarithmic behavior of the result observed in this study is Monod.

The growth model found can be writing as: V = 0.0078 ln (C) -0.0368. Where V represents the velocity of bacterial growth (A / h) and C the concentration of consumption (mg / L). (fig. 5).

Microorganisms play an important role in the biodegradation of chemicals in natural ecosystems. The ability of bacteria in water, soil or sediment to degrade PAHs depends on the complexity of the PAH chemical structure and the extent of enzymatic adaptation by indigenous bacteria in response to chronic exposure to aromatic hydrocarbons. The degradation of xenobiotics may result from catabolism by individual strains of microorganisms or from combined metabolism by microbial communities (Heitkamp et al., 1988).

These two bacteria had the highest OD 525nm in the presence of aromatic compounds. Isolated bacteria in the presence of low molecular weight compounds, such as naphthalene in comparison with high molecular weight compounds like pyrene and phenanthrene, achieved the highest optical density during a short time.

In recent decades, the ability of bacteria for degrading PAH compounds has been documented by extensive studies. The indigenous bacteria in contaminated areas are continuously in contact with aromatic compounds. These bacteria are able to degrade these substances in their surroundings because they possess all the necessary enzymes which are needed to degrade PAHs (Nnamchi et al., 2006).

The biodegradation of low molecular weight (two-and three-ring) PAHs occurred much more rapidly and extensively than high molecular weight (four-, five- and six-ring) hydrocarbons (Li et al., 2007). Shafiee et al. (2006) after analysis of PAHs during the ten day incubation reported that phenanthrene completely, anthracene 60%, pyrene 80%, fluorene 30% and fluoranthene 20% were decomposed by soil bacteria.

4. CONCLUSIONS

This study showed that two species of bacteria, which are able to sediments on naphthalene as a carbon and energy source, were isolated from the terrestrial and aquatic sites in the area near the Panama Canal. These bacteria were identified as Rhodococcus equi and Corynebacterium propinquum. Our experiments demonstrated that naphthalene degrading microorganisms are not restricted to oil polluted sites. This finding supports the fact that crude oil degrading microorganisms are widely distributed in the environment and, therefore, can be "easily" collected from sites with no apparent history of crude oil pollution, as it was in our case.

The final identification by the API method are two main strains of Rhodococcus equi and Corynebacterium propinquum. Furthermore a kinetic model, without logarithmic growth inhibition of Rhodococcus equi, using naphthalene as the sole carbon source was obtained. The model can be written as: $V = 0.0078 \ln (C) - 0.0368$, where V represents the velocity of bacterial growth (A / h) and C the concentration of consumption (mg / L).

5. ACKNOWLEDGMENT

The authors thank the Naval Air Service of Panama, Panama Ports Authority, Laboratory of Sanitary Engineering, Faculty of Civil Engineering at the Universidad Tecnologica de Panamá and the National Secretariat of Science, Technology and Innovation (SENACYT) for their collaboration and participation in various campaigns of sampling and analysis of samples of some environmental parameters as well as funding for the research.

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