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AROMATIC HYDROCARBON DEGRADATION OF BIOFILM FORMED BY MICROORGANISMS ON CELLULOSE MATERIAL AT 50 LITRE MODULES

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SUMAMRY

Biofilms are defined as community of microorganisms which are irreversibly or reversibly attached on solid surfaces. These microorganisms are embedded in a self-produced exopolysaccharide matrix, and exhibit different growth and bioactivity compared with planktonic cells. With their high biomass density, stability, and potential for biodegradation of recalcitrant compounds contained in oil contaminated wastewater such as aromatic hydrocarbons. Aromatic hydrocarbons are the main constituents of petroleum and its refined products. These compounds are also quantitatively the main environmental pollutants worldwide. In this report, cellulose material was used as a carrier for forming biofilm by microorganisms to remove of these components. Cellulose material is considered as inexpensive, available, sustainable, little waste production and can be recycled. As a result, the microorganisms were successful to adhere on cellulose material at 50 liter module with cell density of $4.3x10^8$ CFU/ml after 7 day-incubation. Under the scanning electron microscope with the 1500 magnification, the microbial cells had a very high density, closely linked together and firm adhesion on the cellulose material. The mixture species biofilm attached on cellulose carrier at 50 liter module had the ability to degrade 80.1, 78.3, 60.0, 98.5 and 91.2% of anthracene, fluorene, naphthalene, phenol and pyrene after 7 days, respectively. The obtained results showed that biofilm formed by multiple bacterial strains attached on cellulose material may considerably increase the degrading efficiency of aromatic hydrocarbon compounds. The results also indicated that cellulose material is suitable carrier to choose in removal of aromatic hydrocarbon contaminated wastewater. These results are considered as new approach to apply microbial films on cellulose material to degrade oil polluted waste-water in the environment.

Keywords*: Aromatic hydrocarbons, biodegradation, biofilm, cellulose material, microorganisms*

INTRODUCTION

Petroleum hydrocarbons are introduced into the environment due to their extensive use as fuels and chemicals. But already before their industrial use, locally significant accumulations of these hydrocarbons must have occurred in the biosphere via natural seeps (Spormann, Widdel, 2000). Many techniques are utilized to mitigate and cleanse petroleum hydrocarbon pollution in the environment (Obuekwe, Ale-Muttawa, 2001). Conventional physical and chemical methods could rapidly remove the majority of leaked compounds, but in most cases, the removal seemed to transfer contaminants from one environment medium to another, even producing toxic by-products. More importantly, petroleum hydrocarbons could not be completely cleaned up by physical and chemical methods (Gavrilescu, 2010).

Biodegradation is a technique utilizing organisms – especially microorganisms – to aid in removal of persistant substances from polluted area (Head *et al*., 2006). Recently, application of biofilm forming microorganisms in degradation of these components is considered as a new approach (Liang *et al*., 2009). Considering the broad choices, low cost, simple process and less impact on microbial activity (Oh *et al*., 2000; Lee *et al*., 2010), biofilm formed by microorganisms on carriers has been proved to be an effective strategy to apply functional microorganism for biodegradation (Wang *et al*., 2012; Mollaei *et al*., 2010).

Biofilm is a biologically active matrix of cells and extracellular products attached to a solid surface such as sand particles, glass, rocks etc… or interface (Watnick, Kolter, 2000). It has recently been pointed out that environmental microorganisms exist predominantly as biofilms and gain high tolerance to physical, chemical, and biological stresses (Gorbushina, Broughton, 2009). The close physical associations of microbial community members in biofilms lead to interactions at the genetic level. Gene transfer occurs both at intra- and inter-species levels and plays an important role in biofilm evolution and genetic diversity (Stewart, Costerton 2001). Thus, forming biofilms is considered a natural strategy of microorganisms to construct and maintain a favorable niche in stressful environments (Thompson *et al*., 2005; Shemesh *et al*., 2010).

In the present study, a bench scale cellulose–biofilm system was used in order to investigate its capacity to decontaminate aromatic hydrocarbon-containing waste water from the petroleum storage company. In that system, ten different bacterial and two yeast strains were cultured on the cellulose particles (Fig. 2A). They were originally isolated and identified from the oil contaminated water and sediment samples collected in coastal zones in Vietnam. Cellulose is considered as inexpensive, available, sustainable, little waste production and can be recycled material. To our knowledge, cellulose–biofilm system has been studied with respect to its application involving immobilization or bioconversions, which may be particularly useful both in bioremediation and in biotransformation systems.

Table 1. Characteristics of several microorganisms isolated from coastal zones in Vietnam. Anthracene; 2: Diesel oil; 3: Naphthalene; 4: Phenol; 5: Pyrene; -: no growth; +: growth; ++: good growth.

	Hydrocarbon utilization					Biofilm formation
	1	2	3	4	5	
Acinetorbacter sp. QN1	+	++	+	++	+	++
Bacillus sp. B8	++	++	++	++	++	++
Bacillus sp. C2	+	++	++	++	++	+
Microbacterium sp. B15	++	++	+	+	+	++
Ochrobactrum sp. DGP2	++	+	++	++	++	++
Paracoccus sp. DG25	++	++	+	++	+	++
Pseudomonas sp. B6	++	+	+	+	+	+
Rhodococcus sp. BN5	+	++	++	++	+	++
Rhizobium sp. DG22	+	++	++	++	++	+
Serratia sp. DX3	+	+	+	++	+	++
Debaryomyces sp. QNN1	+	+	+	+	+	++
Rhodotorula sp. QNB3	++	++	++	++	+	++

MATERIALS AND METHODS

Materials

Microbial strains

The strains used in this study (Table 1) were isolated from oil contaminated water in Vietnam, including Quang Ninh, Hai Phong, Thanh Hoa, Quang Ngai, Khanh Hoa and Vung Tau (Nhi Cong *et al.*, 2014). Assimilation tests and sequencing of the gene 16S rRNA and ribosomal ITS region revealed that these strains were *Acinetorbacter, Bacillus, Pseudomonas, Rhodococcus, Serratia, Candida, Debaryomyces, Rhodotorula, Trichosposon,* etc ...

The strains were demonstrated to be safe for environment (data not shown). The isolates exhibited an excellent ability to degrade alkanes, anthracene, phenol, pyrene.

Cellulose materials

Cellulose was selected for this study because of their large surface area (from 800 to 1000 m^2/g) and high adsorption capacity (0.5 g/cm^3). Their characteristics are shown in Table 2. Carriers were pre-treated by soaking in diluted hydrochloric acid

(2–4%) for 2 days, rinsing several times with distilled water, drying at 40°C for 12 h, cooling to room temperature and finally soaking in sterilized water to saturation.

Methods

Biofilm formation test

The experiment was performed using the method described by Shimada *et al*. (2012). In details, an overnight culture was diluted to OD_{600} = 0.3 and inoculated (1% - v/v) into 300 µL of Malt or nutrient broth media in a 1.5-mL micro-centrifuge tube (TC131615, Nippon Genetics, Tokyo, Japan). The tube was kept standing at 30°C for two days, and then the pellicles and the medium were removed. Afterward, the tube was gently rinsed with distilled water and filled with 500 µL of a 1% crystal violet (CV) solution. After 25 min, the CV solution was removed, and the tube was washed with distilled water again. The CV attached to the biofilm was extracted by 400 µL of acetone and quantified by measuring its absorbance at 570 nm. The *Acinetobacter calcoaceticus* P23 was used as a positive control, and the tube without an inoculum was used as a negative control (Yamaga *et al.,* 2010). Each experiment was performed in triplicate. The strain forming the strongest CV-stained biofilm was selected for further experiments.

System design

Each 50 liter-module included 3 carrier sheets placed inside the tank, air sytem and mixing system. At the beginning, the microbial strains were cultured on carrier. Then, 50 liters oily wastewater was added, the treatment processes had been set up for 7 days under laboratory conditions (Fig. 1).

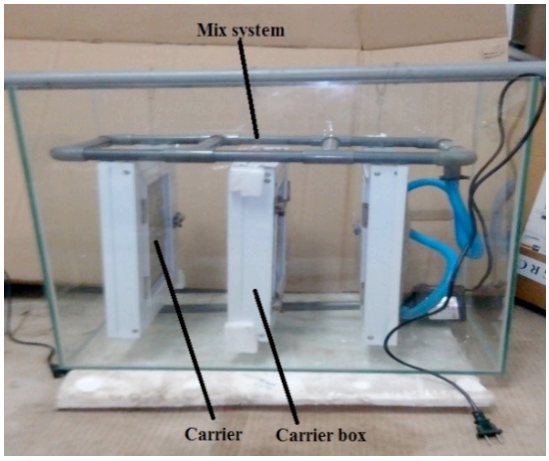

Figure 1. A 50 liter – module used in this study.

Assessment of microbial cell immobilization on carriers by indentifying the most portable number (MPN)

The MPN method was described in detail by Oblinger, Koburger (1975).

SEM (Scanning electron microscope) observation

Pellicles were gently placed on a glass plate that had been coated with poly-L-lysine. The specimen was fixed with glutaraldehyde and OsO_4, dehydrated in ethanol, isoamyl acetate and critical CO_2, and sputter coated with platinum (Glauert, 1975). Observations were performed with a Hitachi S800 scanning electron microscope.

Analysis of petroleum hydrocarbon degradation by high-performance liquid chromatography (HPLC)

The analysis was performed on a Hewlett-Packard (Bad Homburg, Germany) HPLC apparatus 1050 M equipped with a quaternary pump system, a diode array detector 1040 M series I, and an HP Chemstation. The separation was achieved with a LiChroCart 125-4 RP-18 end-capped (5-µm) column (Merck, Darmstadt, Germany). Elution profile is characterized by an initial solvent composition of 30% methanol and 70% phosphoric acid (0.1%), reaching 100% methanol after 14 min at a flow rate of 1 ml/minute.

These experiments were conducted independently at least three times and calculated standard deviation (SD) values.

RESULTS AND DISCUSSION

Microorganism density on cellulose carrier

By observation, the color of samples was changed after 5 and 7 day-incubation. This result indicated that all the cultured strains degraded hydrocarbon components containing in oil polluted waste water. To confirm this prediction, all samples were examined by CFU method and observed under scanning electronic microscope (SEM). The results were showed in Fig. 2 and Table 2. The number of microorganisms attached on cellulose material after 7 day-incubation was quite high (4.3×10^8 CFU/cm^3). Under SEM observation, the density of microorganisms adhered on the carrier was affirmed with this result.

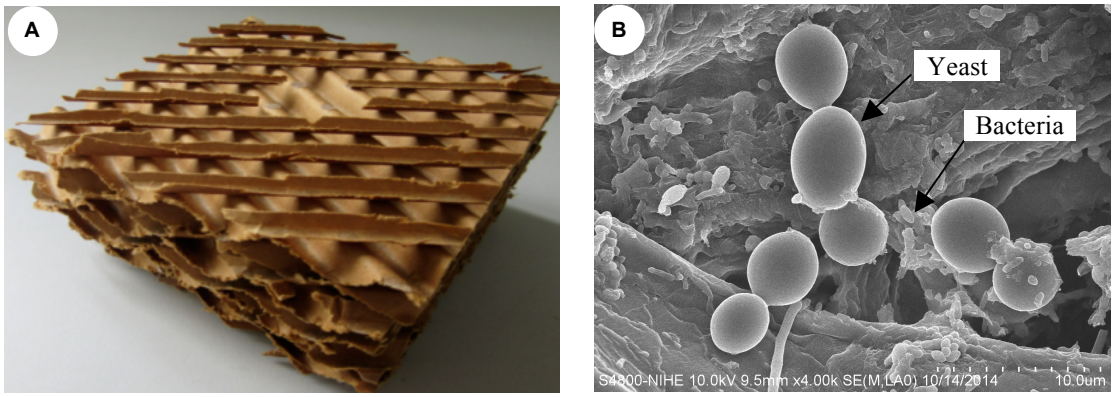

Figure 2. Cellulose material (A) and microorganisms attached on the material under SEM

Table 2. The number of microorganisms attached on cellulose material.

Sample	Number of microoganisms (CFU/cm^3)
Sample at the beginning	4.3 x10^9
Sample after 5 days	4.3x10^8
Sample after 7 days	4.3 x10^8

Hydrocarbon degradation by microorganism adhered on cellulose carrier

The samples were analyzed by HPLC and the results are described in Table 3. The results indicated that this biofilm of mixture species attached on cellulose carrier at 50 liter module has the ability to degrade 80.1, 78.3, 60.0, 98.5 and 91.2% of anthracene, fluorene, naphthalene, phenol and pyrene after 7 days, respectively.

There are many publications concerning about aromatic hydrocarbon degradation by biofilm and suspended microorganisms. Shimada *et al.* (2012) reported that single biofilm formed by *Pseudomonas stutzeri* T102 strain degraded 81% of naphthalene after 9 week-incubation with the initial amount of 100 ppm while planktonic cells degraded only 52%. Cui *et al.* (2014) published the planktonic *Martelella* sp. strain AD-3 could completely degraded anthracene (40 ppm) after 6 days of culture. A functional strain F14, which was constructed through protoplast fusion between *Sphingomonas* sp. GY2B and *Pseudomonas* sp. GP3A, was demonstrated to degrade 46% of pyrene (100 ppm) after 10 day-incubation (Lu *et al.,* 2014).

Table 3. Hydrocarbon degradation productivity by biofilm forming microorganisms attached on cellulose carrier.

Substrate	Remained amount (mg/l)			Degradation productivity (%)	
	0 hour	5 days	7 days	5 days	7 days
Anthracene	50.19	15.12	9.98	69.84	80.12
Fluorene	49.19	20.13	10.67	59.08	78.31
Naphthalene	50	25.11	20.01	49.78	59.98
Phenol	50	1.02	0.3	97.96	99.40
Pyrene	85.19	8.28	7.49	90.28	91.21

Recently, using material carriers adhered by immobilized microorganisms to increase petroleum hydrocarbon degradation have been reported. Liang *et al*. (2009) used activated carbon and zeolite as biocarriers for indigenous hydrocarbon-degrading bacteria to promote crude oil. As the results, biocarrier enhanced the biodegradation of crude oil, with 48.89% removal, compared to natural

attenuation with 13.0% removal, biostimulation (nutrient supplement only) with 26.3% removal, and bioaugmentation (free-living bacteria) with 37.4 % removal. Lin *et al*. (2014) used cotton fibers as crude oil sorbent as well as a biocarrier for bacteria immobilization. The efficiency of saturated hydrocarbons degradation by the immobilized bacterial cells increased about 30% compared to the planktonic bacterial cells. A hypothesis model was proposed to explain the mechanism that the biocarrier improved the oxygen, nutrient mass transfer and water holding capacity of the soil, which were the limiting factors for biodegradation of non-aqueous phase liquid contaminants such as crude oil in soil (Liang *et al*., 2009).

However, to our knowledge, there are not many articles on biofilm forming organisms attached on carriers to degrade aromatic hydrocarbons in Vietnam. La Thi Thanh Phuong *et al*. (2003) reported the isolation of the bacterial strain *Sphingomonas yanoikuyae* MXL-9 from sediment samples taken from the White Tiger oilfield. The strain had capacity of anthracene degradation with 61.4% with the initial amount of 12 mg/l after 7 day-incubation. Nguyen Ngoc Bao *et al*. (2007) published two bacterial strains, BDNR1 và BDNR4 isolated from dioxin contaminated sediment samples taken from the site of the past American military base in Da Nang airport. The BDNR1 had capacity to degrade 50% of anthracene, naphthalene; 86.2% pyrene; and the BDNR4 had capacity to degrade 50% of anthracene, 21.3% of naphthalene and 61.5% pyrene with the the initial amount of 100 mg/l each substrate after 7 day-cultivation. Therefore, the results of this report suggest the potential usefulness of biofilm formed by microorganisms on cellulose carriers to achieve efficient and sustainable bioremediation of polluted soil and water in our country.

CONCLUSIONS

A 50 liter module containing cellulose carriers was conducted from mixture of biofilm forming microorganisms which could well degrade petroleum hydrocarbons. The mixture biofilm attached on cellulose carriers had the ability to degrade 80.1, 78.3, 60.0, 98.5, 0 and 91.2% of anthracene, fluorene, naphthalene, phenol and pyrene after 7 days, respectively.

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KHẢ NĂNG PHÂN HỦY HYDROCARBON THƠM CỦA MÀNG SINH HỌC ĐƯỢC HÌNH THÀNH BỞI VI SINH VẬT GẮN TRÊN GIÁ THỂ CELLULOSE Ở HỆ THỬ NGHIỆM DUNG TÍCH 50 LÍT

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TÓM TẮT

Màng sinh học (biofilm) là một tập hợp các vi sinh vật được gắn trên các bề mặt vật thể rắn. Các vi sinh vật trong biofilm thường tự tiết ra lớp màng polysaccharide ngoại bào và cho thấy sự sinh trưởng cũng như các hoạt tính sinh học khác với ở trạng thái tế bào tự do. Trong màng sinh học, các vi sinh vật thường có mật độ sinh khối cao, ổn định và có khả năng phân hủy các hợp chất khó phân hủy có trong nước thải nhiễm dầu như các chất hydrocarbon thơm. Hydrocarbon thơm là các thành phần chính có trong dầu mỏ và các sản phẩm của dầu mỏ. Các hợp chất này được xem là các yếu tố gây ô nhiễm môi trường rất nghiêm trọng. Trong bài báo này, chúng tôi sử dụng vật liệu dạng cellulose để làm chất mang cho các vi sinh vật tạo màng sinh học bám vào nhằm tăng cường khả năng phân hủy các hợp chất đó. Vật liệu dạng cellulose được xem là có giá thành rẻ, sẵn có, có thể tái sử dụng, thân thiện với môi trường và không gây ô nhiễm thứ cấp. Kết quả chúng tôi thu được như sau: các vi sinh vật đã được gắn trên vật liệu này ở mô hình có dung tích 50 lít với mật độ $4,3x10^8$ CFU/ml sau 7 ngày nuôi cấy. Dưới kính hiển vi điện tử quét với độ phóng đại 1500 lần, các tế bào vi sinh vật đã được gắn trên vật liệu mang và có mật độ cao, liên kết chặt chẽ với nhau. Màng sinh học gắn trên vật liệu mang trong mô hình này có khả năng phân hủy 80,1, 78,3, 60,0, 98,5 và 91,2 % các hợp chất anthracene, fluorene, naphthalene, phenol và pyrene sau 7 ngày thử nghiệm. Kết quả này đã chứng minh được màng sinh học được tạo thành từ hỗn hợp các chủng vi khuẩn gắn trên vật liệu mang cellulose sẽ làm tăng hiệu quả phân hủy các hợp chất hydrocarbon thơm. Đồng thời còn cho thấy vật liệu mang cellulose rất phù hợp để gắn các vi khuẩn nhằm loại bỏ các hợp chất hữu cơ này trong nước thải. Các kết quả này cho thấy tiềm năng của việc ứng dụng

vật liệu dạng cellulose có gắn các vi sinh vật tạo màng sinh học trong xử lý nước thải ô nhiễm dầu trong thực tế ở nước ta hiện nay.

Từ khóa: *Hydrocarbon thơm, màng sinh học, phân hủy sinh học, vật liệu dạng cellulose, vi sinh vật*